Neglected Tropical Diseases

Methods and Principles in Medicinal Chemistry

Neglected Tropical Diseases

Drug Discovery and Development

Edited by
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Contents

A Personal Foreword

There is a great need for new affordable, effective therapeutics for neglected tropical diseases (NTDs). The World Health Organization (WHO) defines NTDs as a diverse group of communicable diseases that prevail in tropical and subtropical conditions – affecting more than one billion people and costing developing economies billions of dollars every year. Populations living in poverty without adequate sanitation and in close contact with infectious vectors and domestic animals and livestock are those worst affected. The designation of "neglected" refers to the limited resources available for controlling and treating these diseases, including the discovery and development of new medicines.

With an eye toward providing a primer to the research community about the various NTDs and challenges in drug discovery for them, this book provides summaries of therapeutic discovery and development efforts for NTDs with a focus on the medicinal chemistry aspects of the programs. The authors for each chapter include experts in medicinal chemistry and biology. Each chapter describes the unmet medical needs, current therapies, available discovery tools including *in vitro* and *in vivo* assays and current medicinal chemistry approaches to address unmet medical needs. There are chapters on 10 NTDs as well as 2 emerging viral diseases, Zika and Ebola. The chapter on Zika virus discusses repurposing knowledge from the NTD dengue. We also included an introductory chapter on drug discovery strategies for NTDs.

The aim of drug discovery and development is to identify new medicines to satisfy the unmet medical need of patients. This is easier said than done. Drug discovery and development is very expensive due to the high failure rate of clinical candidates. It is well documented that most candidate medicines will fail, and the cost of failure contributes to the high cost of medicines. The poor efficiency of drug discovery is due in part to the inability to *a priori* predict if a drug will be efficacious (e.g. will it work or not?). Great advances have been made in reducing attrition due to pharmacokinetics (the ability of the drug to get to the site of action) and safety (toxicity), but not efficacy. The challenge to predicting efficacy is significant due to the complexity of pathophysiology; medicines must account for genetics, molecular mechanisms that translate to specific action as well as the dynamic heterogeneous physiological environment. The only way to confirm efficacy is to evaluate compounds in human proof-of-concept studies. These studies are costly in part because prior to the proof-of-concept studies, the compounds must be shown to be safe. Because of the high likelihood of failure

due to unanticipated toxicity or lack of efficacy, in order to reduce likelihood of project failure, it is necessary to take many (costly!) "shots on goal."

Therein lies the challenge for drug discovery and development for neglected diseases. The funding is insufficient to support many "shots on goal." It is important to recognize that there are no fundamental scientific or technological differences between discovery and development for neglected and non-neglected infectious diseases. What emerges from the chapters in this book is that validated discovery tools are available for the NTDs to support identification of active compounds. These include *in vitro* assays using the infectious organisms, as well as reliable animal models in most cases.

Not surprisingly, all the contributors identified funding as the most significant liability of their disease area, consistent with the neglected designation. One highly cost-effective approach to mitigate this is to repurpose knowledge and compounds from other diseases. Repurposing was highlighted in all the chapters as the most logical strategy.

Where funds should be spent is an important question to consider. Should the funds be spent on more knowledge of the fundamental biology hoping that translates to new candidate mechanisms and targets? Or better optimization of candidates to get more viable shots on goal? Or perhaps more repurposing of medicines previously approved for other indications? An apparent advantage of NTDs is the availability of phenotypic screening with models of infected diseases. At first glance, these models should provide good representation of the biology and identify appropriate new mechanisms of action. The limited success in follow-up on active compounds identified by phenotypic screens begs the questions: Why has there not been more success with actives from phenotypic screens? Do the screening assays not represent the relevant pathological state of the infected organism? Are the candidates not sufficiently optimized for drug-like properties due to limited medicinal chemistry resources? How much of the efforts are spent revaluating flawed compounds and mechanisms in which the flaws were not reported? The answers to these questions will help ensure that funding is used more efficiently in the future.

What are the opportunities for medicinal chemistry in drug discovery and development for NTDs? Drug discovery and development requires acquisition of disease knowledge, creation or invention of new compounds, and optimization and development of the invention to product. The acquisition of knowledge is the domain of the biologist and funded by government organizations, such as the US National Institutes of Health, while product development involving clinical studies must be funded by pharmaceutical companies or public private partnerships and nonprofit organizations such as Medicines Development for Global Health and the Drugs for Neglected Diseases Initiative. These organizations have been responsible for the recent successes with moxidectin for onchoceriasis and fexinidazole for sleeping sickness. The invention of the new molecule and optimization to a product is the domain of the medicinal chemist. The chapters in this book provide some excellent case studies of the optimization toward clinical candidates. Clearly there is much need and opportunities for medicinal chemists to invent new medicines for NTDs. The challenge is to identify quality starting points and funding sources for the medicinal chemistry optimization.

We acknowledge all the contributing authors for sharing their knowledge and perspectives on neglected tropical diseases. We thank the series editors Gerd Folkers, Hugo Kubinyi, and Raimund Mannhold for the opportunity to address this topic, and Frank Weinreich and Stefanie Volk at Wiley-VCH for the support and commitment.

May 2019

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Preface

The diverse group of neglected tropical diseases (NTDs) prevails in a great many countries with tropical and subtropical conditions [1]. It is estimated that over 1 billion people are infected with NTDs, with a further 1 billion at risk. These diseases are the most common afflictions of the world's poorest people. However, some of the NTDs, such as tuberculosis, affect populations globally, including US populations. NTDs have a terrible impact on health, impede child growth and development, harm pregnant women, and often cause long-term debilitating illnesses. The fight against NTDs costs developing countries billions of dollars every year. Despite their significance, relatively little financial support has been provided to address NTDs, compared to the burden of ill health that they cause.

Twenty NTDs have been identified and classified by the World Health Organization (WHO). The majority of NTDs has particular characteristics in common [2]:

(i) they preferentially afflict poor people, who lack access to the safe water, sanitation, and basic health services required in order to protect themselves against infection by bacteria, viruses, and other pathogens; (ii) many are chronic; the damage they cause can be irreversible; (iii) NTDs can elicit severe pain and life-long disabilities; (iv) people with NTDs are often stigmatized and excluded from society, which in turn can affect their mental health.

The infectious agents responsible include the following:

Protozoa	Chagas disease, human African trypanosomiasis, leishmaniasis
Bacteria	Buruli ulcer, leprosy, trachoma, yaws
Helminth	Cysticercosis, dracunculiasis, echinococcosis, trematodiasis, lymphatic filariasis, onchocerciasis, schistosomiasis, and helminthiases
Viruses	Dengue, chikungunya, rabies

The biological diversity of NTDs means that the control or elimination strategies also are very diverse. The availability of new safe and effective drugs for NTDs could provide public health benefit for overall global health; but because these diseases are found primarily in developing countries, existing incentives have been insufficient to encourage development of new drug therapies. While there are plenty of publications focusing on the large health and economic impact on

both the developing and developed world, scientific work dealing with medicinal chemistry aspects of NTDs is less comprehensive. Such work is needed to support the global development programs for discovery and development of new drugs for treatment and prevention or the use of old drugs applying repurposing strategies for tropical disease drug discovery [3]. The importance of supporting such research programs is indicated by the FDA Guidance for Industry, published in 2014 [4]. Thus, to fill this gap, two opinion leaders in this field, David Swinney and Michael Pollastri, accepted our invitation to organize such a volume.

The series editors thank David Swinney and Michael Pollastri for organizing this volume and for working with such excellent authors. Last, but not least, we thank Frank Weinreich and Stefanie Volk from Wiley-VCH for their valuable contributions to this project and to the entire book series.

References

1 Fenwick, A. (2012). The global burden of neglected tropical diseases. *Public Health* 126: 233–236.

2 Molyneux, D.H., Savioli, L., and Engels, D. (2017). Neglected tropical diseases: progress towards addressing the chronic pandemic. *Lancet* 389: 312–325.

3 Klug, D.M., Gelb, M.H., and Pollastri, M.P. (2016). Repurposing strategies for tropical disease drug discovery. *Bioorg. Med. Chem. Lett.* 26: 2569–2576.

4 Guidance for Industry: Neglected Tropical Diseases of the Developing World: Developing Drugs for Treatment or Prevention, *U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER)*, July 2014.

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1

Drug Discovery Strategies for Neglected Tropical Diseases: Repurposing Knowledge, Mechanisms and Therapeutics to Increase Discovery Efficiency

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1.1 Introduction

Neglected tropical diseases (NTDs) are a diverse group of communicable diseases that prevail in tropical and subtropical conditions in 149 countries. These diseases include infections by bacteria, protozoans, helminths, and viruses. Analyses have estimated that NTDs affect more than one billion people and cost developing economies billions of dollars every year [1, 2]. Populations living in poverty, without adequate sanitation, and in close contact with infectious vectors and domestic animals and livestock are those worst affected. We refer readers to the websites of the WHO and CDC for more specifics on the individual diseases [3, 4].

Six of the infections caused by NTDs (dracunculiasis, lymphatic filariasis, onchocerciasis, schistosomiasis, soil-transmitted helminths, and trachoma) can be controlled or even eliminated through mass administration of safe and effective medicines (mass drug administration, MDA), or other, effective interventions. Along with therapeutic interventions, efforts to control the vectors (e.g. mosquitoes, black flies) that transmit these diseases and to improve basic water, sanitation, and hygiene are highly effective strategies against these NTDs [4].

There are still many NTDs that cannot be controlled, due to their mechanism of transmission, or their presence in zoonotic reservoirs, among other reasons. Thus, there is a need for new affordable, effective therapeutics in addition to the plans to control the disease vectors and improve basic water, sanitation, and hygiene.

1.2 First-line Therapies for NTDs and Mechanisms of Action

Most medicines currently used to treat NTDs were discovered many decades ago, despite having limitations (Table 1.1). For example, suramin used for the treatment of human African trypanosomiasis (HAT) was discovered almost 100 years

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Table 1.1 First-line therapies for NTDs and how they were discovered.

First-line	Diseases	Mechanism	Year
Albendazole	Ascariasis, hookworm, echinococcosis, lymphatic filariasis	Tubulin inhibitor	1987
Ivermectin	Lymphatic filariasis, onchocerciasis	Ion channel	1981
Praziquantel	Schistosomiasis, foodborne trematodiasis, Taencasis/cysticerosis	Membrane disruption	1982
Benznidazole	Trichuriasis, chagas	Free radical toxicity	1966
Nifurtimox	Chagas, HAT	Oxidative stress	1970
Pentamidine	HAT	Cross-link DNA	1937
Suramin	HAT	Disrupt energy metabolism	1920
Melarsoprol	HAT	Trypanothione and pyruvate kinase inhibition	1949
Eflornithine	HAT	Ornithine decarboxylase inhibitor	1990
Amphotericin B	Leishmaniasis	Membrane disruption	1953
Miltefosine	Leishmaniasis	Membrane disruption	2002
Rifampicin	Buruli ulcer, leprosy	RNA polymerase	1971
Streptomycin	Buruli ulcer	Protein synthesis inhibition	1943
Dapsone	Leprosy	Dihydropteroate synthase inhibitor	1937
Clofazimine	Leprosy	DNA chelator	1969
Azithromycin	Trachoma, YAWS	Protein synthesis inhibition	1988
Triclabendazole	Foodborne trematodiasis, fascioliasis	Tubulin inhibitor	1989
Niclosamide	Taencasis/cysticerosis	Disrupt energy metabolism	1960s

ago and is still used, albeit a number of newer medicines are now available [5, 6]. Strikingly, most NTD medicines were discovered prior to the 1990s, when molecular biology, molecular genetics, and associated technologies became central to medicine and drug discovery.

The mechanisms of action of these medicines involve disruption of processes essential to an organism's survival. These actions include disruption of microtubules (albendazole, triclabendazole) [7], ion flux (ivermectin) [8], oxidative stress (benznidazole, nifurtimox) [6, 9], disruption of energy production (suramin, niclosamide) [10], inhibition of protein synthesis (streptomycin and azithromycin) [11], inhibition of RNA synthesis (rifampicin) [12], disruption

of membrane integrity (praziquantel [13, 14], amphotericin B [15], miltefosine [16, 17], clofazimine [18]), and inhibition of production of essential metabolites (eflornithine, dapsone) [5, 19].

Most of these functions are not unique to the infectious agents. Selectivity over human homologs is required to achieve a useful safety profile. Differences in binding affinity between the microbe and human homologs provide the selectivity for some (albendazole, ivermectin), but not all, of the medicines. Perhaps, most interesting is that for some of the therapeutics, selectivity is thought to be achieved by the existence of compensatory mechanisms in humans. Greater free radical quenching in human cells versus parasite contribute the selectivity for benznidazole and nifurtimox [15]. Alternative uptake mechanisms for folic acid in hosts contribute to safety of dapsone [19]. Other exploitable differences include compound disposition (e.g. high-affinity uptake systems in trypanosomes by pentamidine) [6], and composition of membranes, which is a key selectivity feature for the function of amphotericin B [15].

1.3 Drug Discovery Efficiency

Drug discovery is an endeavor with very high attrition rates [20]. The high attrition rates are particularly detrimental for drug discovery for NTDs, owing to the disproportionately low research investment in this activity. As such, processes need to be employed to reduce the risk of attrition. Two important aspects relevant to medicinal chemistry are the strategies that provide therapeutic candidates and the critical components to identification and optimization of candidates with a greater chance of success. Drug discovery strategies are first addressed, followed by a discussion of the critical components of the drug discovery process and opportunities for repurposing.

1.3.1 Drug Discovery Process

The process of drug discovery and development is an iterative learn-and-confirm cycle addressing an unmet medical need (Figure 1.1) [21]. The process can be thought of as four stages that require different expertise and tools to define and test the therapeutic hypothesis.

1. Basic research creates new knowledge and **understanding of disease** that leads to tools created for discovery. This phase is most often accomplished in academia and government agencies. Some of the tools important to discovery that are created from basic research include models of disease, clinical relevant biomarkers, predictive phenotypic markers for use in screening assays, as well as potential mechanisms of intervention and drug targets.
2. The aim of the discovery/invention phase is to identify a potential therapeutic and its corresponding mechanism of action to be tested in patients. The strategies used for discovery, including assay formats and endpoints, are informed by the knowledge and tools created in basic research (discussed in Section 1.3.2). The invention phase has historically been the domain of the

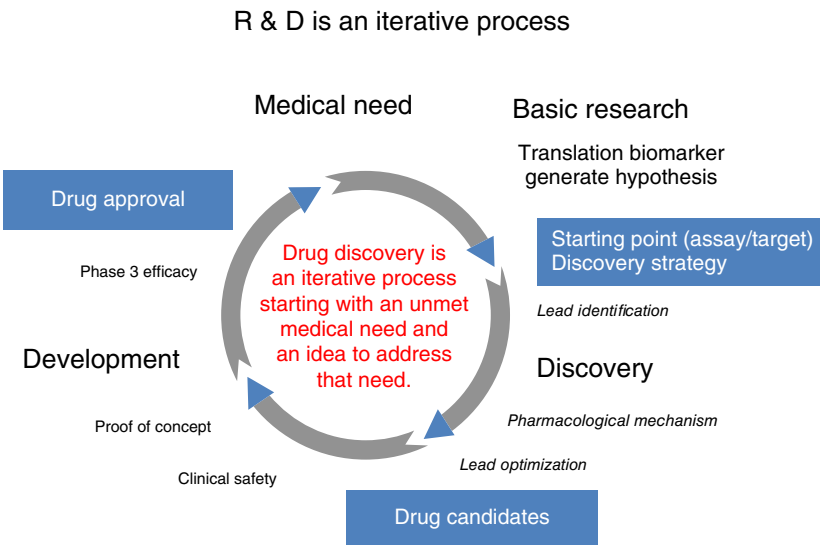


Figure 1.1 Drug discovery and development cycle. The process of drug discovery can be thought of as an iterative learn-and-confirm cycle with specific milestones. The process of discovery and development of a new medicine is initiated in response to an unmet medical need to treat a disease. Physiological, genetic, and chemical knowledge provides an understanding of the disease. This knowledge will lead to the identification of translation biomarkers and assays to enable discovery and invention of new medicines. These molecules will then be optimized for biopharmaceutic properties and safety to provide a drug candidate. At this point, the process of drug discovery is complete and the molecule should succeed or fail based on its own merit. Opportunities to improve efficiency in drug discovery will increase the probability that clinical candidates will make it to registration. The left-hand side of the circle (from 6 to 12 o'clock) is the development phase of drug discovery, which involves testing for safety and efficacy in humans leading to registration. Multiple iterations are generally required before a medicine with sufficient efficacy at a safe dose is discovered, tested in humans, and registered.

pharmaceutical and biotech industries, although academic institutions are now frequently inventing new medicines. The invention is typically identified by evaluation of potential drugs in biological assays that measure a response related to the clinical outcome. The modalities evaluated can be of organic chemical, biological, and genetic material prepared synthetically or isolated from natural substances (e.g. natural products). The modalities for NTDs are all chemical in nature. Part of the reason for this is that the cost and stabilities of biological and genetic therapeutics are prohibitive for NTDs.

The active modality and its corresponding mechanism of action provide the **therapeutic hypothesis** that will be tested in patients. For NTDs, the therapeutic hypothesis will be that the molecule will kill the infectious organism and reduce morbidity and/or mortality. The mechanism may not be known until long after the drug is approved, or it may be never known. For example, the mechanism of action of acetaminophen is still not known. The mechanisms of action of most drugs for NTDs were determined long after the drugs were invented.

3. In order to test the therapeutic hypothesis in the clinic, the active modality must be tolerable and have suitable drug-like properties including pharmacokinetics and pharmaceutical to provide sufficient drug concentrations to achieve the response. The **optimization** phase can be facilitated by knowledge of the mechanism of action, but this knowledge is not mandatory. The optimization phase is considered the "Valley of Death" due to the high attrition rate. It is resource-intensive and typically conducted in the pharmaceutical industry, although there are now academic and government centers conducting this work. The optimization phase produces a clinical candidate that can then be used to test the therapeutic hypothesis in the clinic.
4. The central feature of the therapeutic hypothesis is predicting a dose–response relationship between mechanism of action and efficacy (or toxicity) in humans [22]. Clinical studies are designed to test a specific molecule for its therapeutic usefulness.

Multiple iterations of learn-and-confirm hypothesis testing are usually required to identify first-in-class medicines. This long-term investment is not feasible for NTDs; drug discovery for these diseases must be more successful, with fewer iterations and fewer failures.

1.3.2 Drug Discovery Strategies

The knowledge available from basic research will inform the drug discovery strategy. Important aspects of the knowledge that impact the drug discovery strategies are knowledge of mechanisms of action and targets, availability of robust phenotypic assays, and structures of active compounds [23, 24].

Medicinal chemistry-dependent drug discovery strategies are commonly differentiated into empirical strategies now known as phenotypic drug discovery (PDD) and hypothesis-driven strategies now commonly described as target-based drug discovery (TDD) [25].

Phenotypic assays measure a phenotype in a physiological system. The term "phenotypic assay" includes all preclinical assay formats that use physiological systems, e.g. animals, cells, and biochemical pathways [24, 26]. Phenotypic assays make few assumptions as to the molecular details of how the system works, provide an empirical method to probe effects in physiological systems, and are mechanistic agnostic. Therapeutics are identified by the effect upon a phenotype and, subsequently, the therapeutics are used to identify the mechanism of action. The identification of active therapeutics is accomplished through empirical trial and error, verifiable by observation rather than by theory. The therapeutics are identified in which disease-relevant phenotypes provide a chain of translation between the observation and clinical response [27, 28]. The phenotype most relevant to NTD is reduction in proliferation and death of the organism.

Empirical, phenotypic assays have always played an important role in drug discovery for NTDs [29, 30]. In his Nobel lecture entitled "Selective inhibitors of dihydrofolate reductase," George H Hitchings Jr. stated, "Those early, untargeted studies led to the development of useful drugs for a wide variety of diseases and has justified our belief that this approach to drug discovery is more fruitful than narrow targeting" [29].

In the last decades of the twentieth century, the emphasis of drug discovery changed to a more reductionist, target-based approach, and phenotypic assays were primarily used to confirm efficacy and evaluate safety. The drug target is a gene product that provides a mechanistic hypothesis to focus discovery research to identify a therapeutic that modulates the protein's activity [31]. A target can be validated with many technologies, including genetics [22]. Molecular technologies such as X-ray structure and computational chemistry are tools that help medicinal chemists in the rational design and optimization of molecules that bind to the target [32]. The central features of TDD are (i) identification and validation of a drug target, (ii) identification of a molecule that binds to that target, (iii) optimization of the selectivity over anti-targets, and (iv) optimization of the biopharmaceutic properties such that the drug concentrations in the body are sufficient to ensure that the drug is bound to the target throughout the dosing interval. This target-based paradigm has been envisioned to provide a more rational approach to drug discovery, analogous to a design and engineering approach [23, 32].

Most medicines for NTDs were discovered decades ago using empirical strategies (PDD) involving testing the ability of compounds to kill the infectious organisms (Table 1.1), essentially agnostic to the mechanism of action. Some of the key components of PDD success are the robustness of the assays and the composition of the screening libraries, both of which are addressed in more detail later [27].

Not all NTDs were discovered via phenotypic screening. Eflornithine was discovered on the basis of the hypothesis that an ornithine decarboxylase (ODC) inhibitor would be efficacious for HAT. Eflornithine is an irreversible inhibitor originally developed for cancer and repurposed for HAT. It was not efficacious for cancer due to the fast resynthesis of the ODC enzyme. Differential activity in the parasite was achieved due to much slower enzyme resynthesis in the trypanosome parasite [5].

1.3.3 PDD versus TDD for NTDs

As already noted, historically, PDD has provided most of the medicines for NTDs. A likely contributor to this success is the feasibility of assays measuring viability of parasites, worms, and bacteria, termed the chain of translatability [27]. The translatability of the microbe viability as a phenotypic measure of infectious disease pathology is very strong. This contrasts with the more uncertain translatability that modulation of a new target will provide selective cytotoxicity.

In general, the choice between a phenotypic (PDD) versus target-based strategy (TDD) for medicinal chemistry-dependent, first-in-class drug discovery is strongly influenced by the robustness, feasibility, and translatability that a phenotype will predict clinical efficacy (its chain of translatability) versus the predictability that a drug target and corresponding molecular mechanism will provide efficacy and selectivity. Molecular mechanisms of small molecules interacting with a target to provide sufficient efficacy and safety are more complex than simple binding. They involve conformational changes, kinetics, and are dependent on physiological context. This was the conclusion of an analysis of first-in-class medicines across all disease areas showing that the majority

of medicinal chemistry-driven medicines were discovered with phenotypic screening [25]. The molecular mechanisms are very difficult to predict and incorporate into reductionist assay formats [33, 34]. It was also noted that TDD was more successful for followers, presumably because the mechanism of action had already been validated [25].

An aspect of discovery strategies for NTDs that is rarely appreciated is that the selectivity of drugs was identified in many cases as a consequence of the empirical nature of the strategy. Differences in binding affinity between the microbe and human target determined the selectivity for some but not all of the medicines. As noted earlier, the selectivity is thought to be achieved by other mechanisms including compensatory mechanisms in humans (e.g. greater free radical quenching [6], alternative uptake mechanism for folic acid [19]), compound disposition (high-affinity uptake systems in parasites) [6], and composition of membranes (amphotericin B) [15].

1.4 Critical Components for Successful Drug Discovery

1.4.1 Finding a Starting Point

Identification of suitable chemical matter for optimization is paramount. In tropical medicine drug discovery, both phenotypic and target-based screens have been applied to a number of small-molecule chemical libraries, including FDA drug libraries [35] and natural products [36], as well as collections arising from industry [37] or product development partnerships such as the MMV, which has released the Malaria Box [38] and Pathogen Box [39], each of which contains 400 Lipinski-compliant chemistries with validated antiparasitic activities. In addition, repurposing of established drugs or preclinical chemotypes that inhibit homologous function in other eukaryotic systems can be a fruitful approach.

1.4.2 Assays Robustness and Hit Selection Criteria

As with drug discovery programs for any other indication, it is essential that screening assays are sufficiently robust and reproducible, and of reasonable throughput, to drive chemical optimization. Assays must have sufficient sensitivity to reproducibly identify modifications that affect a compound's activity, and it would be highly desirable to utilize orthogonal assays that measure the same biological endpoint as the primary assay but utilize a different readout. This can help avoid false-positive results that arise due to assay artefacts.

When selecting and defining a compound hit, different disease indications will have different requirements, overall. However, all programs share the same essential criteria: (i) sufficient potency against the target or pathogen, with some indication of a potential selectivity window. (ii) A hit compound is preferably a member of a series of structurally similar compounds that display differences in activity across 2–3 orders of magnitude. (iii) An assessment of compound ADME properties; while such properties are typically measured, computed

properties can also provide useful insights. (iv) A hit series would contain at least several compounds that meet at least some of the desired criteria measured or computed earlier. This will provide high confidence that the chemical series will be a tractable substrate for medicinal chemistry optimization.

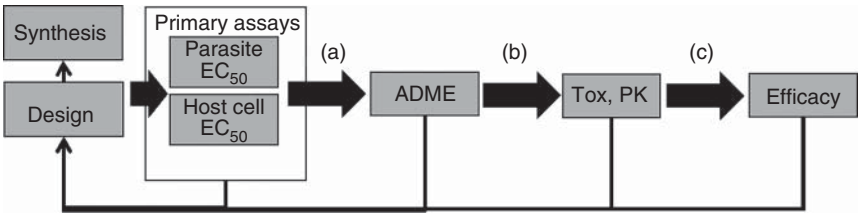
It is essential that all hit compounds are assessed against other metrics of tractability. For example, the employment of a Pan-Assay Interfering compound (PAINs) assessment can identify potentially promiscuous chemotypes that, while appearing to be strong optimization starting points, are artefactual findings. Similarly, any active compounds identified in a screening campaign should be carefully assessed for features that are generally undesirable in a hit compound. These would include highly electrophilic moieties (alkyl halides, aldehydes), hydrolysable features (such as esters or acetals), or any other sort of chemically unstable moiety. Lastly, substructure searches using freely available databases such as PubChem or ChEMBL can often uncover potentially promiscuous or toxic moieties to help inform compound series selection.

It is highly desirable to pursue a chemotype that is readily pursued by organic synthesis (often described as "parallel-enabled"). In particular, the ability to easily and rapidly prepare analogs simultaneously is a major benefit to the speed of an optimization program, and it also allows exploration of a diverse chemical space. While many drugs do indeed trace their roots back to natural products [25], challenges in chemical synthesis of natural product analogs can frequently frustrate analog synthesis while searching for new compounds with appropriate properties.

1.4.3 Optimization Processes

Any successful chemical drug discovery program has, at its center, a well-informed medicinal chemistry effort. Noting that target product profiles for new drugs for many NTDs have been described [40–42], optimization programs must design and employ a series of assays that ensure direction toward the desired endpoint. Rather than an exclusive focus on antiparasitic potency and selectivity, it is critical to include considerations of absorption, distribution, metabolism, and excretion (ADME) properties, pharmacokinetics and pharmacodynamics, and selectivity against important anti-targets, such as hERG.

A project team should design an assay cascade that is fit-to-purpose, both in terms of measuring desired endpoints, as well as in maximizing efficient use of resource (which is frequently limited in NTD drug discovery). An example assay cascade is shown in Figure 1.2, which would lead to a compound that is <100 nM in potency, >100× selective over host cells, with adequate solubility and ADME properties and animal pharmacokinetic exposure, that can be tested in an *in vivo* efficacy experiment. Note that transition to each step of the cascade has defined property cutoffs, in terms of potency and properties. Depending on the goals on a given project, this diagram could be modified to include aspects such as screens in a panel of anti-targets (ion channels, G protein-coupled receptors (GPCRs), kinases, etc.), hERG, or other endpoints that are central to optimization.



(a) $EC_{50} \leq 100$ nM; $TC_{50}/EC_{50} \geq 100$
(b) Aq. sol ≥ 100 μM, low microsomal clearance
(c) Plasma exposure 10x EC_{50}, $t1/2 \geq 3$ h

Figure 1.2 Example project optimization cascade.

1.5 Repurposing Knowledge Mechanisms and Therapeutics

The process of *de novo* drug discovery can be too resource expensive for NTDs. Opportunities to address this deficiency come from repurposing molecules and mechanisms. Repurposing is not a new concept for NTDs. Many of the currently used medicines were repurposed. For example, the benzimidazoles were originally developed as plant fungicides and later as veterinary anthelmintics [43]. The first benzimidazole to be developed and licensed for human use was thiabendazole in 1962. Although thiabendazole was very effective, it was also moderately toxic, which led to enormous efforts by animal health companies to find better and safer compounds. This led to the benzimidazole carbamates, such as mebendazole, flubendazole, oxfendazole, albendazole, and oxibendazole. Subsequently, several veterinary anthelmintics were developed and marketed, including parbendazole, fenbendazole, oxfendazole, and cambendazole. The first benzimidazole carbamate to make it into humans was mebendazole, followed by flubendazole (both Janssen products).

More recent examples of repurposing mechanisms include eflornithine. As noted earlier, eflornithine was discovered on the basis of the hypothesis that an ODC inhibitor would be efficacious for HAT [5]. Eflornithine is an irreversible inhibitor originally developed for cancer and repurposed for HAT and is one of the few therapeutics discovered with TDD.

There is growing optimism in the NTD community that more drugs will become available through repurposing. In 2018, moxidectin was approved by the U.S. FDA for onchocerciasis. Moxidectin, a macrocyclic lactone, was repurposed from animals and clinical studies showed superiority to ivermectin [44]. Fexinidazole, originally developed in the 1980s, was rediscovered in 2005 by DNDi researchers looking for possible antiparasitic compounds. In late 2018, an EMA scientific committee announced its "positive opinion" for fexinidazole, opening the way for individual countries to approve its use in HAT, with the first patients to receive the drug by mid-2019.

Repurposing, known as exaptation, has been an effective source of discovery and invention across many industries. The most obvious and exploited approach for NTDs is to identify molecules that have been developed for another disease

or industry (agriculture, animal health) and then test those against the specific NTDs. This practice is ongoing and the major source of leads for the NTDs, as noted in the chapters on specific NTDs in this book. This is often pursued using a phenotypic approach. A significant bottleneck for this approach is acquiring the funding and infrastructure to test the new hypothesis in the clinic. Organizations such as DND*i*, BMGF, and Welcome Trust provide mechanisms to evaluate promising compounds. However, as noted earlier, the limited resources only allow for the most promising candidates to be evaluated. Sources of candidates for repurposing include selective cytotoxic agents from other infectious diseases including malaria, TB, antibiotics, and HIV. The anticancer pharmacopeia can also provide a source of compounds. Indeed, many compound libraries have been already been evaluated against NTDs, and it is important to follow the criteria described in Section 1.4 to ensure that the actives have sufficient properties to warrant further investment.

In many cases, these evaluations are mechanistically agnostic. A collection of compounds is screened for viability against the pathogenic microbe. The increasing availability of genomic and mechanistic knowledge and bioinformatic and computational biology tools provide opportunities to focus the screening around specific mechanisms and target classes. For example, two species may have homologous essential enzymes such as a MAP kinase or a protease. Therefore, a compound library identified in one species can be used to identify leads in another to provide a starting point for medicinal chemistry optimization.

1.6 Summary

In summary, the expansion of NTD drug discovery, and the progress made to date are encouraging. New programs in this area are bolstered by sophisticated assay technologies, deep understanding of the infectious agent's biology, modern, metric-driven medicinal chemistry campaigns, and excellent disease models in animals. However, because of the resource cost of drug discovery and lack of available resources for NTDs, repurposing compounds and mechanisms provides the best opportunities for new medicines.

References

1 Lee, B.Y., Bartsch, S.M., and Gorham, K.M. (2015). Economic and financial evaluation of neglected tropical diseases. *Adv. Parasitol.* 87: 329–417.
2 Herricks, J.R., Hotez, P.J., Wanga, V. et al. (2017). The global burden of disease study 2013: what does it mean for the NTDs? *PLoS Negl.Trop. Dis.* 11 (8): e0005424.
3 WHO (2018). WHO neglected diseases. https://www.who.int/neglected_diseases/en/.
4 CDC (2018). CDC neglected diseases. https://www.cdc.gov/globalhealth/ntd/index.html.

5 Steverding, D. (2010). The development of drugs for treatment of sleeping sickness: a historical review. *Parasit. Vectors* 3 (1): 15.

6 Gutteridge, W.E. (1985). Existing chemotherapy and its limitations. *Br. Med. Bull.* 41 (2): 162–168.

7 Lacey, E. (1988). The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *Int. J. Parasitol.* 18 (7): 885–936.

8 Arena, J.P., Liu, K.K., Paress, P.S. et al. (1995). The mechanism of action of avermectins in Caenorhabditis elegans: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. *J. Parasitol.* 81 (2): 286–294.

9 Hall, B.S., Bot, C., and Wilkinson, S.R. (2011). Nifurtimox activation by trypanosomal type I nitroreductases generates cytotoxic nitrile metabolites. *J. Biol. Chem.* 286 (15): 13088–13095.

10 Scheibel, L.W., Saz, H.J., and Bueding, E. (1968). The anaerobic incorporation of 32P into adenosine triphosphate by *Hymenolepis diminuta*. *J. Biol. Chem.* 243 (9): 2229–2235.

11 Parnham, M.J., Erakovic Haber, V., Giamarellos-Bourboulis, E.J. et al. (2014). Azithromycin: mechanisms of action and their relevance for clinical applications. *Pharmacol. Ther.* 143 (2): 225–245.

12 Lancini, G., Pallanza, R., and Silvestri, L.G. (1969). Relationships between bactericidal effect and inhibition of ribonucleic acid nucleotidyltransferase by rifampicin in *Escherichia coli* K-12. *J. Bacteriol.* 97 (2): 761–768.

13 Cupit, P.M. and Cunningham, C. (2015). What is the mechanism of action of praziquantel and how might resistance strike? *Future Med. Chem.* 7 (6): 701–705.

14 Thomas, C.M. and Timson, D.J. (2018). The mechanism of action of praziquantel: six hypotheses. *Curr. Top. Med. Chem.* 18 (18): 1575–1584.

15 Kaminski, D.M. (2014). Recent progress in the study of the interactions of amphotericin B with cholesterol and ergosterol in lipid environments. *Eur. Biophys. J.* 43 (10-11): 453–467.

16 Pinto-Martinez, A.K., Rodriguez-Duran, J., Serrano-Martin, X. et al. (2018). Mechanism of action of miltefosine on *Leishmania donovani* involves the impairment of acidocalcisome function and the activation of the sphingosine-dependent plasma membrane Ca(2+) channel. *Antimicrob. Agents Chemother.* (1): 62.

17 Croft, S.L. and Engel, J. (2006). Miltefosine-discovery of the antileishmanial activity of phospholipid derivatives. *Trans. R. Soc. Trop. Med. Hyg.* 100 (Suppl. 1): S4–S8.

18 Cholo, M.C., Steel, H.C., Fourie, P.B. et al. (2012). Clofazimine: current status and future prospects. *J. Antimicrob. Chemother.* 67 (2): 290–298.

19 Brown, G.M. (1971). The biosynthesis of pteridines. *Adv. Enzymol. Relat. Areas Mol. Biol.* 35: 35–77.

20 Paul, S.M., Mytelka, D.S., Dunwiddie, C.T. et al. (2010). How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat. Rev. Drug Discovery* 9 (3): 203–214.

21 Sheiner, L.B. (1997). Learning versus confirming in clinical drug development. *Clin. Pharmacol. Ther.* 61 (3): 275–291.

22 Plenge, R.M., Scolnick, E.M., and Altshuler, D. (2013). Validating therapeutic targets through human genetics. *Nat. Rev. Drug Discovery* 12 (8): 581–594.

23 Lindsay, M.A. (2003). Target discovery. *Nat. Rev. Drug Discovery* 2 (10): 831–838.

24 Lee, J.A., Uhlik, M.T., Moxham, C.M. et al. (2012). Modern phenotypic drug discovery is a viable, neoclassic pharma strategy. *J. Med. Chem.* 55 (10): 4527–4538.

25 Swinney, D.C. and Anthony, J. (2011). How were new medicines discovered? *Nat. Rev. Drug Discovery* 10 (7): 507–519.

26 Lee, J.A. and Berg, E.L. (2013). Neoclassic drug discovery: the case for lead generation using phenotypic and functional approaches. *J. Biomol. Screening* 18 (10): 1143–1155.

27 Moffat, J.G., Vincent, F., Lee, J.A. et al. (2017). Opportunities and challenges in phenotypic drug discovery: an industry perspective. *Nat. Rev. Drug Discovery* 16 (8): 531–543.

28 Swinney, D.C. (2014). The value of translational biomarkers to phenotypic assays. *Front. Pharmacol.* 5: 171.

29 Hitchings, G.H. Jr. (1989). Nobel lecture in physiology or medicine–1988. Selective inhibitors of dihydrofolate reductase. *In Vitro Cell Dev. Biol.* 25: 303–310.

30 Williams, M. (2005). Systems and integrative biology as alternative guises for pharmacology: prime time for an iPharm concept? *Biochem. Pharmacol.* 70 (12): 1707–1716.

31 Simon, G.M., Niphakis, M.J., and Cravatt, B.F. (2013). Determining target engagement in living systems. *Nat. Chem. Biol.* 9 (4): 200–205.

32 Hopkins, A.L. (2008). Network pharmacology: the next paradigm in drug discovery. *Nat. Chem. Biol.* 4 (11): 682–690.

33 Swinney, D.C. (2015). Drug discoveries and molecular mechanism of action. In: *Successful Drug Discovery* (ed. J.F.A.D.P. Rotella), 12–34. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co.

34 Swinney, D.C. (2013). Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clin. Pharmacol. Ther.* 93 (4): 299–301.

35 Abdulla, M.H., Ruelas, D.S., Wolff, B. et al. (2009). Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening. *PLoS Negl.Trop. Dis.* 3 (7): e478.

36 Spivak, A.Y., Keiser, J., Vargas, M. et al. (2014). Synthesis and activity of new triphenylphosphonium derivatives of betulin and betulinic acid against Schistosoma mansoni in vitro and in vivo. *Bioorg. Med. Chem.* 22 (21): 6297–6304.

37 Long, T., Rojo-Arreola, L., Shi, D. et al. (2017). Phenotypic, chemical and functional characterization of cyclic nucleotide phosphodiesterase 4 (PDE4) as a potential anthelmintic drug target. *PLoS Negl.Trop. Dis.* 11 (7): e0005680.

38 Li, S., Schmitz, K.R., Jeffrey, P.D. et al. (2005). Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* 7 (4): 301–311.

39 Perzborn, E., Roehrig, S., Straub, A. et al. (2011). The discovery and development of rivaroxaban, an oral, direct factor Xa inhibitor. *Nat. Rev. Drug Discovery* 10 (1): 61–75.

40 Nwaka, S., Ramirez, B., Brun, R. et al. (2009). Advancing drug innovation for neglected diseases-criteria for lead progression. *PLoS Negl.Trop. Dis.* 3 (8): e440.

41 Holenz and Stoy (2018). Advances in Lead Generation. *Bioorg. Med. Chem. Lett.* (in press).

42 DNDi (2018). Target Product Profiles (TPP). https://www.dndi.org/diseases-projects/target-product-profiles/ (accessed 28 February 2019).

43 Lacey, E. (1990). Mode of action of benzimidazoles. *Parasitol. Today* 6 (4): 112–115.

44 Iten, M., Mett, H., Evans, A. et al. (1997). Alterations in ornithine decarboxylase characteristics account for tolerance of Trypanosoma brucei rhodesiense to D,L-alpha-difluoromethylornithine. *Antimicrob. Agents Chemother.* 41 (9): 1922–1925.

Part I

Virus

2

Toward Antiviral Therapies for the Treatment of Zika Virus Infection: Lessons Learned from Dengue Virus

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2.1 Zika Virus: History and Epidemiology

First identified in 1947 in Entebbe, Uganda, the eponymous Zika virus (ZIKV) emerged from the Zika forest decades before the World Health Organization deemed it a global health emergency. The virus was initially isolated from an ailing sentinel rhesus monkey, number 766, placed in the forest by scientists from the Yellow Fever Virus Research Institute. The monkey was one of many intended to function as a host for the local mosquitos known to be vectors for the hemorrhagic fevers the researchers were studying [1]. Serum from the febrile animal was used to inoculate mice and within two weeks the mice were neurotrophically sick. Analysis of the brains of infected mice resulted in isolation of the transmissible agent, referred to as the ZIKV 766 strain and classified as a member of the genus *Flavivirus*, family *Flaviviridae* [2]. The *Flaviviridae* family also includes the viruses known to cause dengue fever, hepatitis C, Japanese encephalitis, West Nile encephalitis, and yellow fever. Within a year of the discovery of the virus in the sentinel monkey, ZIKV was isolated from trapped *Aedes africanus* mosquitoes from the same forest, hinting at its potential link as an arbovirus and the mosquito as the vector [1]. Postulating human infection, even though none was identified in the local population, analysis of the serum of residents of this Ugandan area revealed ~6% seroprevalence of antibodies against ZIKV [3]. ZIKV was present, but the manifestations of the infection were not yet apparent. It was not until 1952 in Uganda and 1953 in Nigeria that three of the first human illnesses attributed to ZIKV were confirmed. These patients presented with only a mild malaise, fever, and headache, which was a noteworthy finding for an illness that would later be confirmed to be in 40% of the Nigerian population [4, 5]. For the next 50+ years ZIKV, thought to be endemic only to Africa, remained relatively obscure while it silently spread around the globe.

Periodic cases of ZIKV infection were reported from confirmed serological samples just over a dozen times after the infections in 1953 [5]. Speculation about those cases is that dengue fever and infection by an alphavirus called

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Chikungunya may have been misdiagnosed as ZIKV infection since their clinical landscapes are so similar. Since clinicians at the time may have been unaware of ZIKV, and laboratory tests to identify it were not yet available [6], ZIKV was easily overlooked in the clinical setting and unhindered by surveillance and vector management regimes. Until the mid-1980s, cases of serologically confirmed ZIKV infection were limited to the African nations where it was initially identified, as well as Tanzania, Egypt, Central African Republic, Gabon, Sierra Leone, Senegal, and Cote d'Ivoire [7–12]. Asian nations were also affected with ZIKV as it was confirmed in Malaysia, Thailand, Vietnam, Philippines, Indonesia, Pakistan, and India [13–18].

Most of the viral samples were isolated from mosquitos, mainly from the *Aedes* genus. ZIKV was isolated from *Aedes aegypti*, *Aedes albopictus, Aedes lutecephalus, Aedes furcifer, Aedes apicoargenteus, Aedes hensilli,* and *Aedes taylori* [19–23]. These isolations confirmed the sylvatic transmission cycle between nonhuman primates and mosquitoes [24]. Humans then entered the transmission cycle, becoming an alternate host and thus allowing an uninterrupted human-mosquito-human progression.

This logic of transmission held true as the first major ZIKV outbreak occurred in 2007 on Yap Island in the Federated States of Micronesia, where monkeys are absent. The illness infected almost 75% of the island's inhabitants, causing rash, conjunctivitis, and joint pain, but, strikingly, no hospitalizations or deaths were reported [25]. Duffy et al. hypothesized that the *Aedes hensilli* mosquito was the likely transmission vector. At this point, the bigger picture began to emerge as it was recognized that widespread air travel and plentiful mosquito vectors could spread ZIKV throughout Oceania and potentially worldwide. This is exactly what occurred approximately 12 months later and 10 000 miles away when a US scientist studying mosquitoes in Senegal returned home to Colorado and fell ill within a week. Over the next month, the man experienced rash, fatigue, prostatic symptoms, and headaches. After one month, his wife presented with similar signs of rash, chills, headache, and joint pain, providing the first documented case of sexual transmission, or non-vector-borne transmission, of a virus known previously to be transmitted only by mosquitoes [26].

The rapid spread of ZIKV and the advancement of the understanding of its individuality opened the door for its phylogenetic analysis. It was soon discovered that more than one strain of ZIKV existed and isolation of these strains revealed distinct ZIKV lineages as well [25]. Isolated from the rhesus macaque in Uganda, strain MR-766 is the ancestor of the IbH 30656 strain from Nigeria in 1964 and the ArD 41519 strain from Senegal in 1984. These represent the African lineages. Divergence occurred to the Asian lineage with P6-740 from Malaysia in 1966, EC Yap from Micronesia in 2007, and, most recently, FS13025, a strain from Cambodia, in 2010 [6].

In hindsight, the Yap Islands outbreak signaled the onset of a global pandemic of a newly emerging virus as it spread to French Polynesia and then to the Americas. French Polynesia comprises 118 individual islands over 1600 mi^2 in the South Pacific Ocean. These 118 islands are divided into five subgroups of islands, only 67 of which are inhabited. The islands form part of a larger complex of countries including Micronesia and Melanesia, collectively referred to as Oceania.

An outbreak of ZIKV began in late 2013 in French Polynesia as clinicians saw an uptick in patients reporting dengue-like syndrome and rashes. Five months after the initial reports, over 30 000 cases of ZIKV had occurred, affecting 11.5% of the population [27]. The French Polynesian experience was mirrored by concurrent outbreaks in New Caledonia (1400 cases), Cook Islands (932 cases), and finally Easter Island (173 cases) [28]. Analysis of the ZIKV strains circulating during these outbreaks showed that the strain in French Polynesia was more closely associated with the FS13025 strain from Cambodia than the EC Yap strain [29] linking it to the Asian ZIKV lineage.

The outbreak in Oceania was important because it served to further elucidate the damaging characteristics of ZIKV. For example, a study conducted in French Polynesia during the ZIKV outbreak indicated that almost 3% of blood donors tested positive for the virus, raising the risk of transmission through blood transfusion [30]. In parallel, ZIKV was isolated from the semen of a man in French Polynesia seeking treatment for bloody seminal fluid, providing further evidence that ZIKV could be sexually transmitted [31]. Furthermore, in addition to the array of usual dengue-like symptoms, increases in neurological and autoimmune difficulties were reported [27]. French Polynesia health officials logged 74 cases among the reported ZIKV patients with complications due to neurological infection, 42 cases of Guillain–Barré syndrome (GBS), and just over two dozen more cases of encephalitis, meningoencephalitis, paresthesia, or facial paralysis [32, 33]. Lastly, an increased number of fetuses and infants in French Polynesia from 2014 to 2015 were reported with central nervous system, brainstem, and cerebral malformations, which included microcephaly [34, 35].

The presence of ZIKV on Easter Island marked its definitive migration to the Americas. However, awareness of the epidemic by the general public spiked only after it exploded in Brazil. Reports of an exanthematous illness consisting of joint pain, conjunctivitis, rash, and fever began surfacing in Natal, the state of Rio Grande do Norte, Brazil, in March 2015. Suspecting dengue or chikungunya, researchers did not initially screen for ZIKV. As the illness spread to other northeastern states in Brazil, serum samples from ailing patients were analyzed, and in May 2015, Brazil's National Reference Laboratory confirmed the presence of ZIKV [36, 37]. The Pan American Health Organization (PAHO) issued an immediate epidemiological alert regarding the risk of ZIKV spreading throughout the Americas. PAHO emphasized that due to the extensive distribution of the *Aedes* mosquito, coupled with continual travel by people throughout the region, surveillance, detection, and vector control would be critical for impeding the epidemic [38]. As of December 2015, Brazil's Ministry of Health estimated 440 000–1 300 000 cases of suspected ZIKV infection [38]. Simultaneous to the Brazilian outbreak, ZIKV was hopscotching through the Americas with its presence confirmed in Colombia, El Salvador, Guatemala, Mexico, Paraguay, and Venezuela [38]. Viral sequencing showed >90% nucleotide identity with the French Polynesian strain [39].

Also around the close of 2015, Brazil's Ministry of Health reported an unusual rise in cases of microcephaly in the northeastern states of Pernambuco, Paraiba, and Rio Grande do Norte. In Pernambuco, microcephaly had previously been reported at an annual rate of ~10 cases, but the number of recent cases had

reached 141, which led PAHO to issue another epidemiological alert regarding microcephaly risks associated with ZIKV [40]. In January 2016, Brazilian authorities reported that the incidence of ZIKV, as monitored over the past five years, had risen from 163 to 3530 cases per year, or from 5.6 to 121.7 cases per 100 000 live births. Gravely, this also included 46 deaths [41].

The World Health Organization declared ZIKV to be a worldwide public health crisis in February 2016 [42]. Unobstructed global travel and conduits for commerce potentiated the swift proliferation of vector mosquitoes and infected humans. After its explosion in Brazil, the ZIKV migration continued and eventually reached the United States. As of 22 March 2017, the Centers for Disease Control and Prevention had substantiated 5158 cases of ZIKV in the United States, and a total of 38 212 in US Territories. Of the cases within the United States, 4861 patients acquired the virus while traveling to other affected countries, 222 patients (216 in Florida and 6 in Texas) acquired the virus from mosquito transmission while in the United States, and the remaining 75 cases were attributed to sexual transmission, congenital infection, laboratory transmission, or an unknown path. The worldwide spread of ZIKV infection was nearly complete, and thus intensified the efforts for global surveillance, prevention, and vector mediation to occur.

2.2 Detection, Clinical Presentation, and Medical Need

Both the clinical presentation of ZIKV infection and its associated medical need have changed dramatically over the past decade as the understanding about the effects of ZIKV has increased. This has been aided by the fact that ZKV was initially most likely misdiagnosed for dengue virus, or another *Flaviviridae* circulating in the areas of its transmission. The current procedures for an accurate diagnosis of ZIKV require a polymerase chain reaction (PCR) test. Rapid antibody-based diagnostic tests are not considered sufficiently specific because ZIKV antibodies cross-react with those of other circulating flavivirus antibodies [43]. Until the 2007 French Polynesian outbreak, ZIKV caused few, if any, hospitalizations. However, during and subsequent to that outbreak, neurological problems associated with ZIKV infection were reported with increasing frequency as it spread.

Symptoms of ZIKV infection in the human population consist of rash (exanthema), occasional fever, headache, joint pain (arthralgia), conjunctivitis, and muscle pain (myalgia) [44]. The symptoms are generally reported as mild by those affected and persist for a few days to a few weeks. It has been proposed that exposure of the African and Asian populations to ZIKV led to immunity in young children in these regions. Conversely, in the Americas where the populations are immunologically unprotected, ZIKV has evolved to become more genomically stable, allowing successful transmission via sexual contact, and firmly establishing the human as its host [45]. The human hosts in greatest danger from ZIKV infection are the pregnant woman and her fetus. Once a pregnant woman is infected with ZIKV, the virus can be transmitted to the fetus. In light of intrauterine transmission and the frequency of reported neurological

effects, a disease previously considered mild has transformed to a public health crisis. An infected fetus may suffer a multitude of abnormalities comprising microcephaly, lack of brain-fold advancement (lissencephaly), thickening or convolution of the scalp, rigidity of muscles (hypertonia), overactive nervous system or autonomic dysreflexia, disproportionate facial development, basal ganglia calcifications affecting movement, as well as cardiac, digestive, genital, and urinary insufficiencies [46–48].

Pregnant women and their fetuses are not the only group at risk. A 20-fold increase in cases of GBS occurred during the 2007 ZIKV outbreak in French Polynesia [32]. The general age range of those affected was 35–57 years, with both sexes being equally at risk. The clinical features of this syndrome are limb weakness, facial palsy, unsteady gait, and difficulty swallowing and breathing. In severe cases, patients are admitted to the intensive care unit and require mechanical ventilation; some cases result in death [49]. Subsequent reviews of the GBS cases reported during that outbreak have revealed a causative association between the ZIKV outbreak and GBS [50].

A confounding factor to ZIKV management is the presence of dengue virus in the areas where ZIKV is spreading. It has recently been confirmed that hosts who harbor immunity to dengue virus may see an increase in not only infection rates of ZIKV but in severity of symptoms as well [51]. The repercussions of ZIKV infection, coupled with the absence of approved therapies or vaccines, indicate a substantial unmet medical need. The severity and permanence of the developmental abnormalities associated with *in utero* ZIKV infection will likely strain the medical and public health communities for an unknown number of years. A first line of defense in the efforts to curb the impacts of ZIKV is to provide prevention education to the public. Second lines of defense include vector control and limiting person-to-person transmission via harm reduction education. On the pharmaceutical front, drugs that are safe for use in pregnant women will be mandatory. In addition, work will be needed to explore how ZIKV infects different neuronal cell populations and glands, and develop novel therapeutic and prophylactic drugs to combat it. This will be in conjunction with vaccine development.

2.3 ZIKV Replication Cycle

The replication of the ZIKV follows the same succession as that of the other flaviviruses. The viral particle is a relatively small structure, ~50 nm in diameter, and can be categorized as a positive-sense single-stranded RNA (ssRNA) virus [1]. Each particle is made up of a viral genome tucked inside a capsid (C) protein surrounded by a lipid membrane punctuated with envelope (E) protein dimers in association with membrane (prM) proteins (Figure 2.1).

The virus attaches to a target cell in the host via an interaction between its envelope proteins and specific host cell receptors (Figure 2.2). Attachment facilitates engulfment of the virus into the cell. Host receptors responsible for attachment and engulfment are members of the phosphatidylserine receptors, such as TIM (T-cell immunoglobulin and mucin domain) and TAM (TYRO3,

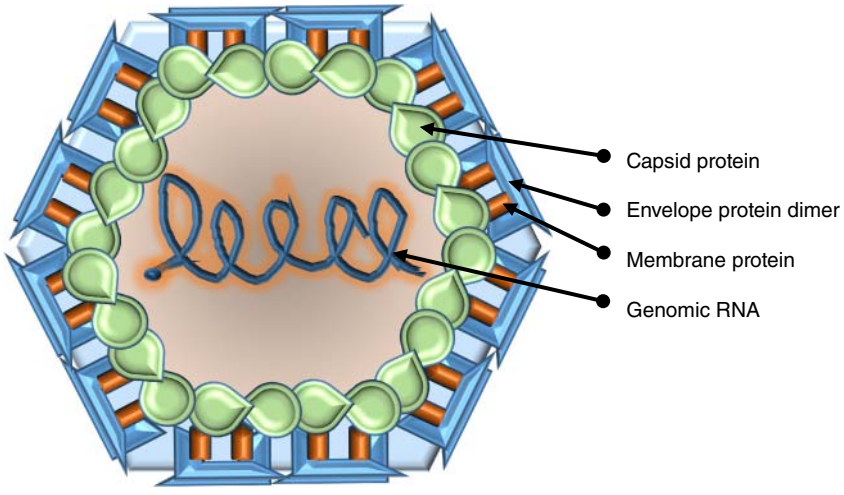


Figure 2.1 Structure of a Zika virus particle. The Zika virion is an enveloped icosahedral particle with an RNA genome at its core surrounded by a capsid protein inside of a dimerized envelope protein associated with membrane protein.

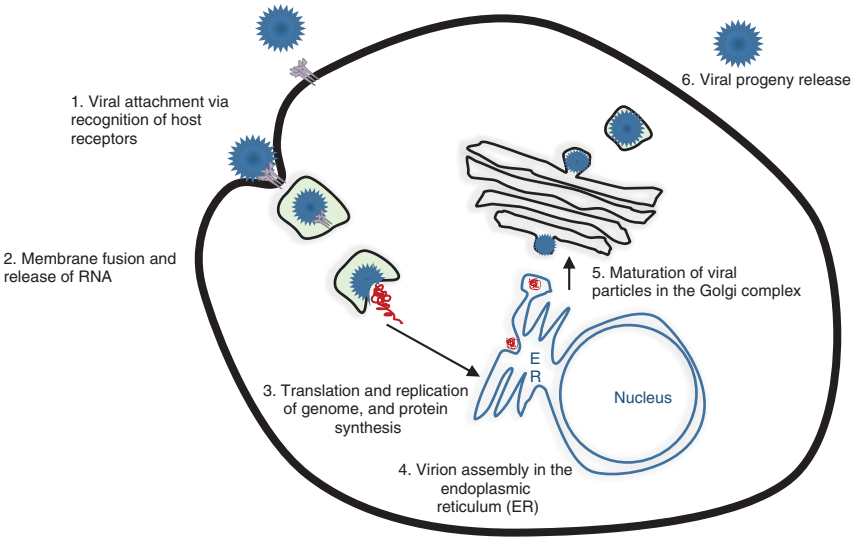


Figure 2.2 Zika virus replication cycle. The virus attaches to the envelope protein and enters the cell by endocytosis. Viral and host membrane fusion release viral RNA and allow ssRNA translation and formation of structural and nonstructural proteins. RNA replication and assembly occurs in the endoplasmic reticulum (ER); and after virion maturation at the Golgi apparatus, the mature virus particle is released from the host cell via exocytosis.

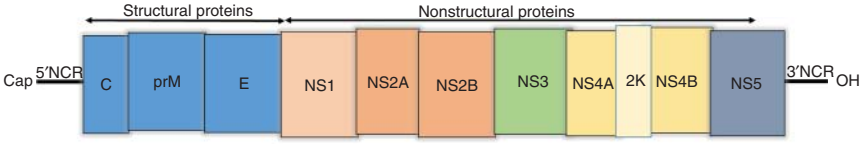


Figure 2.3 Zika virus genome organization. Structural and nonstructural (NS) protein organization of the Zika polyprotein. NS1 provides immunomodulation and guidance of RNA replication in congress with NS2A; NS2B is a cofactor to NS3 protease activity. NS3A/B encodes protease and helicase enzymes responsible for viral cleavage, replication, and membrane processing. NS4A/B directs membrane reordering associated with viral replication and inhibition of antivirals. Peptide 2K is a signal peptide for NS4B. NS5 provides methyl transferase for capping and RNA-dependent RNA polymerase (RdRp) functions [55–57] NCR, noncoding region; C, capsid; prM, precursor membrane.

AXL, and MER) as well as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) [52]. Following this, the virus membrane fuses with the membrane of a host endosome and the viral genome is released into the host's cytoplasm. Once inside the host cytoplasm, the ssRNA genome comprising 10 794 nucleotides is translated by the host's machinery into a polyprotein and is further processed into the three structural proteins, C, E, and prM, as well as seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. In addition, this polyprotein is bordered by two noncoding regions termed the 5′ and 3′ noncoding regions [53, 54] (Figure 2.3). The polyprotein cleavage allows the NS proteins responsible for replication and the viral processing license to these functions. Replication of the new viral particles occurs at the endoplasmic reticulum (ER) where double-stranded DNA results, which is then transcribed to the ssRNA genome of the virus. The immature virion is assembled at the ER before traveling to the Golgi apparatus for complete maturation and release from the cell via exocytosis. A distinctive characteristic of ZIKV is that during viral replication, the virus causes a radical disconcertion of the host's cytoskeleton, which creates a fortress-like confine to surround and protect it during these processes [58]. Another exceptional attribute of ZIKV is its rapid and explosive spread from Asia to the Americas, associated with more severe disease symptoms. It has been proposed that a single amino acid change in the NS1 protein is responsible for the enhanced ZIKV infectivity, and that the single change could be correlated with the more pathogenic form of the recent ZIKV outbreaks [59].

2.4 Lessons Learned from Dengue Antiviral Research

Over the past 20 years, many molecular targets and their inhibitors have been reported for the dengue virus. These molecules fall into two categories distinguished by whether they target host or viral proteins. Only five small-molecule drug candidates have progressed to human clinical trials and none have demonstrated virologic or symptomatic efficacy in patients. The following section

summarizes past efforts on dengue antiviral research. More complete reviews on dengue virus inhibitors are also available [60, 61].

2.4.1 Host Targeting Agents

Dengue, like all viruses, relies heavily on host machineries to complete its replication cycle. It is therefore expected that small molecules targeting host proteins could indirectly inhibit the dengue virus life cycle. The main advantage of host protein-targeting agents is the broad antiviral spectrum they generally confer, often encompassing multiple virus families. Another strength of host protein-targeting agents, at least in principle, is that they are less prone to mutation-induced drug resistance. One of the main classes of dengue host protein targets is dihydroorotate dehydrogenase (DHODH), a mitochondrial enzyme involved in *de novo* pyrimidine biosynthesis. DHODH inhibitors such as brequinar have been reported to interfere with *in vitro* replication of dengue and other viruses [62–64]. The antiviral effect of DHODH inhibitors is reversed in cell culture by supplementing the culture medium with exogenous uridine, which might explain why DHODH inhibitors do not inhibit dengue virus replication *in vivo*, since intracellular uridine pools can be replenished through diet [64]. Another prominent class of host protein targets is α-glucosidase I, an enzyme involved in maturation of viral envelope glycoproteins. Celgosivir is the butyl prodrug of catanospermine, a natural product that inhibits α-glucosidase I. Celgosivir inhibits dengue virus replication *in vitro* and is efficacious in a lethal mouse model [65, 66]. Although celgosivir was well tolerated when given as a 400-mg loading dose and 200 mg twice daily for five days in patients with dengue, the drug did not reduce viral load or fever [67, 68]. Other host protein-targeting agents that have been evaluated without success in dengue-infected patients include the antimalaria drug chloroquine [69], the corticosteroid drug prednisolone [70], and the cholesterol synthesis inhibitor lovastatin [71]. One of the main lessons from these failed attempts to develop anti-dengue drugs is the difficulty of timely patient enrollment into trials evaluating interventions in such an acute viral infection.

2.4.2 Direct Antiviral Agents

By analogy to antiviral research previously conducted on human immunodeficiency virus and HCV, one could consider the protease and the polymerase as the two main viral molecular targets. The two classes of polymerase inhibitors are nucleoside analogs and non-nucleoside analogs. Most dengue virus protease inhibitors use peptidomimetics, but their major shortcomings prevent advanced nonclinical development: low permeability and stability and lack of selectivity through inhibition of host proteases. Other classes of direct-acting agents include capsid inhibitors and NS4B binders (for a more complete review, see [61]). The only direct antiviral agent to have reached clinical trial evaluation in dengue-virus-infected patients is balapiravir, the ester prodrug of R1479 (4′azido-cytidine). Balapiravir, which was originally developed against HCV infection, was only tolerated for a short treatment duration [72].

In dengue-virus-infected patients, the cytidine analog was found to be safe but ineffective when tested for five days at doses up to 3000 mg [73]. The following sections review the dengue virus inhibitors already tested against ZIKV.

2.5 *In Vitro* Tools for Anti-ZIKV Drug Discovery

2.5.1 Cell-Based Assays

Cell-based ZIKV assays provide important tools in the discovery effort of ZIKV antivirals. Primarily, the assays are used to simultaneously screen compounds for antiviral activity and cytotoxicity. In addition, cell-based assays provide insights into the mechanism of action of anti-ZIKV drugs. Based on experiences with closely related dengue virus, ZIKV antiviral assays were established and published soon after the 2015–2016 ZIKV epidemic in the Americas. The foundation of these assays was the early characterization of ZIKV [1, 74]. Assay development was facilitated by the broad cellular tropism of ZIKV and the fact that ZIKV can be propagated to high titers in multiple cell lines [75, 76], including human cell lines from tissues such as A549 (human lung carcinoma cells), Huh7 (human hepatoma) cells, U87 (human glioblastoma cells), and EA.hy.926 (human vascular endothelial cells) [75, 76]. ZIKV can also be propagated in rhesus and African green monkey kidney cells (LLC-MK2 or Vero) [74, 77]. The choice of the cell line is important for antiviral drugs that depend on cell-line-specific metabolic activation, such as phosphorylation of nucleosides to the pharmacologically active metabolite (such as nuclesoside triphosphates). For example, the marketed anti-HCV drug sofosbuvir demonstrated anti-ZIKV activity with micromolar EC_{50} concentrations in baby hamster kidney cells, human neuroblastoma cells (SH-Sy5y), and Huh7 cells but not in African green monkey kidney (Vero) cells, where it was inactive [78].

Most of the ZIKV antiviral assays rely on cytopathic effect, immunostaining, or quantification of the viral genome (RT-PCR) as readouts [76, 77]. For example, a combination of immunostaining and viral yield reduction assays using Vero cells was used to test the anti-ZIKV activity of several nucleoside inhibitors [77, 79]. The majority of antiviral assays described use a limited set of ZIKV strains, often relying on the older African lineages such as the widely used MR-766 strain isolated in 1946 in Uganda [1, 74, 77, 79]. This can be problematic if the compound of interest targets a highly variable part of the virus, in which case validation with contemporary strains is critical.

An alternative to antiviral assays using infectious ZIKV are virus replicon and full reverse genetic systems [80–82]. In addition to increased biosafety, these systems allow easy site-directed modifications of the viral genome and offer the possibility of following viral replication through reporter proteins [80, 81].

In addition to antiviral assays in established cell lines, human stem-cell-derived neural cells and brain organoids have been used to confirm the anti-ZIKV activity of drugs [78, 83]. These experiments can provide insights into the antiviral activity of compounds of interest in relevant primary cells and tissue-like organoids before proceeding to animal efficacy studies.

2.5.2 Biochemical Assays and Tools for Structure-Based Drug Design

Since the start of the ZIKV pandemic in 2015, significant advances have been made in understanding the structures of the ZIKV and its structural and non-structural proteins. Some of these include high-resolution data of ZIKV proteins complexed with inhibitors. The multitude of structures can be used as a valuable tool for the discovery of new molecules and for the optimization and repurposing of existing drug candidates.

2.5.2.1 The NS5 MTase and Polymerase

The NS5 protein is about 900 amino acids (aa) long and is divided into two independent enzymatic domains. NS5 contains the N-terminal MTase domain of about 30 kDa (aa 1–265), followed by a short linker connecting the C-terminal RNA-dependent RNA polymerase (RdRp) domain of about 70 kDa (aa 278–903) (Figure 2.4). The crystal structure of the ZIKV NS5-MTase has recently been solved, revealing features closely associated with other flaviviruses such as dengue and Japanese encephalitis virus [84–86]. The structure of the RdRp domain adopts a classic right-hand structure with clearly identifiable palm, fingers, and thumb subdomains as well as a priming loop that protrudes from the thumb subdomain [86]. The RdRp domain binds two zinc ions in the thumb domain (residues H714, C730, and C849) and in the finger domain (residues G439, H443, C448, and C451) [84]. The MTase domain is in close contact with the RdRp domain through a hydrophobic set of residues (Pro113, Leu115, and Trp121 from the MTase; Tyr350, Phe466, and Pro854 from the RdRp). This close contact suggests a linkage between the two enzymatic activities. The full-length NS5 protein can be expressed and purified from *Escherichia coli* using standard affinity tags at either end of the protein, using methods previously described for dengue NS5 [87, 88]. Like other DNA and RNA polymerases, the RdRp activity of ZIKV NS5 is metal dependent, with a preference for manganese over

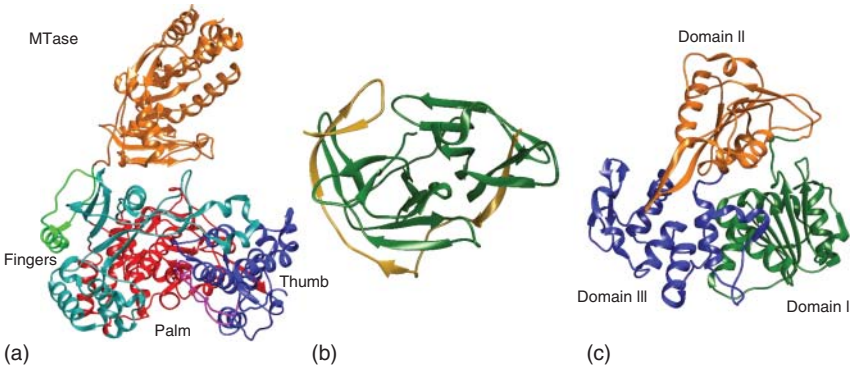


Figure 2.4 Structures of Zika virus proteins. (a) The structure of ZIKV NS5 methyltransferase (MTase)-polymerase in a ribbon format shows the MTase (orange) with the right-handed structure of the RdRp domain identifying fingers, palm, and thumb (PDB 5U0B). (b) The structure of the unlinked ZIKV NS2B:NS3 protease showing the NS2B in gold and NS3 in green (PDB 5GPI). (c) The structure of the ZIKV NS3 helicase showing the domain organization: domain I (green; residues 182–327), domain II (orange; residues 328–480), and domain III (blue; residues 480–617) (PDB 5JMT).

magnesium [87]. Recombinant ZIKV NS5 protein can synthesize fluorescent and radiolabeled RNA using either short or long heteropolymeric RNA templates, as well as long homopolymeric poly(rC) templates annealed to the rG13 primer [88, 89]. This activity relies on the catalytic GDD motif within the RdRp domain, which was verified by site-directed mutagenesis [88, 89]. Although the truncated C-terminal polymerase domain of NS5 is also capable of synthesizing RNA, its activity is reduced compared to full-length NS5 [89]. However, recombinant full-length NS5 and the isolated N-terminal domain (aa 1–264) carry identical levels of 2′-*O*-MTase enzymatic activity using a short $GpppAC_4$ oligonucleotide substrate, suggesting that the RdRp domain plays no role in this function [90]. Similar to the West Nile virus and dengue virus proteins, ZIKV NS5 also carries an N7-MTase activity only when the RNA substrate contains the conserved flavivirus SLA hairpin structure [90].

2.5.2.2 The NS2B–NS3 Protease

Similar to other flavivirus proteases, ZIKV NS3 has two enzymatic domains consisting of a protease domain and a helicase domain [91, 92]. This enzyme complex processes structural and NS proteins along with host proteases. The functional form of the ZIKV protease is formed by the NS2B–NS3 complex (Figure 2.4). Similar to related viral proteases, it has been shown that the mature form of the enzyme complex is composed of the C-terminal membrane–associated residues of ZIKV NS2B and the N-terminal domain of NS3 (aa 1–170). The structure of this complex has recently been solved using a Gly_4-Ser-Gly_4 linker to connect the NS2B with NS3 [93]. This protein adopts an open conformation in its uninhibited form, where NS2B is largely disordered and has minimal interactions with NS3 protease. The inhibited form causes a structural rearrangement of the C-terminal components of NS2B, causing it to wrap around the NS3 protease and interact with the inhibitor itself to form the closed conformation. This linked complex can be expressed in *E. coli* and purified as an active, recombinant protein that has approximately 20-fold higher activity than its West Nile virus counterpart [93, 94] when tested using a typical flavivirus protease substrate benzoyl-norleucinelysine-lysine-arginine 7-amino-4-methylcoumarine. It has been suggested that the Gly_4-Ser-Gly_4 linker region may change the binding of substrate and inhibitor. It may also introduce an artificial steric hindrance; this resulted in the design, expression, and characterization of an unlinked NS2B:NS3 protease [95]. The unlinked NS2B:NS3 protease adopts a primarily closed conformation, while local structural dynamics exist at the interface of the NS2B:NS3. To understand whether this new protease construct could serve as a platform for antiviral drug design, fragment-based screening was used to identify a small molecule, EN300 (1*H*-benzo[*d*]imidazol-1-yl) that thermally stabilized the unlinked protease [95]. Solution nuclear magnetic resonance studies revealed binding of EN300 to NS3 alone and not to NS2B and did not indicate large-scale conformational changes upon binding.

2.5.2.3 The NS3 Helicase

The RNA helicase of ZIKV NS3 consists of the C-terminal region of NS3 (residues 171–617) similar to other flaviviruses [96, 97]. Flavivirus helicases are responsible for unwinding RNA and ATP hydrolysis. The ZIKV NS3 helicase has recently

been crystallized, revealing structural features similar to other flavivirus helicases [96]. The ZIKV helicase is composed of three domains (Figure 2.4). Domain I contains the conserved P loop, which is important for nucleotide triphosphate (NTP) binding and catalysis. The residues of the P loop tend to be highly conserved between flavivirus species, although structural conformations of the P loop vary significantly among flavivirus species, indicating its intrinsic flexibility [96, 97]. Domains I and II are structurally similar but differ significantly in sequence. Domains I and II consist of a set of four helices, several loops, and stacked β-sheets in between them. Domain III is built from a four-helix bundle with several antiparallel beta strands. The NTPase active site, where binding of ATP and metal (Mn^{2+} or Mg^{2+}) occurs, is situated at the bottom of the cleft between domains I and II, whereas RNA is retained in a positively charged tunnel separating domains I/II from domain III [98]. The positive charges on the tunnel are hypothesized to stabilize the sugar-phosphate backbone through electrostatic interactions. This tunnel is of optimal size for single-stranded nucleic acid to run in its extended form from domain II to domain I.

2.6 Animal Models for Evaluating *In Vivo* Efficacy

Since the ZIKV outbreaks in the Americas in 2015, numerous animal models have been reported, providing valuable insights into ZIKV infection, transmission, and pathogenesis. The advantages and limitations of these models, predominately using mice or nonhuman primates, have been recently summarized [99, 100]. Here, we focus on the use of ZIKV animal efficacy models for antiviral drug discovery and discuss the requirements, characteristics, and limitations of the existing models.

An animal efficacy model for the evaluation of direct-acting antiviral drugs is ideally characterized by (i) small animal size to reduce cost, keep drug substance requirements low, and allow sufficient sample size within a treatment arm; (ii) a degree of pathogenesis caused by viral replication; (iii) active, measurable viral replication caused by a low viral inoculum via a clinically relevant route of infection; and (iv) objectively quantifiable outcomes such as tissue viral load or viremia, weight loss, disease severity, and/or death. Prior validation of the efficacy model using an antiviral compound that resulted in meaningful reductions of viral load and other outcomes is preferred. The choice of animal species further depends on the metabolism and pharmacokinetic behavior of the antiviral drug. Exposure levels of the drug or its active metabolite (for example, the NTP for nucleoside analogs) in the target organs vary substantially from species to species. Finally, ethical concerns must be considered when using nonhuman primates as the test species.

Several immunocompromised mouse models fulfill many of the ideal characteristics. Learning from experience with other flaviviruses such as the West Nile virus [101] and dengue virus [102], infection of interferon α/β/γ receptor knockout mice AG129 with ZIKV caused death as early as six days postinfection, weight loss of approximately 10–20%, and viremia and viral load in multiple tissues [103, 104]. Several experimental factors such as age of the mice at the time of

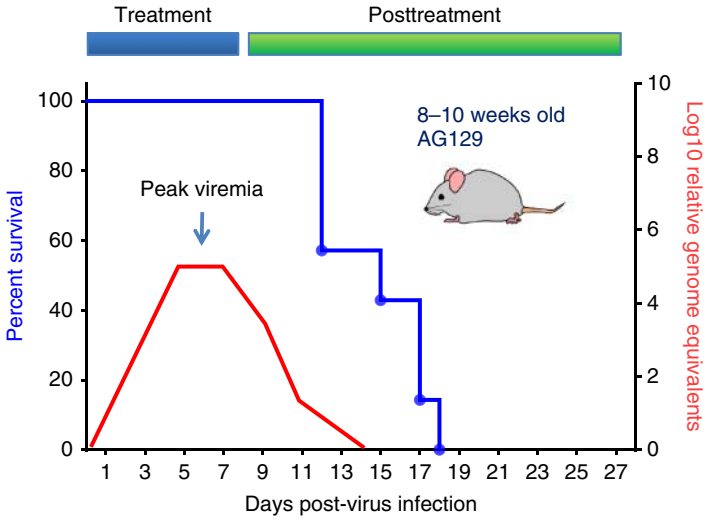


Figure 2.5 Standard mouse efficacy model for ZIKV infection. On day 0, 8- to 10-week-old AG129 mice are infected with a subcutaneous dose of ZIKV (1000 PFU/animal). Viremia peaks around days 6–7 at which time, mice start to exhibit pronounced clinical signs of infection, such as weight loss and neurological disease. The model is 100% lethal, starting two to three weeks after virus challenge. For efficacy studies, small molecules are typically administered daily beginning on the same day as virus inoculation and continuing for up to eight days (treatment). Mice are observed after treatment ends for up to 20 days (posttreatment).

inoculation, route of inoculation (subcutaneous, intraperitoneal, or via foot pad), and viral inoculum influence the time of peak viremia and death [79, 103, 105]. A depiction of an animal efficacy model is shown in Figure 2.5 (based on [105]). In this model, 8- to 10-week-old AG129 mice were infected subcutaneously with 1000 PFU/animal with the Malaysian isolate P6-740. Peak serum viremia was observed around day 7 postinfection and most mice die by day 18. Treatment with an antiviral drug typically begins either at the time of inoculation or four hours before inoculation and continues for up to eight days.

The choice of ZIKV isolate might affect the time of disease onset and death: The majority of AG129 mice infected with 1×10^3 PFU/animal of a Malaysian ZIKV strain (P 6–740) started to lose weight by approximately day 15 and died by day 16–21 post-infection, while AG129 mice infected with 1×10^5 PFU of Polynesian Zika isolate (H/PF/2013) died by day 8 [103, 105, 106]. Using the African isolate MR766, roughly inoculum-proportional mortality was observed in AG129 with all mice dying by day 14 at the highest inoculum of 2×10^4/animal while 25% of mice survived infection with 0.2 PFU/animal by day 20 postinfection [107]. Disease progression and time of death caused by infection with MR766 could be delayed but not fully prevented by treating animals with the nucleoside inhibitor 7-deaza-2′-*C*-methyladenosine (7DMA) [79]. In this model, 7DMA caused modest reductions of serum viremia [79]. Other compounds used to validate ZIKV mouse efficacy models are NITD008 (60% survival versus 0% survival for the vehicle control) and BCX4430 (90% survival versus 0% survival for the vehicle control) [105, 108]. However, experimental differences such as viral strain, age of

mice, and inoculum size and site must be considered when comparing results from different models.

Similar to the AG129 mice, which lack the interferon receptor, mice with other defects in the interferon α/β signaling cascade such as Ifnar1$^{-/-}$ or Irf3$^{-/-}$ Irf5$^{-/-}$ Irf7$^{-/-}$ triple-knockout mice demonstrated rapid death, weight loss, and disease signs such as progressive limb weakness and paralysis upon infection with ZIKV [109]. In contrast, immunocompetent mice survived the infection with no apparent pathogenesis and minimal or no viral RNA detected in several tissues [104, 109]. An immunosuppressed state can also be achieved through treatment with anti-Ifnar1 antibodies, allowing infection with ZIKV followed by a delay in weight gain and mortality [110]. This model was validated using sofosbuvir, an FDA-approved nucleoside analog polymerase inhibitor of the related flavivirus HCV. Therapeutic administration of sofosbuvir in the drinking water starting one day postinfection partially protected ZIKV-infected mice. Sofosbuvir-treated animals also gained weight faster than did the control animals [110]. Unfortunately, the effect of sofosbuvir on viral replication was not assessed.

For further confirmation of anti-ZIKV activity or for compounds where mice might not be an option due to poor pharmacokinetic behavior, other ZIKV animal models are available. These models use primarily nonhuman primates and have been developed predominately to study pathogenesis, transmission, or vaccine efficacy. Following infection of rhesus macaques subcutaneously with ZIKV, viral RNA was detected in plasma, urine, saliva, and spinal fluid [111]. Similar observations were made by other groups; some also noted elevated body temperature and liver enzymes [112, 113].

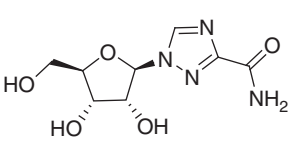
A possible alternative to nonhuman primates are hamsters with a defect in the interferon response pathway. Subcutaneous infection of STAT2 knockout hamsters with ZIKV isolate P6-740 caused death in approximately 60% of animals and modest reductions in body weight [114]. ZIKV RNA was detected in the brain, spinal cord, testes, kidney, and spleen and appeared to be proportional to the viral inoculum [114]. Unfortunately, this model was not further validated with a known antiviral drug.

2.7 ZIKV NS5 RdRp and MTase Inhibitors

Nucleoside analogs represent an important class of molecules that often have broad antiviral spectrum across one or multiple virus families. Therefore, it is logical that broad-spectrum ribonucleoside analogs known to inhibit RNA viruses such as HCV, dengue virus, or even influenza virus, be tested against ZIKV.

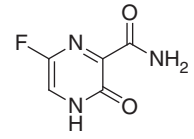
2.7.1 Ribavirin and T-705 (Favipiravir)

Ribavirin (Figure 2.6), a guanosine analog first identified as a very broad antiviral agent over 40 years ago, has *in vitro* potency against 16 different DNA and RNA viruses [115, 116]. Although ribavirin is currently approved for the treatment of chronic HCV and severe respiratory syncytial virus (RSV) infection, it was originally developed against influenza based on its efficacy in a mouse model of



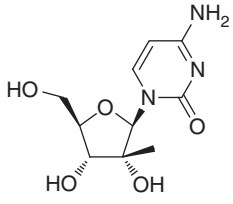
Ribavirin

ZIKV EC_{50} = 13 μM
CC_{50} > 400 μM
Reference [79]



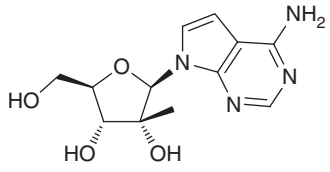
T-705

ZIKV EC_{50} = 22 μM
CC_{50} > 600 μM
Reference [79]



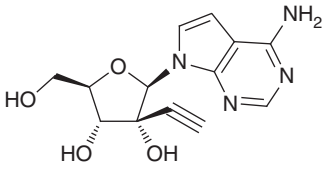
2CMC

ZIKV EC_{50} = 10 μM
CC_{50} = 28 μM
(a) Reference [79]



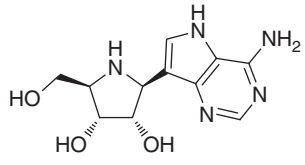
7DMA

ZIKV EC_{50} = 20 μM
CC_{50} > 350 μM
Reference [79]



NITD008

ZIKV EC_{50} = 140–950 μM
(b) Reference [108]



BCX4430

ZIKV EC_{50} = 19–38 μM
(c) Reference [105]

Figure 2.6 Structures of ZIKV NS5 RdRp inhibitors. (a) Ribavirin = a guanosine analog; T-705 = favipiravir (a guanosine analog); 2CMC = xxx; 7DMA = nucleoside inhibitor 7-deaza-2′-*C*-methyladenosine; Source: Zmurko et al. 2016 [79]. Reproduced with permission of PLOS. (b) NITD008 = an adenosine analog; Source: Deng et al. 2016 [108]. Reproduced with permission of OUP. (c) BCX4430 = an adenosine analog. Source: Julander et al. 2017 [105]. Reproduced with permission of Elsevier.

influenza [117, 118]. The use of ribavirin in the clinic for the treatment of HCV infection has diminished due to availability of newer and more potent agents. In addition, RSV infections are rarely treated with ribavirin because of the inconvenient route of administration requiring aerosols, lack of clear evidence for efficacy, and significant safety concerns associated with anemia and risk of teratogenicity. Nevertheless, ribavirin continues to be tested *in vitro* against new and potentially life-threatening emerging viruses. It is therefore not surprising that ribavirin was one of the first nucleoside analogs to be reported as potent

against ZIKV replication in cell culture [79]. Ribavirin also abrogated viremia in ZIKV-infected STAT-1-deficient mice [119].

T-705 (favipiravir) is a more recently developed analog of ribavirin approved (for emergency use only) in Japan for the treatment of pandemic influenza infection since 2014 [120] (Figure 2.6). As with ribavirin, T-705 exerts a very broad antiviral spectrum against various RNA viruses including ZIKV with an EC_{50} value of around 20 μM [79]. Further work recently showed that structural analogs of T-705 also inhibit ZIKV replication *in vitro*, although none of the other molecules displayed greater potency than T-705 [121]. Although no *in vivo* data have yet been reported, mathematical models predict that T-705 might be efficacious in nonclinical animal models either as monotherapy or combined with interferon α [122, 123].

2.7.2 2′-C-Methylated Nucleosides

The antiviral activity of 2′-modified nucleosides was evaluated in a series of *in vitro* assays where Vero cells were infected with ZIKV [77]. In this experiment, 2′-C-methylated nucleosides conferred the highest level of antiviral activity, with EC_{50} values ranging from 5.5 to 45 μM depending on the heterocyclic base moiety (Figure 2.6). This result is not completely unexpected, given the known antiviral spectrum of 2CMC and 7DMA (Figure 2.6), two molecules originally developed against HCV infection but also known to inhibit dengue and other flaviviruses [124–127]. Treatment of ZIKV-infected AG129 mice with 7DMA at 50 mg/kg/d via oral gavage for 10 days reduced viremia and delayed virus-induced morbidity and mortality [79]. Other anti-HCV 2′-modified nucleosides, including 2′-fluoro,2′-*C*-methyl and 2′-*O*-methyl nucleosides, did not inhibit *in vitro* replication of ZIKV [77]. The lack of antiviral activity of 2′-fluoro, 2′-*C*-methyl nucleosides such as the FDA-approved HCV drug sofosbuvir, could be attributed to a deficiency in intracellular triphosphate formation in Vero cells, combined with a suboptimal interaction between the NTP and ZIKV polymerase. In other cell types such as Huh7, sofosbuvir inhibited ZIKV replication with EC_{50} values of 8–25 μM, a concentration range significantly higher than that required to inhibit HCV [88, 110, 128]. In a biochemical study using recombinant RdRp assembled into an elongation complex, both dengue and ZIKV polymerases recognized all tested 2′-modified NTPs as chain terminators, but the two enzymes incorporated 2′-*C*-methyl CTP much more efficiently than 2′-fluoro,2′-*C*-methyl CTP/UTP and 2′-*O*-methyl CTP [87]. This result was supported by two independent studies where 2′-fluoro, 2′-*C*-methyl UTP (sofosbuvir triphosphate) was 10- to 100-fold less potent than its non-fluorinated nucleotide counterparts against ZIKV polymerase [88]. The greater selectivity of dengue and ZIKV polymerase compared with HCV polymerase against sofosbuvir triphosphate indicates that, despite early encouraging reports [78, 129], sofosbuvir might not be potent enough to be effectively repurposed for treatment of ZIKV or dengue virus infection. In addition, the monophosphate prodrug moiety of sofosbuvir was optimized to target the liver, which limits its potential use against viruses infecting and replicating in other organs.

2.7.3 NITD008

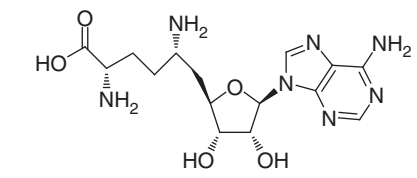
The adenosine analog NITD008 (Figure 2.6) is perhaps the most studied and best characterized dengue virus inhibitor so far, but its development toward clinical evaluation was interrupted by an unfavorable safety profile [130]. NITD008 blocks the replication of dengue virus in cell culture and in mice through inhibition of the RdRp activity of NS5 [130, 131]. The host enzyme responsible for the first phosphorylation step resulting in the conversion of NITD008 to its monophosphate form is adenosine kinase, a protein found in many cell types [132]. Replacing the adenine base by cytosine or guanosine suppressed the antiviral potency of NITD008, most likely due to a loss of intracellular phosphorylation by adenosine kinase [132]. In Vero cells, NITD008 inhibited the replication of ZIKV with an EC_{50} value ranging from 0.14 to 0.24 μM depending on the virus strain [108]. In ZIKV-infected AG129 mice, treatment with 50 mg/kg/d NITD008 for five days protected 50% of the infected animals from death and none of the surviving mice developed neurological symptoms [108]. The survival benefit in treated mice was associated with a 2.6-fold decrease in mean peak viremia on day 2 postinfection compared with the untreated group. Despite these impressive efficacy results in mice, severe toxicity following 14 days of low doses of NITD008 in rats and dogs would prevent further development of the compound for ZIKV infection [130].

2.7.4 BCX4430

The adenosine analog BCX4430 (Figure 2.6) was originally developed as an inhibitor of Ebola virus based on its protective effect in a mouse model of Ebola and Marburg virus diseases and in a lethal nonhuman primate model of the Marburg virus [133]. In cell culture, BCX4430 displayed broad-spectrum potency against various RNA viruses, which was attributed to the inhibition of viral RdRp by its triphosphate form. BCX4430 was also highly active in a hamster model of yellow fever virus, another flavivirus closely related to dengue virus and ZIKV [134]. As expected, BCX4430 also inhibited the replication of ZIKV in cell culture at concentrations that did not cause any cytopathic effect [105]. Treatment of ZIKV-infected AG129 mice with BCX4430 provided up to 80% protection from mortality and no weight loss at the maximum dose of 300 mg/kg/d twice daily for seven days, whereas ribavirin had no protective effect. Given the current interest in developing BCX4430 for the treatment of Ebola virus infection [135], additional studies may also be conducted in parallel to further evaluate the potential use of this compound against ZIKV.

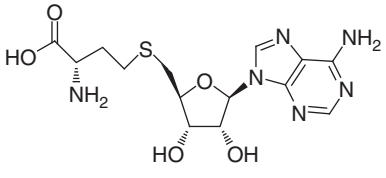
2.7.5 MTase Inhibitors

Active site and allosteric inhibitors of the dengue virus MTase domain have been reported [136, 137] and these small molecules were also tested as potential inhibitors of 2′*O*-MTase activity of the ZIKV MTase domain [90]. The MTase reaction product *S*-adenosyl-L-homocysteine inhibited ZIKV MTase with IC_{50} values of 0.4 μM with a GpppAC4 RNA substrate (2′-O-cap methylation), and



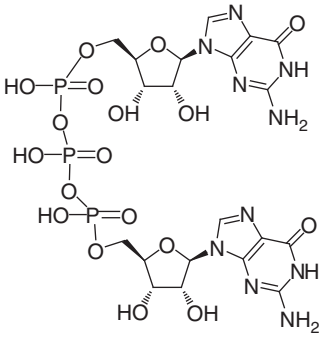
Sinefungin

ZIKV MTase IC_{50} = 1.2 μM
Reference [90]



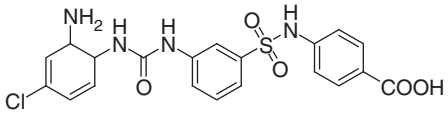
SAH

ZIKV MTase IC_{50} = 0.4 μM
Reference [90]



GpppG

ZIKV MTasel C_{50} = 72 μM
Reference [90]



Compound 2

ZIKV MTase IC_{50} = 33 μM
Reference [90]

Figure 2.7 Structures of ZIKV NS5 MTase inhibitors. Source: Coutard et al. 2017 [90]. Reproduced with permission of ASM.

7.3 μM with an A27 RNA (internal 2′-O-methylation). In comparison, the inhibition potency of sinefungin in the same assay using the two different RNAs was 1.2 and 1.6 μM, respectively (Figure 2.7). A number of other cap analogs were also tested in this assay, among which GpppG was the most potent with an IC_{50} value of 72/405 μM (Figure 2.7). Finally, six allosteric inhibitors designed from a fragment-based screening campaign against dengue virus MTase were 2- to 10-fold more potent against ZIKV. Although the improved enzyme inhibition potency could not be rationalized, it provided proof of concept that dengue virus MTase inhibitors could also inhibit the ZIKV protein counterpart. Although small molecules targeting the MTase of dengue virus or ZIKV remain at very early stages of drug discovery, structure-based approaches might provide avenues to improve ligand binding and inhibition potency [138].

2.8 NS3 Protease and Helicase Inhibitors

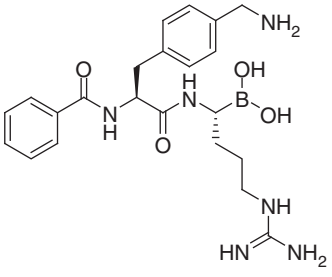
There remain many other targets besides NS5 for antivirals including ZIKV NS2B-NS3 and NS3 helicase. NS2B-NS3 plays a critical role in processing

of viral proteins, which has catalyzed many efforts to inhibit the NS2B–NS3 complex [139–141]. Three main ZIKV protease constructs were developed for structure-based drug design. One of these constructs, the bZiPro, is assembled from residues 45–96 of NS2B and 1–177 of NS3 [142]. The second construct uses enzymatic self-cleavage during protein expression to yield an unlinked protein complex, termed the eZiPro construct [143]. The third construct is the gZiPro, which uses a nine-residue peptide linker between NS2B and NS3 protease [93]. These constructs exhibit different enzyme kinetics depending on how the NS2B/NS3 is expressed which has consequences for inhibitor development. The unlinked protease has higher k_{cat} values and is a more efficient enzyme than the linked protease. It is thought the linker may play a role in substrate and, potentially, inhibitor access to the active site. Therefore, the bZiPro construct provides an advantage for structure-guided inhibitor design because it has the highest resolution structure, it is in the closed conformation, and structures already exist of the bZipro in complex with inhibitors. While there are no crystal structures of the full-length NS2B/NS3 protein, several structures of the NS3 protease with and without an inhibitor as well as the NS3 helicase have been reported [93, 96–98, 143, 144]. Both represent important druggable targets.

The ZIKV protease was the first ZIKV target to have a crystal structure in complex with an inhibitor. A focus of protease inhibitor development has been substrate-mimicking peptides, also known as peptidomimetics. Tetrapeptides with a different carboxy-terminal electrophile led to the identification of a boronic-acid analog with activity toward DENV-2 protease [145, 146]. This compound was optimized through the replacement of the P2 with a nonnatural arginine mimetic led to the identification of a capped dipeptide boronic acid compound (cn-716) with activity against DENV-2, West Nile virus, and ZIKV protease [147]. This led to the crystal structure of the boronic acid analog in complex with cn-716 with an $IC_{50} = 0.25 \pm 0.02\,\mu M$ and a $K_i = 0.040 \pm 0.006\,\mu M$ [93] (Figure 2.8). Limitations in the clinical development of boronic acid analogs exist due to their off-target effects; a high-resolution structure of protease with inhibitor provided deep insights into the ZIKV protease molecular recognition and structural changes.

Several other lower molecular weight compounds have been identified as ZIKV protease inhibitors. Previous work identified inhibitors of HCV protease during a high-throughput screening of 40 967 compounds [148]. A follow-up analysis of 71 of these compounds identified two compounds, compounds 2 and 3, with 50% inhibitory concentrations of 5.2 and 4.1 μM [139, 149] (Figure 2.8). Drug purposing studies have also identified bromocriptine as a noncompetitive inhibitor of ZIKV NS2B-NS3 protease [150]. It showed an IC_{50} of $21.6 \pm 1.1\,\mu M$. Aprotinin, a serine protease inhibitor used to stop bleeding during surgical procedures, has also been identified as an inhibitor of the NS2B-NS3 protease with a K_i of $361 \pm 19\,nM$ [144].

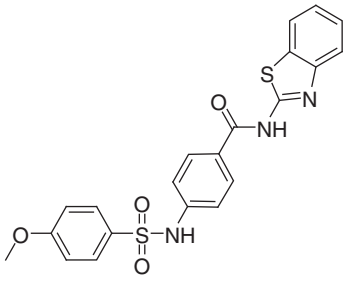
Multiple structures of the NS3 helicase from ZIKV have been reported [96, 98]. These structures, combined with computational methods, have located druggable hot spots within two polar, hydrophobic pockets at a junction between domains 1 and 2 and at a cleft between domains 1 and 3 of the helicase [96]. While helicase inhibitors have been reported for DENV [151–153], to date, there have



cn-716

ZIKV protease IC_{50} = 0.25 μM

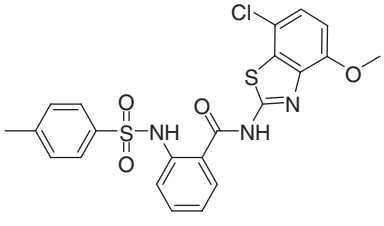
(a) Reference [93]



Compound 2

ZIKV protease IC_{50} = 5.2 μM

(b) Reference [139]



Compound 3

ZIKV protease IC_{50} = 4.1 μM

Reference [139]

Figure 2.8 Structures of ZIKV NS3 protease inhibitors. (a) Source: Lei et al. 2016 [93]. Reproduced with permission of Science Mag. (b) Source: Lee et al. 2017 [139]. Reproduced with permission of Elsevier.

been no direct functional or structural studies to probe such inhibitors against ZIKV helicase; therefore, future work must address this.

2.9 Other Classes of Small Molecules against ZIKV

Other ZIKV proteins have been targeted for antiviral efforts. The stem region of the envelope protein was conserved among flaviviruses, which prompted the design of synthetic peptides intended to prevent a conformational change and trimeric form in the DENV envelope leading to cell fusion [154]. The same strategy has been applied to ZIKV, resulting in an antiviral peptide, Z2, that prevented vertical transmission of ZIKV in pregnant mice [155]. The same molecule was also efficacious in the lethal AG129 mouse model. Over the past two years, several host-targeting agents that inhibit ZIKV replication have been described, including *N*-(4-hydroxyphenyl) retinamide (4-HPR) and a nuclear import inhibitor also potent against dengue and West Nile virus replication

[156, 157]. It is hypothesized that 4-HPR exerts its antiviral activity by disrupting the interaction between ZIKV NS5 and the host transport factor importin α/β1 [158, 159]. Treatment of ZIKV-infected AG129 mice with 4-HPR resulted in approximately 1 $\log_{10}$ reduction in serum viremia [158]. The polyphenol virucidal agent ECGC is known to inhibit a broad panel of DNA and RNA viruses by blocking cell entry [160, 161]. The antiviral activity of ECGC, a green tea extract, also extends to ZIKV; however, further development is limited by its metabolic instability and lack of permeability [162].

2.10 Conclusions and Future Directions on ZIKV Inhibition

ZIKV has recently gained global public attention after its introduction to the American continent, resulting in numerous cases of severe neurological disorders in patients. Although it is still too early to expect the approval of any anti-ZIKV antiviral therapy anytime soon, progress made in dengue antivirals has accelerated knowledge and applications toward ZIKV antiviral research. As described in this chapter, several previously identified dengue inhibitors also demonstrate *in vitro* antiviral potency against ZIKV. Moreover, a few of these molecules are efficacious when tested in small animal models for ZIKV infection. Of note, most of these molecules are nucleoside analogs targeting the RNA polymerase of ZIKV. Are antiviral results from animal efficacy models for ZIKV predictive of potential human clinical efficacy? It is at this point very speculative to extrapolate animal efficacy data to human clinical settings, due to differences in kinetics and distribution of virus replication and symptoms between different species. Moreover, ZIKV and other flavivirus infections are short, acute, and often self-limiting, thereby greatly limiting the window for therapeutic intervention. An alternative option could be to consider prophylactic treatments with small molecules, especially for individuals traveling to endemic regions. This intervention model could be similar to what is currently done for malaria drugs. One of the main target populations for ZIKV treatments are pregnant women, which represents an additional challenge due to high safety requirements. For these reasons, it is important to continue to pursue ZIKV vaccine approaches in parallel with small-molecule treatments. One potential benefit of small-molecule treatments is the opportunity to develop a pan-flavivirus drug. The ambitious goal to discover and develop molecules capable of inhibiting ZIKV, dengue, and other flaviviruses addresses an important unmet medical need for millions of patients across the globe.

References

1 Dick, G.W.A., Kitchen, S.F., and Haddow, A.J. (1952). Zika virus (I). Isolations and serological specificity. *Trans. R. Soc. Trop. Med. Hyg.* 46 (5): 509–520.

2 Dick, G.W. (1952). Zika virus. II. Pathogenicity and physical properties. *Trans. R. Soc. Trop. Med. Hyg.* 46 (5): 521–534.

3 Dick, G.W.A. (1953). Epidemiological notes on some viruses isolated in Uganda (Yellow fever, Rift Valley fever, Bwamba fever, West Nile, Mengo, Semliki forest, Bunyamwera, Ntaya, Uganda S and Zika viruses). *Trans. R. Soc. Trop. Med. Hyg.* 47 (1): 13–48.
4 MacNamara, F.N. (1954). Zika virus : a report on three cases of human infection during an epidemic of jaundice in Nigeria. *Trans. R. Soc. Trop. Med. Hyg.* 48 (2): 139–145.
5 Fagbami, A.H. (1979). Zika virus infections in Nigeria: virological and seroepidemiological investigations in Oyo State. *J. Hyg. (Lond.)* 83 (2): 213–219.
6 Haddow, A.D., Schuh, A.J., Yasuda, C.Y. et al. (2012). Genetic characterization of Zika virus strains: geographic expansion of the Asian lineage. *PLoS Negl.Trop. Dis.* 6 (2): e1477.
7 Renaudet, J., Jan, C., Ridet, J., et al. (1978). A serological survey of arboviruses in the human population of Senegal. *Bull. Soc. Pathol. Exot. Filiales* 71 (2): 131–140.
8 Robin, Y. and Mouchet, J. (1975). Serological and entomological study on yellow fever in Sierra Leone. *Bull. Soc. Pathol. Exot. Filiales* 68 (3): 249–258.
9 Jan, C., Languillat, G., Renaudet, J., et al. (1978). A serological survey of arboviruses in Gabon. *Bull. Soc. Pathol. Exot. Filiales* 71 (2): 140–146.
10 Akoua-Koffi, C., Diarrassouba, S., Bénié, V.B., et al. Investigation surrounding a fatal case of yellow fever in Cote d'Ivoire in 1999. *Bull. Soc. Pathol. Exot. 2001.* 94 (3): 227–230.
11 Saluzzo, J.F., Gonzalez, J.P., Herve, J.P., and Georges, A.J. (1981). Serological survey for the prevalence of certain arboviruses in the human population of the south-east area of Central African Republic (author's transl). *Bull. Soc. Pathol. Exot. Filiales* 74 (5): 490–499.
12 Smithburn, K.C., Taylor, R.M., Rizk, F., and Kader, A. (1954). Immunity to certain arthropod-borne viruses among indigenous residents of Egypt. *Am. J. Trop. Med. Hyg.* 3 (1): 9–18.
13 Olson, J.G., Ksiazek, T.G., Suhandiman, and Triwibowo. (1981). Zika virus, a cause of fever in Central Java, Indonesia. *Trans. R. Soc. Trop. Med. Hyg.* 75 (3): 389–393.
14 Smithburn, K.C. (1952). Neutralizing antibodies against certain recently isolated viruses in the sera of human beings residing in East Africa. *J. Immunol.* 69 (2): 223–234.
15 Darwish, M.A., Hoogstraal, H., Roberts, T.J., et al. (1983). A sero-epidemiological survey for certain arboviruses (Togaviridae) in Pakistan. *Trans. R. Soc. Trop. Med. Hyg.* 77 (4): 442–445.
16 Smithburn, K.C. (1954). Neutralizing antibodies against arthropod-borne viruses in the sera of long-time residents of Malaya and Borneo. *Am. J. Hyg.* 59 (2): 157–163.
17 Pond, W.L. (1963). Arthropod-borne virus antibodies in sera from residents of South-East Asia. *Trans. R. Soc. Trop. Med. Hyg.* 57: 364–371.
18 Hammon, W.M., Schrack, W.D. Jr., and Sather, G.E. (1958). Serological survey for a arthropod-borne virus infections in the Philippines. *Am. J. Trop. Med. Hyg.* 7 (3): 323–328.

19 Weinbren, M.P. and Williams, M.C. (1958). Zika virus: further isolations in the Zika area, and some studies on the strains isolated. *Trans. R. Soc. Trop. Med. Hyg.* 52 (3): 263–268.

20 Marchette, N.J., Garcia, R., and Rudnick, A. (1969). Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. *Am. J. Trop. Med. Hyg.* 18 (3): 411–415.

21 Grard, G., Caron, M., Mombo, I.M., et al. (2014). Zika virus in Gabon (Central Africa) – 2007: a new threat from *Aedes albopictus*? *PLoS Negl.Trop. Dis.* 8 (2): e2681.

22 Diallo, D., Sall, A.A., Diagne, C.T., et al. (2014). Zika virus emergence in mosquitoes in southeastern Senegal, 2011. *PLoS One* 9 (10): e109442.

23 Ledermann, J.P., Guillaumot, L., Yug, L., et al. (2014). *Aedes hensilli* as a potential vector of Chikungunya and Zika viruses. *PLoS Negl.Trop. Dis.* 8 (10): e3188.

24 Haddow, A.J., Williams, M.C., Woodall, J.P., et al. (1964). Twelve isolations of Zika virus from Aedes (Stegomyia) Africanus (Theobald) taken in and above a Uganda forest. *Bull. World Health Organ.* 31: 57–69.

25 Duffy, M.R., Chen, T.H., Hancock, W.T., et al. (2009). Zika virus outbreak on Yap Island, Federated States of Micronesia. *N. Engl. J. Med.* 360 (24): 2536–2543.

26 Foy, B.D., Kobylinski, K.C., Chilson Foy, J.L., et al. (2011). Probable non-vector-borne transmission of Zika virus, Colorado, USA. *Emerg. Infect. Dis.* 17 (5): 880–882.

27 Roth, A., Mercier, A., Lepers, C., et al. (2014). Concurrent outbreaks of dengue, chikungunya and Zika virus infections – an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. *Euro Surveill.* 19 (41).

28 Musso, D., Nilles, E.J., and Cao-Lormeau, V.M. (2014). Rapid spread of emerging Zika virus in the Pacific area. *Clin. Microbiol. Infect.* 20 (10): O595–O596.

29 Cao-Lormeau, V.M., Roche, C., Teissier, A., et al. (2014). Zika virus, French polynesia, South pacific, 2013. *Emerg. Infect. Dis.* 20 (6): 1085–1086.

30 Musso, D., Nhan, T., Robin, E., et al. (2014). Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. *Euro Surveill.* 19 (14).

31 Musso, D., Roche, C., Robin, E., et al. (2015). Potential sexual transmission of Zika virus. *Emerg. Infect. Dis.* 21 (2): 359–361.

32 Oehler, E., Watrin, L., Larre, P., et al. (2014). Zika virus infection complicated by Guillain-Barre syndrome--case report, French Polynesia, December 2013. *Euro Surveill.* 19 (9).

33 European Centre for Disease Prevention and Control (2015). Zika virus epidemic in the Americas: potential association with microcephaly and Guillain-Barré syndrome, 10 December 2015.

34 Ioos, S., Mallet, H.P., Leparc Goffart, I., et al. (2014). Current Zika virus epidemiology and recent epidemics. *Med. Mal. Infect.* 44 (7): 302–307.

35 McNeil, D., Saint Louis, C. and St Fleur, N. (2016). Short answers to hard questions about Zika virus. *New York Times*.

36 Campos, G.S., Bandeira, A.C., and Sardi, S.I. (2015). Zika virus outbreak, Bahia. *Braz. Emerging Infect. Dis.* 21 (10): 1885–1886.

37 Zanluca, C., deMelo, V.C.A., Mosimann, A.L.P., et al. (2015). First report of autochthonous transmission of Zika virus in Brazil. *Mem. Inst. Oswaldo Cruz.* 110 (4): 569–572.

38 Hennessey, M., Fischer, M., and Staples, J.E. (2016). Zika virus spreads to new areas – region of the Americas, May 2015-January 2016. *MMWR Morb. Mortal. Wkly. Rep.* 65 (3): 55–58.

39 Lanciotti, R.S., Lambert, A.J., Holodniy, M., et al. (2016). Phylogeny of Zika virus in Western Hemisphere, 2015. *Emerg. Infect. Dis.* 22 (5): 933–935.

40 Pan American Health Organization, W.H.O. (2015). Regional Office for the Americas. Epidemiological Alert Increase of microcephaly in the northeast of Brazil. [Epidemiological Alert] 2015 November 17 2015. http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&Itemid=270&gid=32285&lang=en (accessed 28 February 2019).

41 Pan American Health Organization, W.H.O. (2016). Regional Office for the Americas. Epidemiological alert: neurological syndrome, congenital anomalies, and Zika virus infection. 2016 January 17 2016. http://www.paho.org/hq/index.php?option=com_content&view=category&layout=blog&id=1218&Itemid=2291 (accessed 28 February 2019).

42 Pan American Health Organization, W.H.O. (2016). WHO Director-General summarizes the outcome of the Emergency Committee regarding clusters of microcephaly and Guillain-Barré syndrome. 2016 1 Feb 2016. http://www.who.int/mediacentre/news/statements/2016/emergency-committee-zika-microcephaly/en/ (accessed 28 February 2019).

43 St George, K., Sohi, I.S., Dufort, E.M., et al. (2017). Zika virus testing considerations: lessons learned from the first 80 real-time reverse transcription-PCR-positive cases diagnosed in New York state. *J. Clin. Microbiol.* 55 (2): 535–544.

44 Cerbino-Neto, J., Mesquita, E., Souza, T.L. et al. (2016). Clinical manifestations of Zika virus infection, Rio de Janeiro, Brazil, 2015. *Emerg. Infect. Dis.* 22 (7): 1318–1320.

45 Klase, Z.A., Khakhina, S., Schneider, A.D.B., et al. (2016). Zika fetal neuropathogenesis: etiology of a viral syndrome. *PLoS Negl. Trop. Dis.* 10 (8): e0004877.

46 Cavalheiro, S., Lopez, A., Serra, S., et al. (2016). Microcephaly and Zika virus: neonatal neuroradiological aspects. *Childs Nerv. Syst.* 32: 1057–1060.

47 Driggers, R.W., Ho, C.-Y., Korhonen, E.M., et al. (2016). Zika virus infection with prolonged maternal viremia and fetal brain abnormalities. *N. Engl. J. Med.* 374 (22): 2142–2151.

48 Mlakar, J., Korva, M., Tul, N., et al. (2016). Zika virus associated with microcephaly. *N. Engl. J. Med.* 374 (10): 951–958.

49 Parra, B., Lizarazo, J., Jiménez-Arango, J.A., et al. (2016). Guillain–Barré syndrome associated with Zika virus infection in Colombia. *N. Engl. J. Med.* 375 (16): 1513–1523.

50 Paploski, I.A.D., Prates, A.P.P.B., Cardoso, C.W., et al. (2016). Time lags between exanthematous illness attributed to Zika virus, Guillain–Barré

syndrome, and microcephaly, Salvador. *Braz. Emerging Infect. Dis.* 22 (8): 1438–1444.

51 Paul, L.M., Carlin, E.R., Jenkins, M.M., et al. (2016). Dengue virus antibodies enhance Zika virus infection. *Clin. Transl. Immunol.* 5 (12): e117.

52 Perera-Lecoin, M., Meertens, L., Carnec, X., et al. (2013). Flavivirus entry receptors: an update. *Viruses* 6 (1): 69–88.

53 Kuno, G. and Chang, G.-J.J. (2007). Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. *Arch. Virol.* 152 (4): 687–696.

54 Chambers, T.J., Hahn, C.S., Galler, R., and Rice, C.M. (1990). Flavivirus genome organization, expression, and replication. *Annu. Rev. Microbiol.* 44: 649–688.

55 Bollati, M., Alvarez, K., Assenberg, R., et al. (2010). Structure and functionality in flavivirus NS-proteins: perspectives for drug design. *Antiviral Res.* 87 (2): 125–148.

56 Saiz, J.-C., Vázquez-Calvo, Á., Blázquez, A.B., et al. (2016). Zika virus: the latest newcomer. *Front. Microbiol.* 7: 496.

57 Galán-Huerta, K.A. et al. (2016). The Zika virus disease: an overview. *Medicina Universitaria* 18 (71): 115–124.

58 Cortese, M., Goellner, S., Acosta, E.G., et al. (2017). Ultrastructural characterization of Zika virus replication factories. *Cell Rep.* 18 (9): 2113–2123.

59 Liu, Y., Liu, J., Du, S., et al. (2017). Evolutionary enhancement of Zika virus infectivity in *Aedes aegypti* mosquitoes. *Nature* 545 (7655): 482–486.

60 Low, J.G., Ooi, E.E., and Vasudevan, S.G. (2017). Current status of dengue therapeutics research and development. *J. Infect. Dis.* 215 (suppl_2): S96–S102.

61 Lim, S.P., Wang, Q.-Y., Noble, C.G., et al. (2013). Ten years of dengue drug discovery: progress and prospects. *Antiviral Res.* 100 (2): 500–519.

62 Hoffmann, H.-H., Kunz, A., Simon, V.A., et al. (2011). Broad-spectrum antiviral that interferes with de novo pyrimidine biosynthesis. *Proc. Natl. Acad. Sci.* 108 (14): 5777–5782.

63 Bonavia, A., Franti, M., Pusateri Keaney, E., et al. (2011). Identification of broad-spectrum antiviral compounds and assessment of the druggability of their target for efficacy against respiratory syncytial virus (RSV). *Proc. Natl. Acad. Sci.* 108 (17): 6739–6744.

64 Wang, Q.Y., Bushell, S., Qing, M., et al. (2011). Inhibition of dengue virus through suppression of host pyrimidine biosynthesis. *J. Virol.* 85 (13): 6548–6556.

65 Watanabe, S., Rathore, A.P., Sung, C., et al. (2012). Dose- and schedule-dependent protective efficacy of celgosivir in a lethal mouse model for dengue virus infection informs dosing regimen for a proof of concept clinical trial. *Antiviral Res.* 96 (1): 32–35.

66 Rathore, A.P., Paradkar, P.N., Watanabe, S., et al. (2011). Celgosivir treatment misfolds dengue virus NS1 protein, induces cellular pro-survival genes and protects against lethal challenge mouse model. *Antiviral Res.* 92 (3): 453–460.

67 Low, J.G., Sung, C., Wijaya, L., et al. (2014). Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. *Lancet Infect. Dis.* 14 (8): 706–715.

68 Sung, C., Wei, Y., Watanabe, S., et al. (2016). Extended evaluation of virological, immunological and pharmacokinetic endpoints of CELADEN: a randomized, placebo-controlled trial of celgosivir in dengue fever patients. *PLoS Negl.Trop. Dis.* 10 (8): e0004851.

69 Tricou, V., Minh, N.N., Van, T.P., et al. (2010). A randomized controlled trial of chloroquine for the treatment of dengue in Vietnamese adults. *PLoS Negl.Trop. Dis.* 4 (8): e785.

70 Tam, D.T., Ngoc, T.V., Tien, N.T., et al. (2012). Effects of short-course oral corticosteroid therapy in early dengue infection in Vietnamese patients: a randomized, placebo-controlled trial. *Clin. Infect. Dis.* 55 (9): 1216–1224.

71 Whitehorn, J., Nguyen, C.V.V., Khanh, L.P., et al. (2016). Lovastatin for the treatment of adult patients with dengue: a randomized, double-blind. *Placebo-Controlled Trial. Clin. Infect. Dis.* 62 (4): 468–476.

72 Ma, H., Jiang, W.R., Robledo, N., et al. (2007). Characterization of the metabolic activation of hepatitis C virus nucleoside inhibitor beta-D-2′-deoxy-2′-fluoro-2′-*C*-methylcytidine (PSI-6130) and identification of a novel active 5′-triphosphate species. *J. Biol. Chem.* 282 (41): 29812–29820.

73 Nguyen, N.M., Tran, C.N., Phung, L.K., et al. (2013). A randomized, double-blind placebo controlled trial of balapiravir, a polymerase inhibitor, in adult dengue patients. *J. Infect. Dis.* 207 (9): 1442–1450.

74 Way, J.H., Bowen, E.T., and Platt, G.S. (1976). Comparative studies of some African arboviruses in cell culture and in mice. *J. Gen. Virol.* 30 (1): 123–130.

75 Barr, K.L., Anderson, B.D., Prakoso, D., and Long, M.T. (2016). Working with Zika and Usutu viruses *in vitro*. *PLoS Negl.Trop. Dis.* 10 (8): e0004931.

76 Vicenti, I., Boccuto, A., Giannini, A., et al. (2017). Comparative analysis of different cell systems for Zika virus (ZIKV) propagation and evaluation of anti-ZIKV compounds in vitro. *Virus Res.* 244: 64–70.

77 Eyer, L., Nencka, R., Huvarova, I., et al. (2016). Nucleoside inhibitors of Zika virus. *J. Infect. Dis.* 214 (5): 707–711.

78 Sacramento, C.Q., deMelo, G.R., de Freitas, C.S., et al. (2017). The clinically approved antiviral drug sofosbuvir inhibits Zika virus replication. *Sci. Rep.* 7: 40920.

79 Zmurko, J., Marques, R.E., Schols, D., et al. (2016). The viral polymerase inhibitor 7-deaza-2′-*C*-methyladenosine is a potent inhibitor of *in vitro* Zika virus replication and delays disease progression in a robust mouse infection model. *PLoS Negl.Trop. Dis.* 10 (5): e0004695.

80 Xie, X., Zou, J., Shan, C., et al. (2016). Zika virus replicons for drug discovery. *EBioMedicine* 12: 156–160.

81 Mutso, M., Saul, S., Rausalu, K., et al. (2017). Reverse genetic system, genetically stable reporter viruses and packaged subgenomic replicon based on a Brazilian Zika virus isolate. *J. Gen. Virol.* 98 (11): 2712–2724.

82 Shan, C., Xie, X., Muruato, A.E., et al. (2016). An infectious cDNA clone of Zika virus to study viral virulence, mosquito transmission, and antiviral inhibitors. *Cell Host Microbe* 19 (6): 891–900.

83 Lanko, K., Eggermont, K., Patel, A., et al. (2017). Replication of the Zika virus in different iPSC-derived neuronal cells and implications to assess efficacy of antivirals. *Antiviral Res.* 145: 82–86.

84 Godoy, A.S., Lima, G.M., Oliveira, K.I. et al. (2017). Crystal structure of Zika virus NS5 RNA-dependent RNA polymerase. *Nat. Commun.* 8.

85 Wang, B., Tan, X.-F., Thurmond, S., et al. (2017). The structure of Zika virus NS5 reveals a conserved domain conformation. *Nat. Commun.* 8.

86 Zhao, B., Yi, G., Du, F., et al. (2017). Structure and function of the Zika virus full-length NS5 protein. *Nat. Commun.* 8.

87 Potisopon, S., Ferron, F., Fattorini, V., et al. (2017). Substrate selectivity of Dengue and Zika virus NS5 polymerase towards 2′-modified nucleotide analogues. *Antiviral Res.* 140: 25–36.

88 Xu, H.T., Hassounah, S.A., Colby-Germinario, S.P., et al. (2017). Purification of Zika virus RNA-dependent RNA polymerase and its use to identify small-molecule Zika inhibitors. *J. Antimicrob. Chemother.* 72 (3): 727–734.

89 Lu, G., Bluemling, G.R., Collop, P., et al. (2017). Analysis of ribonucleotide 5′-triphosphate analogs as potential inhibitors of Zika virus RNA-dependent RNA polymerase by using nonradioactive polymerase assays. *Antimicrob. Agents Chemother.* 61 (3).

90 Coutard, B., Barral, K., Lichiere, J., et al. (2017). Zika virus methyltransferase: structure and functions for drug design perspectives. *J. Virol.* 91(5).

91 Shi, Y. and Gao, G.F. (2017). Structural biology of the Zika virus. *Trends Biochem. Sci* 42 (6): 443–456.

92 Luo, D., Vasudevan, S.G., and Lescar, J. (2015). The flavivirus NS2B–NS3 protease–helicase as a target for antiviral drug development. *Antiviral Res.* 118: 148–158.

93 Lei, J., Hansen, G., Nitsche, C., et al. (2016). Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor. *Science* 353 (6298): 503–505.

94 Erbel, P., Schiering, N., D'Arcy, A., et al. (2006). Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. *Nat. Struct. Mol. Biol.* 13 (4): 372–373.

95 Zhang, Z., Li, Y., Loh, Y.R., et al. (2016). Crystal structure of unlinked NS2B-NS3 protease from Zika virus. *Science* aai9309.

96 Jain, R., Coloma, J., García-Sastre, A., and Aggarwal, A.K. (2016). Structure of the NS3 helicase from Zika virus. *Nat. Struct. Mol. Biol.* 23 (8): 752–754.

97 Tian, H., Ji, X., Yang, X., et al. (2016). Structural basis of Zika virus helicase in recognizing its substrates. *Protein Cell* 7 (8): 562–570.

98 Tian, H., Ji, X., Yang, X., et al. (2016). The crystal structure of Zika virus helicase: basis for antiviral drug design. *Protein Cell* 7 (6): 450–454.

99 Morrison, T.E. and Diamond, M.S. (2017). Animal models of Zika virus infection, pathogenesis, and immunity. *J. Virol.* 91 (8).

100 Pawitwar, S.S., Dhar, S., Tiwari, S., et al. (2017). Overview on the current status of Zika virus pathogenesis and animal related research. *J. Neuroimmune Pharmacol.* 12 (3): 371–388.

101 Samuel, M.A. and Diamond, M.S. (2005). Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. *J. Virol.* 79 (21): 13350–13361.

102 Shresta, S., Kyle, J.L., Snider, H.M., et al. (2004). Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. *J. Virol.* 78 (6): 2701–2710.

103 Aliota, M.T., Caine, E.A., Walker, E.C., et al. (2016). Characterization of lethal Zika virus infection in AG129 mice. *PLoS Negl. Trop. Dis.* 10 (4): e0004682.

104 Rossi, S.L., Tesh, R.B., Azar, S.R., et al. (2016). Characterization of a novel murine model to study Zika virus. *Am. J. Trop. Med. Hyg.* 94 (6): 1362–1369.

105 Julander, J.G., Siddharthan, V., Evans, J., et al. (2017). Efficacy of the broad-spectrum antiviral compound BCX4430 against Zika virus in cell culture and in a mouse model. *Antiviral Res.* 137: 14–22.

106 Shao, Q., Herrlinger, S., and Zhu, Y.-N. (2017). The African Zika virus MR-766 is more virulent and causes more severe brain damage than current Asian lineage and Dengue virus. *Development*.

107 Saudi, M., Zmurko, J., Kaptein, S., et al. (2016). Synthetic strategy and antiviral evaluation of diamide containing heterocycles targeting dengue and yellow fever virus. *Eur. J. Med. Chem.* 121: 158–168.

108 Deng, Y.Q., Zhang, N.N., Li, C.F., et al. (2016). Adenosine analog NITD008 is a potent inhibitor of Zika virus. *Open Forum Infect. Dis.* 3 (4): ofw175.

109 Lazear, H.M., Govero, J., Smith, A.M., et al. (2016). A mouse model of Zika virus pathogenesis. *Cell Host Microbe* 19 (5): 720–730.

110 Bullard-Feibelman, K.M., Govero, J., Zhu, Z., et al. (2017). The FDA-approved drug sofosbuvir inhibits Zika virus infection. *Antiviral Res.* 137: 134–140.

111 Dudley, D.M., Aliota, M.T., Mohr, E.L., et al. (2016). A rhesus macaque model of Asian-lineage Zika virus infection. *Nat. Commun.* 7: 12204.

112 Osuna, C.E., Lim, S.Y., Deleage, C., et al. (2016). Zika viral dynamics and shedding in rhesus and cynomolgus macaques. *Nat. Med.* 22 (12): 1448–1455.

113 Li, X.F., Dong, H.L., Huang, X.Y., et al. (2016). Characterization of a 2016 clinical isolate of Zika virus in non-human primates. *EBioMedicine* 12: 170–177.

114 Siddharthan, V., VanWettere, A.J., Li, R., et al. (2017). Zika virus infection of adult and fetal STAT2 knock-out hamsters. *Virology* 507: 89–95.

115 Sidwell, R.W., Huffman, J.H., Khare, G.P., et al. (1972). Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* 177 (4050): 705–706.

116 Witkowski, J.T., Robins, R.K., Sidwell, R.W., and Simon, L.N. (1972). Design, synthesis, and broad spectrum antiviral activity of

1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. *J. Med. Chem.* 15 (11): 1150–1154.

117 Wilson, S.Z., Knight, V., Wyde, P.R., et al. (1980). Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. *Antimicrob. Agents Chemother.* 17 (4): 642–648.

118 Wyde, P.R., Wilson, S.Z., Gilbert, B.E., and Smith, R.H. (1986). Protection of mice from lethal influenza virus infection with high dose-short duration ribavirin aerosol. *Antimicrob. Agents Chemother.* 30 (6): 942–944.

119 Kamiyama, N., Soma, R., Hidano, S., et al. (2017). Ribavirin inhibits Zika virus (ZIKV) replication in vitro and suppresses viremia in ZIKV-infected STAT1-deficient mice. *Antiviral Res.* 146: 1–11.

120 Furuta, Y., Gowen, B.B., Takahashi, K., et al. (2013). Favipiravir (T-705), a novel viral RNA polymerase inhibitor. *Antiviral Res.* 100 (2): 446–454.

121 Cai, L., Sun, Y., Song, Y., et al. (2017). Viral polymerase inhibitors T-705 and T-1105 are potential inhibitors of Zika virus replication. *Arch. Virol.*

122 Best, K., Guedj, J., Madelain, V., et al. (2017). Zika plasma viral dynamics in nonhuman primates provides insights into early infection and antiviral strategies. *Proc. Natl. Acad. Sci. U.S.A.* 114 (33): 8847–8852.

123 Pires de Mello, C.P., Tao, X., Kim, T.H., et al. (2017). Zika virus (ZIKV) replication is substantially inhibited by novel favipiravir and interferon-alpha combination regimens. *Antimicrob. Agents Chemother.*

124 Benzaria, S., Bardiot, D., Bouisset, T., et al. (2007). 2′-C-methyl branched pyrimidine ribonucleoside analogues: potent inhibitors of RNA virus replication. *Antiviral Chem. Chemother.* 18 (4): 225–242.

125 Carroll, S.S., Tomassini, J.E., Bosserman, M., et al. (2003). Inhibition of hepatitis C virus RNA replication by 2′-modified nucleoside analogs. *J. Biol. Chem.* 278 (14): 11979–11984.

126 Julander, J.G., Jha, A.K., Choi, J.A., et al. (2010). Efficacy of 2′-*C*-methylcytidine against yellow fever virus in cell culture and in a hamster model. *Antiviral Res.* 86 (3): 261–267.

127 Olsen, D.B., Eldrup, A.B., Bartholomew, L., et al. (2004). A 7-deaza-adenosine analog is a potent and selective inhibitor of hepatitis C virus replication with excellent pharmacokinetic properties. *Antimicrob. Agents Chemother.* 48 (10): 3944–3953.

128 Retallack, H., Di Lullo, E., Arias, C., et al. (2016). Zika virus cell tropism in the developing human brain and inhibition by azithromycin. *Proc. Natl. Acad. Sci. U.S.A.* 113 (50): 14408–14413.

129 Reznik, S.E. and Ashby, C.R. Jr., (2017). Sofosbuvir: an antiviral drug with potential efficacy against Zika infection. *Int. J. Infect. Dis.* 55: 29–30.

130 Yin, Z., Chen, Y.L., Schul, W., et al. (2009). An adenosine nucleoside inhibitor of dengue virus. *Proc. Natl. Acad. Sci. U.S.A.* 106 (48): 20435–20439.

131 Latour, D.R., Jekle, A., Javanbakht, H., et al. (2010). Biochemical characterization of the inhibition of the dengue virus RNA polymerase by beta-D-2′-ethynyl-7-deaza-adenosine triphosphate. *Antiviral Res.* 87 (2): 213–222.

132 Chen, Y.L., Yin, Z., Duraiswamy, J., et al. (2010). Inhibition of dengue virus RNA synthesis by an adenosine nucleoside. *Antimicrob. Agents Chemother.* 54 (7): 2932–2939.

133 Warren, T.K., Wells, J., Panchal, R.G., et al. (2014). Protection against filovirus diseases by a novel broad-spectrum nucleoside analogue BCX4430. *Nature* 508 (7496): 402–405.

134 Julander, J.G., Bantia, S., Taubenheim, B.R., et al. (2014). BCX4430, a novel nucleoside analog, effectively treats yellow fever in a Hamster model. *Antimicrob. Agents Chemother.* 58 (11): 6607–6614.

135 Taylor, R., Kotian, P., Warren, T., et al. (2016). BCX4430 – a broad-spectrum antiviral adenosine nucleoside analog under development for the treatment of Ebola virus disease. *J. Infect. Public Health* 9 (3): 220–226.

136 Benmansour, F., Trist, I., Coutard, B., et al. (2017). Discovery of novel dengue virus NS5 methyltransferase non-nucleoside inhibitors by fragment-based drug design. *Eur. J. Med. Chem.* 125: 865–880.

137 Coutard, B., Decroly, E., Li, C., et al. (2014). Assessment of Dengue virus helicase and methyltransferase as targets for fragment-based drug discovery. *Antiviral Res.* 106: 61–70.

138 Hercik, K., Brynda, J., Nencka, R., et al. (2017). Structural basis of Zika virus methyltransferase inhibition by sinefungin. *Arch. Virol.* 162 (7): 2091–2096.

139 Lee, H., Ren, J., Nocadello, S., et al. (2017). Identification of novel small molecule inhibitors against NS2B/NS3 serine protease from Zika virus. *Antiviral Res.* 139: 49–58.

140 Rut, W., Zhang, L., Kasperkiewicz, P., et al. (2017). Extended substrate specificity and first potent irreversible inhibitor/activity-based probe design for Zika virus NS2B-NS3 protease. *Antiviral Res.* 139: 88–94.

141 Cao, X., Li, Y., Jin, X., et al. (2016). Molecular mechanism of divalent-metal-induced activation of NS3 helicase and insights into Zika virus inhibitor design. *Nucleic Acids Res.* 44 (21): 10505–10514.

142 Kang, C., Keller, T.H., and Luo, D. (2017). Zika virus protease: an antiviral drug target. *Trends Microbiol.* 25 (10): 797–808.

143 Phoo, W.W., Li, Y., Zhang, Z., et al. (2016). Structure of the NS2B-NS3 protease from Zika virus after self-cleavage. *Nat. Commun.* 7.

144 Chen, X., Yang, K., Wu, C., et al. (2016). Mechanisms of activation and inhibition of Zika virus NS2B-NS3 protease. *Cell Res.* 26 (11): 1260.

145 Boldescu, V., Behnam, M.A., Vasilakis, N., and Klein, C.D. et al. (2017). Broad-spectrum agents for flaviviral infections: dengue, Zika and beyond. *Nat. Rev. Drug Discovery* 16 (8): 565–586.

146 Yin, Z., Patel, S.J., Wang, W.-L., et al. (2006). Peptide inhibitors of Dengue virus NS3 protease. Part 1: Warhead. *Bioorg. Med. Chem. Lett.* 16 (1): 36–39.

147 Nitsche, C., Zhang, L., Weigel, L.F., et al. (2016). Peptide–boronic acid inhibitors of flaviviral proteases: medicinal chemistry and structural biology. *J. Med. Chem.* 60 (1): 511–516.

148 Lee, H., Zhang, L., Weigel, L.F., et al. (2013). High-throughput screening (HTS) and hit validation to identify small molecule inhibitors with activity against NS3/4A proteases from multiple hepatitis C virus genotypes. *PLoS One* 8 (10): e75144.

149 Saiz, J.-C. and Martín-Acebes, M.A. (2017). The race to find antivirals for Zika virus. *Antimicrob. Agents Chemother.* 61 (6): e00411–e00417.

150 Chan, J.F., Chik, K.K., Yuan, S., et al. (2017). Novel antiviral activity and mechanism of bromocriptine as a Zika virus NS2B-NS3 protease inhibitor. *Antiviral Res.* 141: 29–37.

151 Basavannacharya, C. and Vasudevan, S.G. (2014). Suramin inhibits helicase activity of NS3 protein of dengue virus in a fluorescence-based high throughput assay format. *Biochem. Biophys. Res. Commun.* 453 (3): 539–544.

152 Byrd, C.M., Grosenbach, D.W., Berhanu, A., et al. (2013). Novel benzoxazole inhibitor of dengue virus replication that targets the NS3 helicase. *Antimicrob. Agents Chemother.* 57 (4): 1902–1912.

153 Mastrangelo, E., Pezzullo, M., De Burghgraeve, T., et al. (2012). Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS3 helicase activity: new prospects for an old drug. *J. Antimicrob. Chemother.* 67 (8): 1884–1894.

154 Schmidt, A.G., Yang, P.L., and Harrison, S.C. (2010). Peptide inhibitors of flavivirus entry derived from the E protein stem. *J. Virol.* 84 (24): 12549–12554.

155 Yu, Y., Deng, Y.Q., Zou, P., et al. (2017). A peptide-based viral inactivator inhibits Zika virus infection in pregnant mice and fetuses. *Nat. Commun.* 8: 15672.

156 Fraser, J.E., Watanabe, S., Wang, C., et al. (2014). A nuclear transport inhibitor that modulates the unfolded protein response and provides *in vivo* protection against lethal dengue virus infection. *J. Infect. Dis.* 210 (11): 1780–1791.

157 Tay, M.Y., Fraser, J.E., Chan, W.K., et al. (2013). Nuclear localization of dengue virus (DENV) 1-4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. *Antiviral Res.* 99 (3): 301–306.

158 Pitts, J.D., Li, P.C., de Wispelaere, M., Yang, P.L. (2017). Antiviral activity of *N*-(4-hydroxyphenyl) retinamide (4-HPR) against Zika virus. *Antiviral Res.* 147: 124–130.

159 Wang, C., Yang, S.N.Y., Smith, K., et al. (2017). Nuclear import inhibitor *N*-(4-hydroxyphenyl) retinamide targets Zika virus (ZIKV) nonstructural protein 5 to inhibit ZIKV infection. *Biochem. Biophys. Res. Commun.* 493 (4): 1555–1559.

160 Calland, N., Albecka, A., Belouzard, S., et al. (2012). (-)-Epigallocatechin-3-gallate is a new inhibitor of hepatitis C virus entry. *Hepatology* 55 (3): 720–729.

161 Song, J.M., Lee, K.H., and Seong, B.L. (2005). Antiviral effect of catechins in green tea on influenza virus. *Antiviral Res.* 68 (2): 66–74.

162 Carneiro, B.M., Batista, M.N., Braga, A.C.S., et al. (2016). The green tea molecule EGCG inhibits Zika virus entry. *Virology* 496: 215–218.

3

Developing Therapeutics for Ebola Virus Disease: A Multifaceted Approach

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3.1 Overview of Ebola Virus Disease (EVD)

Ebola virus disease (EVD), previously known as Ebola hemorrhagic fever, is a rare and deadly disease caused by infection with a virus of the family *Filoviridae*, genus *Ebolavirus*. There are five species within the genus, four of which cause disease in humans: Ebola virus (*Zaire ebolavirus*, EBOV); Sudan virus (*Sudan ebolavirus*, SUDV); Taï Forest virus (*Taï Forest ebolavirus,* TAFV), formerly *Côte d'Ivoire ebolavirus*; and Bundibugyo virus (*Bundibugyo ebolavirus*, BDBV). The fifth species, Reston virus (*Reston ebolavirus*, RESTV), causes disease in nonhuman primates (NHPs) but not in humans. EBOV and SUDV were first discovered in 1976 in what is now known as the Democratic Republic of the Congo and South Sudan, respectively [1, 2]. Since then, human EVD outbreaks by EBOV and SUDV have occurred across multiple nations of Central Africa until the most recent West African EVD outbreak. TAFV and BDBV have only spilled over into the human population once and twice, respectively [3]. Due to their high pathogenicity and lack of approved therapeutics, working with live ebolaviruses requires the highest level of biocontainment in a biosafety level-4 (BSL-4) laboratory. Following an incubation period averaging 8–10 days, EVD typically presents with nonspecific symptoms including fever, chills, muscle pain, diarrhea, and vomiting [4]. An ensuing maculopapular rash is often observed in the buttocks, trunk, and the upper arms, and eventually becomes more diffuse with the progression of disease. Some, but not all, EVD patients develop multiple foci of mucosal hemorrhage, most prominently in the conjunctiva but also in easy bruising and persistent bleeding from needle puncture sites. Viremia is detectable within the first 72 hours of symptom onset, and remains at high levels (>7 Log_{10} RNA copies/ml serum) in fatal cases [5, 6]. Common laboratory findings include a marked decrease in leukocytes and lymphocytes, with elevated serum levels of liver enzymes. Hypotension, renal failure, and shock often characterize late-stage EVD, along with bleeding from the gastrointestinal tract [5]. Ebolaviruses are transmitted through direct contact with blood or

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body fluids, and semen via broken skin or mucous membranes [7, 8]. Several means of transmission include contact with contaminated needles and syringes used on EVD patients, infected animals (e.g. primates or, potentially, bats), or sexual contact with semen from an EVD survivor. The natural reservoir host(s) of ebolaviruses remains unknown. However, detection of EBOV cross-reactive antibodies in bat serum [9, 10], along with evidence describing bats as the ecological reservoir of a related filovirus Marburg virus [11, 12] implicate bats as the most likely reservoir for ebolaviruses.

3.2 Ebola Virus Diagnostics: Challenges and Innovations

The relative rarity of EVD and the accompanying biosafety concerns of working with these highly pathogenic viruses makes initial EVD diagnosis challenging. Due to the similarity of symptoms to more common infections, clinical recognition of EVD is often difficult, and is compounded by the tendency for outbreaks to begin in remote rural locations. Timely diagnosis is essential for controlling an EVD outbreak, as any delay in initial diagnosis and appropriate patient isolation increases the potential for virus transmission. Furthermore, given the rarity of EVD, few laboratories are able to sustain the biosafety requirements and expertise necessary to safely handle infectious specimens.

Diagnostic tests for EBOVs fall into two categories; testing of acute specimens to identify the virus, viral antigen, or viral nucleic acids and testing of non-acute specimens (e.g. convalescent surveillance specimens) to determine a virus-reactive antibody response. Blood, serum, and plasma are the typical specimen types used for testing. Other specimen types such as oral/tissue swabs from corpses have been used to ensure safe burials [13–15], while semen can be used to determine viral persistence during convalescence [16–22]. Since the specimen type can affect assay sensitivity, internal controls are critical for proper interpretation of assay results [23, 24].

High-performing real-time (or quantitative) reverse transcription polymerase chain reactions (qRT-PCR) have been developed by multiple groups as a standard tool for diagnosing and monitoring acute EBOV cases [25–28]. While all these assays share similar overall techniques, their respective RNA extraction platforms vary widely, which can affect comparisons between the assays with regard to sensitivity and ease of use [28, 29]. Some advantages of qRT-PCR assays are their sensitivity, specificity, and the inactivation of the virus during the initial steps of RNA extraction; while disadvantages include both the requirement for trained laboratorians and for reliable electrical power. To date, efforts to correlate qRT-PCR cycle threshold values to an infectious measurement (i.e. tissue culture infectious dose, plaque-forming units) have been unreliable, since qRT-PCR measures the number of EBOV-specific RNA copies in a specimen and not the infectivity therein. The high specificity of qRT-PCR assays can also be a double-edged sword, as new point mutations or EBOV variants could lead to

false-negative results [30]. For this reason, running qRT-PCR assays against two different viral gene targets can safeguard against this type of situation. However, qRT-PCR can also potentially miss positive cases depending on whether a specimen is collected early on or late in the disease course. This highlights the importance of documenting the dates of symptom onset and specimen collection for proper assay choice and ensuing interpretation of results. Diagnostic laboratories have historically performed confirmatory assays such as traditional RT-PCR [31], antigen detection assays [32–35], viral isolation in cell culture or animal models [36–41], electron microscopy [39, 40, 42], immunohistochemistry (IHC) for tissues [43, 44], and, more recently, whole-genome sequencing (WGS) [45–48]. While many of these confirmatory assays are time intensive and require BSL-4 containment, WGS technology, on the other hand, has both reduced costs and accelerated the sequencing of relatively short viral genomes. WGS can be used not only to confirm the causative agent but also to monitor viral evolution and mutation rates, and to identify additional introductions from the natural reservoir host [45–48].

For non-acute specimens, detection of virus-specific antibodies in patient sera or plasma is a means to identify the disease agent and to monitor disease progression [49, 50]. Typical assays for determining antibody responses include enzyme-linked immunosorbent assays (ELISAs) [35, 49, 51, 52] and indirect immunofluorescence assays (IFAs) [44, 51]. The detection of IgM antibodies indicates a recent infection starting from a few days postinfection (dpi) and lasting several months [49, 52, 53], while IgG antibodies are long-lasting and are indicative of past infection [49, 52, 54, 55]. Multiple assays have been developed to measure both classes of antibodies against EBOV [35, 49, 50, 52, 56]. Methods and interpretation of serology assays vary, and their limitations must be understood to properly use the data generated. Serial dilution of sera can be used to both determine the amount of EBOV-specific antibody present and to confirm infection by evidence of increasing antibody titers over time. Acquiring seronegative specimens from a particular region of interest is crucial to determine appropriate cutoff values for serology, so as to take into account regional background differences due to region-specific endemic disease burdens. Although qRT-PCR is preferable for diagnosis of acute EVD cases due to little to no antibody response in severely ill patients [49], monitoring the rise of IgM and IgG antibodies as viral nucleic acid declines in recovering patients can expedite decisions for patient discharge [57].

Given the remote locations of most EBOV outbreaks, development of rapid diagnostic tests (RDTs) and lateral flow assays (LFAs) would facilitate outbreak detection and management. While LFAs detecting EBOV antigen are not as sensitive as qRT-PCR, their use provides an additional screening tool in low-resource settings [13, 14, 58]. Several proof-of-concept studies for new tests may prove useful as surveillance and research diagnostic tools, including reverse-transcription-loop-mediated isothermal amplification (RT-LAMP) and TaqMan array cards (TACs) [59–62]. In addition, the single-particle interferometric reflectance imaging sensor (SP-IRIS) can be used to count virions in a

specimen, while a newly developed pan-ebolavirus reporter cell line speeds up the process of virus isolation by shortening the timeframe needed to detect a live virus [63, 64]. Clinical biomarkers have been and continue to be evaluated as useful indicators of disease progression to either survival or death [50, 65–71]. While not an EBOV-specific diagnostic assay, the data could be utilized to direct resources toward patients with more severe disease and monitor the effects of potential therapeutic treatments.

3.3 Ebola Virus Genome Structure, Components, and Replication Cycle

The approximately ~19 kb genome of Ebola viruses consists of seven genes flanked by leader and trailer sequences, arranged in the following order from 3′ to 5′: the nucleoprotein gene (NP), the polymerase cofactor gene (VP35), the matrix gene (VP40), the attachment glycoprotein gene (GP) which encodes multiple protein products, the transcriptional activator gene (VP30), the transcriptional control gene (VP24), and the polymerase gene (L). The open reading frames for each gene are flanked by untranslated regions (UTRs), some of which overlap with UTRs of adjacent genes (Figure 3.1).

Ebola virus particles consist of a central nucleocapsid, which is made up of a single-stranded negative-sense viral RNA genome and the ribonucleoprotein complex (RNP) proteins (i.e. the nucleoprotein (NP), the polymerase (L), the polymerase cofactor (VP35), and the transcriptional activator (VP30)). The VP24 protein associates with the RNP complex and serves to condense the RNP as well as to lock the polymerase at the genomic terminus to ensure transcription and replication in the next round of infection [72]. The VP40 matrix protein surrounds the nucleocapsids and drives particle budding by manipulating host cell membranes that are embedded with the viral GP, which is responsible for facilitating particle entry [73].

At the cell surface, ebola virus particles attach to C-type lectins through the GP, or to phosphatidylserine receptors (e.g. TIM-1) through interactions with the viral envelope [74] (Figure 3.1). Virus particles are then taken up into endosomes through endocytosis/micropinocytosis [75], where an acidified environment enables host proteases to cleave the GP into its activated form ($GP_{1,2}$), allowing it to interact with the cellular receptor NPC-1 [76, 77]. This interaction ultimately results in membrane fusion and release of nucleocapsids into the cytoplasm [78]. After entry into the cell, nucleocapsids relax due to dissociation of VP24, which allows the viral RNP complex to perform primary transcription. Transcription of the viral genes leads to expression of the viral proteins required for genome replication as well as for further rounds of transcription and translation. Accumulating the VP24 protein generated then condenses newly formed RNP complexes into nucleocapsids that are packaged at the cell surface where VP40-driven budding occurs [73].

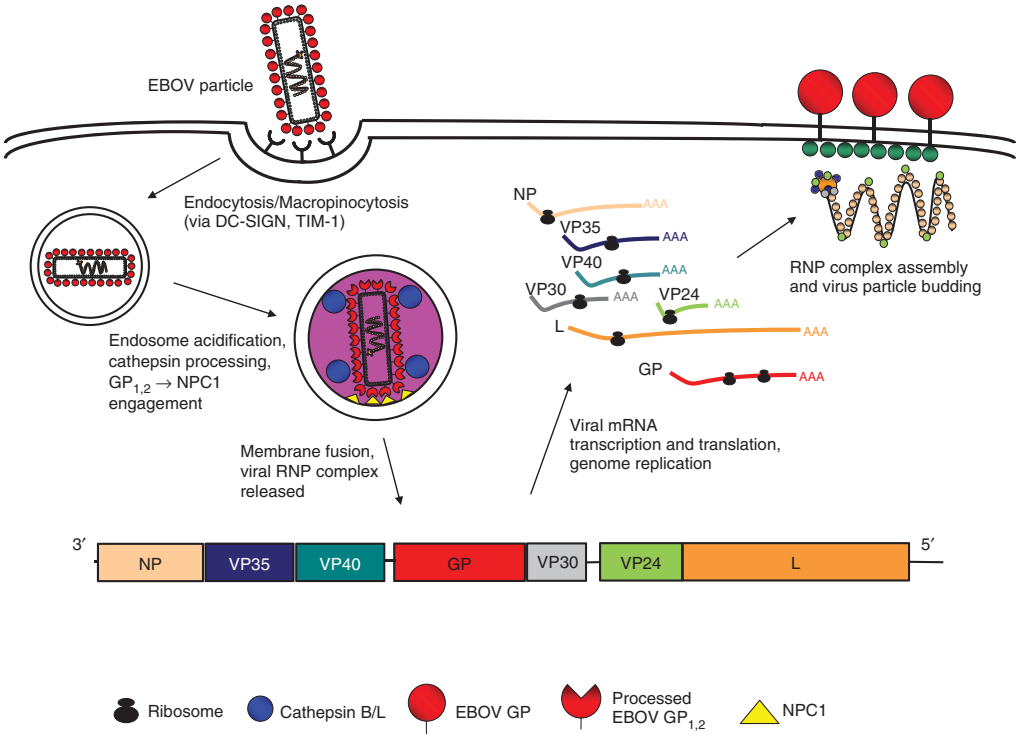

Figure 3.1 Ebola virus (EBOV) replication cycle and genome organization. The EBOV particle attaches to the envelope protein through interactions with C-type lectins and/or phosphatidylserine receptors, and enters the cell either by receptor-mediated endocytosis or by macropinocytosis. Endosomal acidification activates cathepsins B/L which proteolytically cleave EBOV GP into $GP_{1,2}$, which interacts with cellular receptor NPC1. Viral and host membrane fusion releases the EBOV ribonucleocapsid (RNP) complex into the cytoplasm, where mRNA transcription, translation, and genome replication occur. Resulting translated proteins are trafficked toward the host cell membrane, where the virus particles are assembled and eventually released from the cell.

3.4 *In vitro* Toolbox: Cell-Based Assays

Cell-based assays are crucial to driving the discovery of EBOV antivirals, be it in regard to evaluating anti-EBOV activity or to measuring the cytotoxicity of a particular molecule/compound. Although the African green monkey kidney cell line (Vero E6) has historically been used to propagate ebolaviruses, a variety of human cell types are permissible to EBOV replication including Huh7 (human liver hepatoma), HT-1080 (human fibrosarcoma), HeLa (human cervical carcinoma), HFF-1 (human foreskin fibroblasts), and HMVEC (human microvascular endothelial cells) [79–82]. Given that certain types of compounds (i.e. nucleoside analogs) may have varied anti-EBOV potencies depending on the cell type used, it is advisable to confirm compound activity in multiple cell lines that are major targets for EBOV infection, as well as in primary cells such as monocytes and monocyte-derived macrophages [82, 83].

Studies assessing anti-EBOV activity using cell-based antiviral assays with wild-type EBOV typically measure cytopathic effect, plaque-forming units, infectious virus yield, and/or viral protein expression by ELISA [81, 84]. Recent advances in high-content imaging and analysis technology have enabled automated quantification of virus-infected cells by antibody-specific immunostaining [85]. Since the highly pathogenic nature of EBOV requires handling in a BSL-4 laboratory, work with live EBOVs has been relatively limited. Advances made in molecular biology over the past three decades, however, have enabled laboratories to study molecular mechanisms of viral entry, replication, and assembly outside of BSL-4 containment. A number of cell-based antiviral screens have utilized recombinant reporter viral vectors pseudotyped with EBOV glycoprotein in search of entry inhibitors [86–89]. Moreover, virus-like particles (VLPs) and transcription-competent VLPs, which better reflect EBOV particle morphology, have been used in a similar manner [72, 87, 90]. Recombinant plasmid minigenome systems have been developed to screen for replication inhibitors at BSL-2 containment. These systems consist of helper plasmids expressing EBOV replication components (NP, VP35, VP30, and L) along with a truncated genome reporter plasmid flanked by replication promoter leader and trailer sequences, and enable high-throughput screens of inhibitors targeting the EBOV replication complex [91–93]. To facilitate study of the EBOV life cycle in a system that better reflects authentic EBOV, Halfmann and colleagues generated a novel biologically contained VP30-deficient EBOV that replicates only when VP30 is provided in trans (via a stable VP30-expressing cell line), and thus can be used at a lower level of containment (BSL-3) [94]. For BSL-4 containment laboratories, the development of recombinant full-length EBOV genomes engineered to express fluorescent or bioluminescent reporter proteins have enabled high-throughput screens that preclude the additional labor and resources involved in preparing cell monolayers for antibody staining [91, 93, 95, 96].

3.5 *In Vivo* Toolbox: Animal Models for Efficacy Testing

Several animal models have been described for EBOV infection, including rodent and NHP models. More recently, ferrets have also been shown to be

susceptible to EBOV infection [97, 98]. Disease in mice requires serial passaging of ebolaviruses to generate mouse-adapted (MA) variants [99]. Similarly, guinea pig models are dependent on the use of guinea pig–adapted variants [100–103]. Disease in rodent models from wild-type (WT) virus infection is accomplished using animal strains with deficient immune systems, either innate (STAT-1$^{-/-}$ [104], IFNAR$^{-/-}$ [105]) or adaptive (severe combined immunodeficient [SCID] mice [104]). However, even in immunocompromised strains, infection with WT virus is not necessarily terminal or uniform; some virus variants (e.g. EBOV-Kikwit, SUDV-Boneface, SUDV-Gulu, and RESTV) cause nonterminal disease, and some virus species (e.g. BDBV) do not even result in weight loss [105, 106]. Humanized mice – more specifically, mice engrafted with human tissues and/or cells by a variety of approaches, resulting in reconstitution of human immune lymphoid and myeloid cells that are both present and functional to varying degrees – have been described in a series of recent studies as disease models of EBOV infection. In these mice, WT EBOV causes disease and can be uniformly lethal depending on experimental conditions [107–111].

NHPs remain the most accurate platform to model human filovirus infections, and the experience of the 2013–2016 EBOV outbreak demonstrated that they can predict the success of vaccines and therapeutics in humans. EBOV infection has been modeled in various NHP species: African green monkeys (*Chlorocebus* spp.) [15, 112], cynomolgus macaques (*Macaca fascicuaris*) [113], rhesus macaques (*Macaca mulatta*) [113–115], common marmosets (*Callithrix jacchus*) [116], and hamadryas baboons (*Papio hamadryas*) [117, 118]. Many aspects of filovirus pathogenesis can vary between NHP models, including aspects of coagulopathy (such as fibrin deposition, clotting, and hemorrhage), and may contribute to observed differences in efficacy of therapeutic trials in different NHP species [118–121].

Cynomolgus and rhesus macaques are the most commonly used NHP models for intervention studies. Clinical features (of EVD) in cynomolgus macaques mirror those in rhesus macaques. Endpoint disease in cynomolgus macaques tends to occur at 6–7 dpi, and in rhesus monkeys at 5–9 dpi [122]. Because disease is slightly more rapid in cynomolgus macaques, they have been preferred for vaccine studies; conversely, slightly delayed disease in rhesus macaques is favored for evaluating postexposure treatments [123]. Intramuscular (IM) challenge is almost universally used in EVD studies. Other routes of exposure have been evaluated, including oral and conjunctival [124], as well as aerosol studies to investigate intentional virus release or laboratory exposure [125]. Historical studies used a challenge dose of 1000 PFU to mimic needle stick exposure. A protracted disease course may be achieved by dose titration, as time to death was extended to 8–12 dpi in cynomolgus macaques exposed to low-dose IM EBOV challenge (~10 PFU) [126]. Despite the reported susceptibility of the NHP model to lethal disease from low-dose exposure, the 1000 PFU dose continues to be the standard used as a stringent assessment of interventions in NHP models, and often in other models of EVD.

The pathogenesis of EBOV infection has been investigated extensively, yet it remains complex. Because the virus is able to replicate in a wide variety of cell types, viremia leads to massive infection and necrosis of parenchymal cells of the liver, adrenal glands, and other organs. Liver and kidney function

is diminished, as measured by increased tissue enzymes and molecules in the blood. Disease, therefore, likely results from a combination of severe systemic inflammation and substantial tissue damage [119]. The NHP model recapitulates nearly all features of severe human disease, including prodrome signs, clinical analyte abnormalities, organ pathology, and immune dysregulation [123]. In mice, immune responses, virus replication, and overall pathology (including liver dysfunction) appear quite similar to NHP models. The main differences seen in the MA-EBOV mouse model is a lack of fibrin deposition and the lethality of the virus only when it is administered via the intraperitoneal (IP) route [119]. With the exception of an absence of hemorrhage and signs of disseminated intravascular coagulation (DIC) syndrome [112], and some variation in the extent and relative level of virus replication, guinea pig infection with adapted virus results in lesions similar to those reported in both NHPs and humans. Primary target cells of EBOV (macrophages, monocytes, Kupffer cells, and antigen-presenting cells) and common pathological features (e.g. diffuse lymphoid depletion, necrosis, and congestion in the spleens and lymph nodes) in guinea pigs also mirror those seen in NHPs [100]. Ferrets, the most recently described small animal model, demonstrate several characteristics of human filoviral disease following intranasal or intramuscular infection, including lymphopenia, thrombocytopenia, alongside hepatic and splenic pathology [97, 98].

The most efficient and accurate approach to progressive screening of EBOV therapeutic candidates *in vivo* is not known. Both small animal and NHP models of EVD are important in assessing promising therapeutics identified by *in vitro* characterization. Small animal models provide a feasible and economic means to translate demonstrated *in vitro* antiviral activity into a whole organism system. These studies provide important data that help prioritize assessment in NHP studies, which are more resource intensive and limited in size. Small animal models have proved useful for studying basic aspects of replication, pathogenesis, and immune responses, and have also served as critical platforms for evaluating a wide range of vaccines, antibodies, antisense molecules, and antiviral compounds [119]. Notable examples of data from small animal model studies mirroring results observed in NHP studies, and, most importantly, later in clinical application to human disease, include use of the guinea pig model in initial evaluation and characterization of the vesicular stomatitis virus (VSV) EBOV vaccine [127] and of a therapeutic combination of monoclonal antibodies (ZMapp) [128]. However, in some instances, small animal screening did not translate to protection in NHP studies. Venezuelan equine encephalitis replicon particles, recombinant *Vaccinia* virus expressing EBOV GP, and gamma-irradiated whole-virion preparations, all of which successfully protected rodents from lethal infection, failed to protect cynomolgus or rhesus macaques challenged with EBOV [129].

One limitation of currently available animal models is in studying the pathophysiology of disease sequelae. The majority of existing models that recapitulate EVD are extremely sensitive to virus challenge, customarily resulting in uniform requirement for euthanasia due to disease. In human EVD survivors, numerous physiological sequelae have been identified, including cardiopulmonary, ocular, gastrointestinal, cardiopulmonary, reproductive, and dermatologic effects [130].

Ideal treatment options would prevent both primary disease and subsequent sequelae. Adequately addressing this need would benefit from a model that is not uniformly lethal. While the pathogenesis of these sequelae conditions is challenging to investigate in current models, sequelae can be investigated in animals that survive infection as a result of treatment or vaccination. Viral persistence in immune-privileged sites is of particular interest. Recent reports using archived samples from NHP studies, and capturing rare samples from animals recovered from disease and those that were successfully treated, provide data for advanced treatment considerations and rational drug design [131]. The potential confounding effects of comparative physiology in the model (e.g. bioavailability) must be thoroughly considered prior to initiating studies and interpreting data. Selection of an appropriate animal model should be determined by the therapeutic target and the purported mechanism of action of the particular drug being evaluated. Basic antivirals that act by inhibiting replication can be prescreened in rodent models that are susceptible to disease due to widely disseminated and robust viral replication. Immunomodulatory therapeutics may be better screened in chimeric rodent models that incorporate human immune responses in a mouse background. While the utility of newer small animal models, including humanized mice and ferrets, in therapeutic screening has yet to be determined, these models offer promising opportunities. Importantly, the variety of available models permits multiple platform testing prior to clinical trials. This is pivotal for current and future identification of the most promising candidates for treating human disease.

3.6 Therapeutic Strategies

3.6.1 Host-Directed Antivirals

Like many other RNA viruses, EBOVs co-opt cellular systems to facilitate various aspects of their life cycle. To date, several different classes of host-targeted inhibitors have been shown to interfere with EBOV infection (Table 3.1). Improved characterization of the host cell pathway-specific host proteins involved in the EBOV life cycle provide potential targets against which to develop therapeutics.

3.6.1.1 *S*-Adenosyl-Homocysteine Hydrolase Inhibitors

Methylation plays a key role in viral replication, one such example being the 7-position methylation of guanosine in 5′-capped viral mRNAs [161]. One of the earliest host-protein-directed molecules identified with anti-EBOV activity *in vitro* and *in vivo* was carbocyclic 3-deazaadenosine (Ca-c^3 Ado) (Figure 3.2) [84, 132]. Ca-c^3 Ado indirectly inhibits viral mRNA cap methylation by blocking the activity of the enzyme *S*-adenosyl-homocysteine (SAH) hydrolase. Inhibiting SAH hydrolase results in accumulation of SAH, which acts as a negative feedback regulator of both viral and cellular methyltransferases, leading to reduction in viral gene expression [162]. A similar analog, 3-deazaneplanocin A (Figure 3.2), purported to have the same mechanism of action as Ca-c^3 Ado,

Table 3.1 Selected list of host-directed EBOV antivirals.

Host target	Drug ID	Cell-based assay(s) used	*In vivo* model(s) (% survival)[a)]	FDA/clinical trial status for non-EVD indication	References
S-adenosyl-homocysteine hydrolase inhibitor	Ca-c^3 Ado	LV	M (33–100%)	None	[84, 132]
	3-Deazaneplanocin A	LV	M (40–100%); NHP (0%)	None	[84, 132, 133]
Kinases/ phosphatases	BIBX 1382	PSE; LV	NT	None	[79]
	Genistein	PSE; LV	NT	None	[134]
	Tyrphostin AG1478	PSE; LV	NT	None	[134]
	Compound C	PSE; LV	NT	None	[135]
	Apilimod	PSE; VLP; LV	NT	Approved	[136]
	Erlotinib	PSE; LV	M (10–40%)	Approved	[137]
	Sunitinib	PSE; LV	M (30–40%)	Approved	[137]
	Nilotinib	VLP; LV	NT	Approved	[138]
	Imatinib	VLP; LV	NT	Approved	[138]
	1E7-03	MG; LV	NT	None	[139]
Protein folding	Geldanamycin	LV	NT	None	[140]
	Radicicol	LV	NT	None	[140]
	17AAG	LV	NT	None	[140]
	VER-155008	MG	NT	None	[141]
	(-) EGCG	LV	NT	Phase 3	[142]
	IHVR-19029	PSE; LV	M (30–80%)	None	[143, 144]
	UV-4B	LV	M (0–10%); GP (0%); NHP (0%)	None	[145]

Table 3.1 (Continued)

Host target	Drug ID	Cell-based assay(s) used	*In vivo* model(s) (% survival)[a)]	FDA/clinical trial status for non-EVD indication	References
Proteolytic processing	5705213	PSE	NT	None	[146]
	7402683	PSE	NT	None	[146]
	K11777	PSE	NT	None	[147]
	Teicoplanin	PSE	NT	Phase 4	[148, 149]
NPC1	3.0	PSE; LV	NT	None	[77]
	3.47	PSE; LV	NT	None	[77]
Ion channels	Amiodarone	PSE; LV	NT	Approved	[150]
	Tetrandrine	PSE; LV	M (62.5–75%)	None	[151]
	Verapamil	PSE; LV	NT	Approved	[150, 151]
Sphingosine, endosomal calcium/cholesterol regulation (cationic amphiphiles)	Clomiphene	VLP; LV	M (90%)	Approved	[152–154]
	Toremifene	VLP; LV	M (50%)	Approved	[152, 153]
	U18666a	PSE; LV	NT	None	[153, 154]
	Bepridil	PSE; VLP; LV	M (100%)	Approved	[155]
	Setraline	PSE; VLP; LV	M (70%)	Approved	[155]
	Tamoxifen	PSE	NT	Approved	[154]
Host immune response	KIN1408	LV	NT	None	[156]
	IFN-γ	LV	M (57–100%)	Approved	[157]
Translation (eIF4A)	Silvestrol	PSE; LV	NT	None	[158]
Anti-oxidant	CoPP	MG; REP	NT	None	[159]
	NSC 62914	LV	M (50–85%)	None	[160]

a) Range represents data reported from independent studies that may include varying dosing schedules, challenge dose, or species of model.
Abbreviations: EVD, Ebola virus disease; CADs, cationic amphiphilic drugs; eIF4A, eukaryotic initiation factor 4A; LV, live Ebola virus; MG, minigenome; PSE, pseudotyped virus; REP, VP30-deficient replicon EBOV; VLP, virus-like-particle; M, mouse model; GP, guinea pig model; NHP, nonhuman primate model; NT, not tested. Clinical trial statuses were retrieved from https://clinicaltrials.gov.

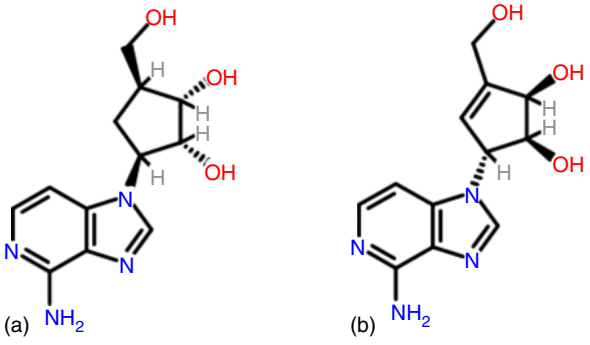

Figure 3.2 *S*-adenosyl-homocysteine hydrolase inhibitors. (a) Carboxylic 3-deazaadenosine [84, 132]. (b) 3-Deazaneplanocin A [84, 132, 133].

surprisingly induced high levels of the innate immune cytokine interferon-alpha in EBOV-infected mice, but did not show the same effect in NHPs [133].

3.6.1.2 Kinases and Phosphatases

Cellular processes are often regulated by kinases and phosphatases to either trigger or impede specific signaling pathways [163]. EBOV particles were shown to activate the phosphoinositol kinase-3 (PI3K)-Akt1 pathway to facilitate entry, as treatment of cells with inhibitors of PI3K, Akt1, or Rac-1 caused virus particle clustering and accumulation in a cytosolic vesicular compartment [164]. Subsequently, additional kinases involved in EBOV entry have been identified (i.e. AMP-activated protein kinase, Axl tyrosine kinase) using targeted antibodies or small molecule inhibitors (i.e. genistein, tyrphostin, compound C, BIBX 1382, apilimod, sunitinib, erlotinib) (Figure 3.3 and Table 3.1) [79, 134–137, 165]; a combination treatment of sunitinib and erlotinib demonstrated partial protection in the mouse model [137]. Several studies have identified specific cellular kinases and phosphatases that directly interact with EBOV proteins. The c-Abl1 tyrosine kinase (TK) has been implicated in phosphorylating VP40, enabling efficient budding of EBOV particles. Inhibition of c-Abl1 TK either by si-RNA knockdown or by inhibitors such as nilotonib and imatinib (Figure 3.3) showed significant decreases in VLP formation, with 20 μM of nilotinib effectively reducing infectious virus yield by more than three orders of magnitude [138]. With regard to viral replication, inhibition of protein phosphatase-1 enzyme (PP1)-mediated VP30 dephosphorylation by compound 1E7-03 (Figure 3.3) decreased viral transcription by way of accumulating the inactive phosphorylated form of VP30 [139]. Using specific peptide-binding inhibitors, however, a subsequent study delineated the recruitment of a related but distinct phosphatase (PP2A-B56) by EBOV NP in order to dephosphorylate VP30 [166].

3.6.1.3 Protein Folding and Processing

Chaperone proteins ensure protein function by facilitating their proper folding and stability, and have critical roles in the EBOV life cycle [167]. Several inhibitors targeting the ATP-binding site of heat shock protein (HSP) 90 have shown anti-EBOV activity with 50% effective inhibition concentrations (EC_{50}) in the high nanomolar to low micromolar range (17AAG, geldanamycin, radicicol)

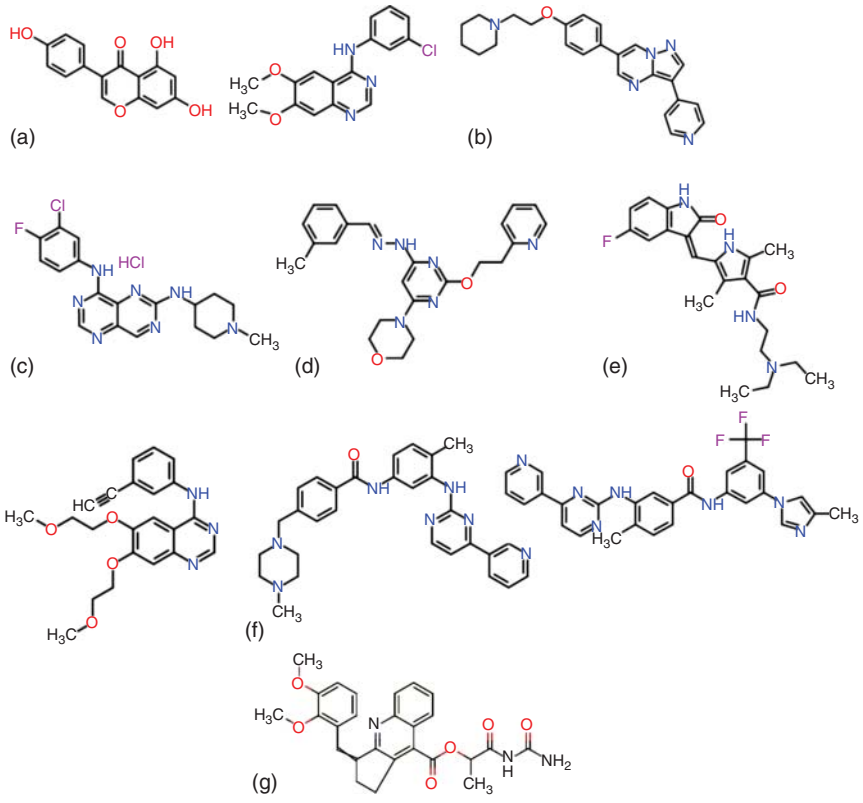

Figure 3.3 Kinase and phosphatase inhibitors. (a) Genistein and Tyrphostin. Source: Kolokoltsov et al. 2012 [134]. Reproduced with permission of Springer. (b) Compound C. Source: Kondratowicz et al. 2013 [135]. Reproduced with permission of ASM. (c) BIBX 1382. Source: Mohr et al. 2015 [79]. Reproduced with permission of Elsevier. (d) Apilimod. Source: Nelson et al. 2017 [136]. Reproduced with permission of PLOS. (e) Sunitinib and Erlotinib. Source: Bekerman et al. 2017 [137] Reproduced with permission of JCI Journal of Clinical Investigation. (f) Imatinib and Nilotinib. Source: Garcia et al. 2012 [138]. Reproduced with permission of Science Translational Medicine. (g) 1E7-03. Source: Ilinykh et al. 2014. [139]. Reproduced with permission of Journal of Biological Chemistry.

(Figure 3.4) [140, 168]. Along similar lines, a novel EBOV NP interaction assay was used together with co-immunoprecipitation and mass spectrometry experiments to identify a number of chaperones that interacted with NP, including HSP 70 and HSP 90. EBOV minigenome assays were then employed to confirm the anti-EBOV activity of an HSP 70 inhibitor (VER-155008) (Figure 3.4) [141]. Inhibition of an endoplasmic reticulum (ER) chaperone protein (HSP5A) either by a small molecule ((−)-epigallocatechin gallate [EGCG]) or by si-RNA knockdown reduced EBOV infection *in vitro*, while phosphorodiamidate morpholino oligomers (PMOs) targeted against HSPA5 expression *in vivo* completely protected mice from lethal EBOV infection [142].

Similar to chaperones, ER α-glucosidases facilitate proper folding and maturation of glycoproteins by ensuring their accurate N-glycosylation [169]. Inhibition

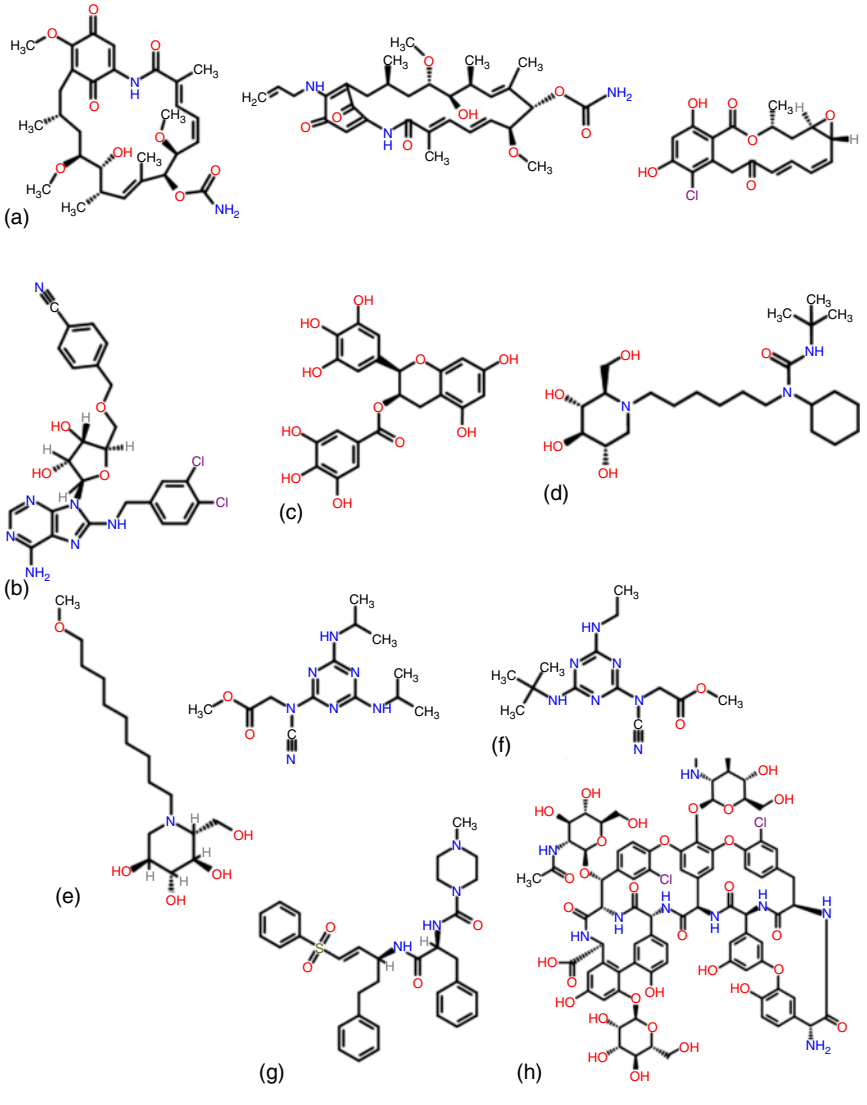

Figure 3.4 Protein folding and processing inhibitors. (a) Geldanamycin, Tanespimycin (17-AAG), and Radicicol. Source: Smith et al. 2010. [140]. Reproduced with permission of Elsevier. (b) VER-155008. Source: Garcia-Dorival et al. 2016 [141]. Reproduced with permission of ACS Publications. (c) (−) Epigallocatechin gallate (EGCG). Source: Reid et al. 2014 [142]. Reproduced with permission of Elsevier. (d) IHVR-19029 [143, 144]. (e) UV-4B. Source: Warfield et al. 2017 [145]. Reproduced with permission of Elsevier. (f) 5705213 and 7402683. Source: Elshabrawyet al. 2014 [146]. Reproduced with permission of ASM. (g) K11777 (K-777). Source: Zhou et al. 2015 [147]. Reproduced with permission of Elsevier. (h) Teicoplanin [148, 149].

of ER α-glucosidases I and II by imino sugar derivatives reduce EBOV GP pseudotyped HIV particle entry *in vitro*, and provide partial protection from EBOV morbidity and mortality in mice [143]. A follow-up study demonstrated synergistic *in vitro* anti-EBOV activity when treatment with a lead iminosugar (IHVR-19029) (Figure 3.4 and Table 3.1) was paired with a nucleoside analog Favipiravir (T-705) (see Section 3.6.2.4 regarding T-705) [144]. When this combination was evaluated *in vivo*, suboptimal doses of both compounds significantly increased the survival rate of infected mice. Treatment with a different imino sugar derivative (UV-4B) against EBOV infection in NHPs, however, did not show clinical or survival benefit, although this result may be attributed to a suboptimal dosing regimen [145].

Proteolytic processing of EBOV GP by endosomal/lysosomal proteases, cathepsins B and L (CTSB/L), is crucial for it to engage with its receptor NPC-1 [76, 77, 170, 171]. In order to identify potential CTSL inhibitors, Elshabrawy and colleagues developed a novel *in vitro* high-throughput screening assay which measured CTSL-mediated cleavage of viral glycopeptides in the presence or absence of a compound [146]. In utilizing this assay as an initial screen alongside a cell-based viral glycoprotein pseudotype entry assay for confirmation, they identified small molecules (5705213 and its derivative 7402683) (Figure 3.4) which showed inhibition against EBOV. Results from enzyme kinetic experiments indicated that 5705213 was a mixed inhibitor of CTSL, with a greater affinity for CTSL alone as compared to when CTSL was bound with a substrate peptide. A separate study queried the broad-spectrum antiviral activity of a vinylsulfone-class cysteine protease inhibitor (K11777) against virus glycoprotein-mediated entry from several virus families, which included the family *Filoviridae* (Figure 3.4). K11777 showed potent activity against entry by EBOV, SUDV, TAFV, and BDBV glycoprotein pseudotyped viruses at EC_{50} values in the high picomolar to low nanomolar range [147]. K11777 has potential for future *in vivo* testing against filovirus infections, since it has acceptable safety and pharmacokinetic profiles as determined by anti-parasite studies in dogs, rodents, and NHPs [172–174]. Subsequently, two independent FDA-approved drug library screens identified the cysteine protease inhibitor teicoplanin (a glycopeptide antibiotic) as an inhibitor of EBOV entry *in vitro* [148, 149] (Figure 3.4), underlining the potential for evaluating cysteine protease inhibitors for efficacy against EBOV in animal models.

3.6.1.4 Non-Proteolytic Endosomal Targets

NPC1 was identified as the EBOV receptor by two studies using different approaches. While one study applied a genome-wide haploid genetic screen [76], the other used a small-molecule hit identified from a compound library screen [77]. In this latter study, the initial hit was a benzylpiperazine adamantane diamide–derived compound (3.0, Figure 3.5) which reduced live EBOV growth by approximately two orders of magnitude at 96 hours postinfection. Further characterization of 3.0 alongside its more potent modified analog 3.47 (Figure 3.5) showed that both compounds directly interact with NPC1, effectively blocking the EBOV $GP_{1,2}$–NPC1 interaction (Figure 3.1). A subsequent study

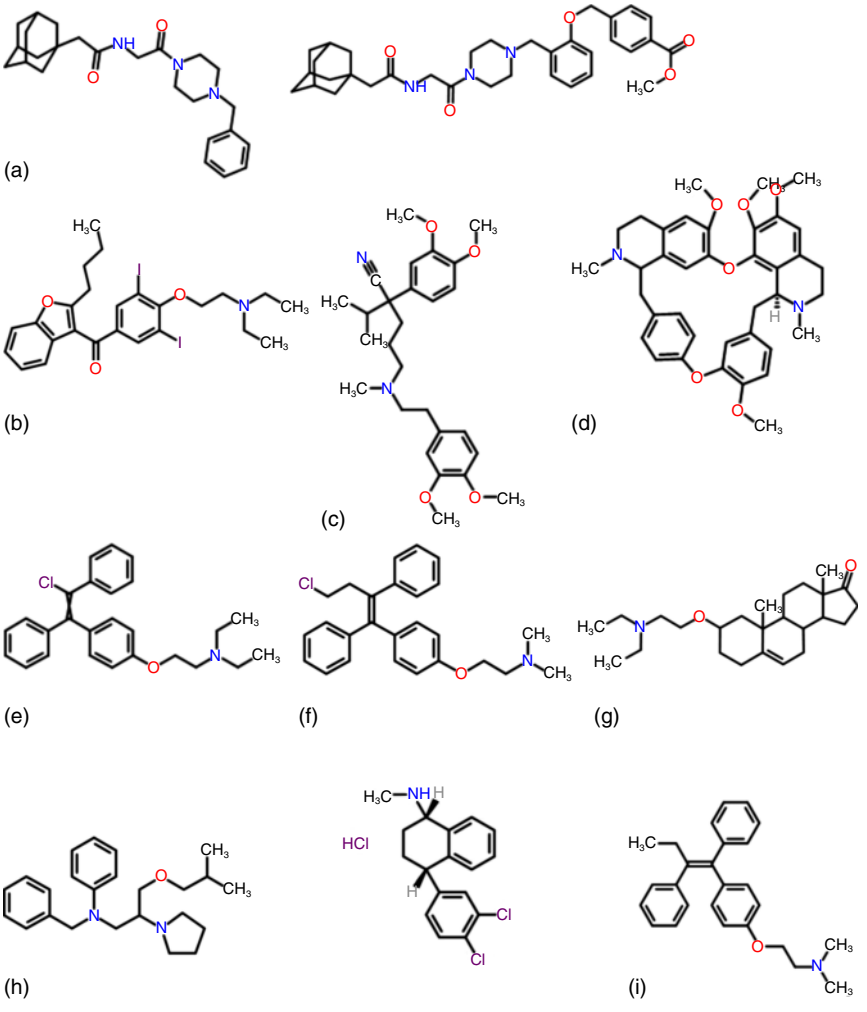

Figure 3.5 Non-proteolytic endosomal targets. (a) 3.0 and 3.47. Source: Cote et al. 2011 [77]. Reproduced with permission of Springer. (b) Amiodarone. Source: Gehring et al. 2014 [150]. Reproduced with permission of OUP. (c) Verapamil [150, 151]. (d) Tetrandrine. Source: Sakurai et al. 2015 [151]. Reproduced with permission of Science Mag. (e) Clomiphene [152–154]. (f) Toremifene [152, 153]. (g) U18666a [153, 154]. (h) Bepridil and SetralineHCl. Source: Johansen et al. 2015 [155]. Reproduced with permission of Science Mag. (i) Tamoxifen [154]. Source: Fan et al. 2017. Reproduced with permission of Springer.

identified two additional EBOV entry inhibitors sharing a similar mechanism of action, highlighting the potential for NPC1 as a viable therapeutic target [175]. A related but alternative strategy is to target host proteins such as T-cell immunoglobulin mucin domain 1 (TIM-1) that physically interact with NPC1. Kuroda and colleagues developed a monoclonal TIM-1 antibody that blocked its interaction with NPC1, which resulted in suppression of EBOV infection and GP-induced viral membrane fusion [176].

Ion channels have crucial roles in regulating endo-lysosomal function [177], and have been effectively targeted by FDA-approved inhibitors to block EBOV entry [150, 151]. Amiodarone (Figure 3.5), a multi-ion channel inhibitor for the treatment of arrhythmia was shown to inhibit EBOV entry at EC_{50} values which were 6- to 10-fold lower than typical therapeutic human serum levels during treatment for arrhythmia [150]. In the same study, inhibition of EBOV entry by the L-type calcium-channel-specific blocker verapamil indicated that calcium flux may specifically have a role in promoting EBOV entry (Figure 3.5). A subsequent study by Sakurai and colleagues not only confirmed this but more specifically identified the role of nicotinic acid adenine dinucleotide phosphate (NAADP)-stimulated two-pore channels (TPCs) in EBOV infection [151]. Furthermore, treatment of mice with the bis-benzylisoquinoline alkaloid tetrandrine (the most potent TPC inhibitor identified in the study) provided significant but not complete protection from mouse-adapted EBOV challenge (Figure 3.5 and Table 3.1).

Multiple independent FDA-approved drug screens have identified selective estrogen receptor modulators (SERMs) as EBOV entry inhibitors [87, 152, 153, 155]. Two of these studies demonstrated that SERMs (i.e. clomiphene, toremifene, bepridil, setraline) (Figure 3.5 and Table 3.1) had *in vivo* anti-EBOV activity in a mouse model, and that the mechanism of action of these SERMs were due to off-target effects affecting a cellular pathway unrelated to estrogen receptor signaling [152, 155]. Another study by the same group identified six additional SERMs that targeted the same biosynthetic pathway as clomiphene. These six inhibitors were all classified as cationic amphiphilic drugs (CADs), which induce endosomal cholesterol accumulation associated with defects in the NPC1 receptor [153]. A subsequent study seeking to clarify the mechanism of action of SERMs linked their anti-EBOV activity with depletion of cellular sphingosine and its downstream effect of accumulating endolysosomal calcium [154]. As opposed to endosomal cholesterol accumulation which was observed only after eight hours of CAD treatment (tamoxifen, clomiphene and U18666a), sphingosine depletion was clearly observed at just one hour post-CAD treatment, which better correlates with inhibition of viral entry that occurs within approximately two hours [178]. This result confirms and clarifies an earlier finding indicating that acid sphingomyelinase activity and sphingosine were crucial for efficient entry by EBOV [179]. It remains to be seen whether ion channel inhibitors and SERMs (as well as other CADs) can provide any protection against EBOV infection in NHPs.

3.6.1.5 Priming Host Immune Responses

An additional host-directed strategy to counter EBOV infection is to boost the host immune response. Upon detection of intracellular RNA from invading pathogens, RIG-I-like receptors (RLRs) promote an antiviral state by initiating innate immune signaling cascades through interferon-regulating factor 3 (IRF3), resulting in the induction of type I and type III interferons (IFN-α/β, IFN-λ) along with their downstream IFN-stimulated genes (ISGs) [180]. Pattabhi and colleagues identified a class of hydroxyquinoline compounds that activate IRF3 and induce antiviral gene expression [156]. Among these compounds, pretreatment

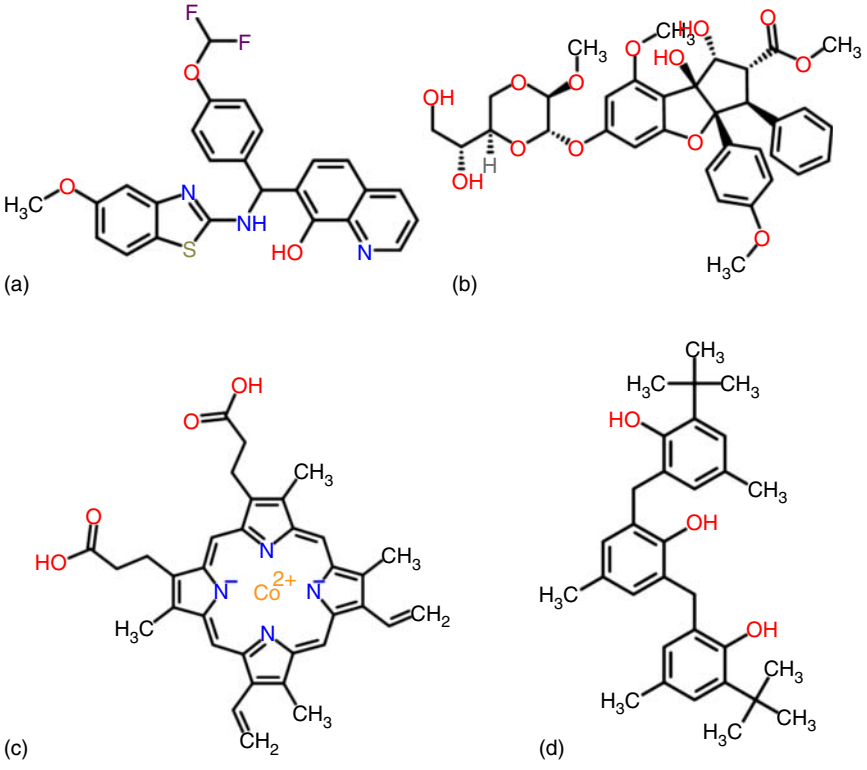

Figure 3.6 Other host-targeted inhibitors. (a) KIN1408. Source: Pattabhi et al. 2015 [156]. Reproduced with permission of ASM. (b) Silvestrol. Source: Biedenkopf et al. 2017 [158]. Reproduced with permission of Elsevier. (c) CoPP. Source: Hill-Batorski et al. 2013 [159]. Reproduced with permission of ASM. (d) NSC 62914. Source: Panchal et al. 2012 [160]. Reproduced with permission of Elsevier.

of cells with KIN1408 (Figure 3.6) was shown to reduce EBOV virus titers by 1.5 log plaque-forming units per mL [156]. In a similar manner, another study documented the *in vitro* anti-EBOV activity of a tetrahydrobenzothiazole compound which induces a type I IFN response [181]. While *in vivo* data against EBOV were lacking for both of these studies, innate immune agonists have shown efficacy against viral infections in small animal models [182, 183]. IFN-β therapy has been shown to prolong survival in EBOV-infected NHPs [184], but results from a human clinical trial in Guinea against EBOV have yet to become available [185].

While the near-ubiquitous intracellular innate immune response is based on general pattern recognition of foreign nucleic acids or antigens, the adaptive immune response is driven by a network of specialized immune cell types (i.e. macrophages, dendritic cells, T lymphocytes, B lymphocytes) working together to mount specific responses (both cell-mediated and humoral) against a particular pathogen [186]. In this regard, one study demonstrated a 1.5 log reduction in EBOV RNA levels in mouse peritoneal macrophages pretreated for 24 hours with type II interferon gamma (IFN-γ) [157]. Moreover, the initiation of IFN-γ treatment in mice anytime between 24 hours preinfection to 24 hours

postinfection with mouse-adapted EBOV provided significant clinical and survival benefit (Table 3.1). Reduction in plasma viremia, however, was only observed when IFN-γ was administered at zero and 24 hours postinfection. Results from this study indicate the potential use of IFN-γ as a postexposure prophylactic for EBOV infection.

3.6.1.6 Other Host Targets

Although the majority of host-directed antivirals inhibit EBOV entry, recent studies have identified elements of the host translational system that can be targeted by small-molecule inhibition. Blocking the hypusination (i.e. post-translational modification) of eukaryotic initiation factor 5A (eIF5A) inhibited the accumulation of VP30, and thereby blocked viral RNP-driven gene expression [187]. Similarly, inhibition of eIF4A-mediated cap-dependent translation initiation by a naturally derived compound silvestrol (Figure 3.6) potently inhibits EBOV gene expression in both primary human macrophages and human hepatoma cells [158]. Silvestrol-related toxicity has not been observed in anti-tumorigenic animal studies at therapeutic doses, which may portend well for future evaluation against EBOV in small animal models [188, 189].

Certain compounds with purported antioxidant activities have also shown anti-EBOV activity. Activating heme-oxygenase 1 (HO-1), an enzyme responsible for catalyzing heme degradation using cobalt protoporphyrin (CoPP) (Figure 3.6) significantly reduced EBOV replication in a dose-dependent manner [159]. A high-throughput screen identified a triphenol compound NSC 62914 (Figure 3.6) which was verified to have both anti-oxidant and anti-EBOV activity *in vitro*. Although it is unclear as to whether the antioxidant activity is linked to the anti-EBOV activity of NSC 62914, prophylactic treatment protected 80% of infected mice, while the post-challenge model protected up to 50% [160] (Table 3.1).

3.6.2 Direct-Acting Antivirals

3.6.2.1 Antibody-Based Therapeutics

Since the development and characterization of the first anti-EBOV neutralizing monoclonal antibody (mAb) KZ52 against EBOV GP [190], there have been numerous studies evaluating the efficacy of mAbs against EBOV in animal models [191]. Although conflicting results for KZ52 efficacy in guinea pigs versus rhesus macaques may have clouded initial prospects of mAbs as therapeutics (Table 3.2) [192, 193], subsequent studies leading up to the West African EVD outbreak clearly demonstrated the potential prophylactic and therapeutic use of mAb cocktails against EBOV in NHPs [196–200, 224, 225]. Amidst the outbreak, one particular mAb cocktail comprised of three antibodies (ZMapp) showed 100% efficacy against EBOV infection in rhesus macaques when treatment was initiated up to five days post-challenge [128, 194] (Table 3.2). However, when ZMapp was evaluated in a randomized, controlled trial during the West African EVD outbreak, there was no significant mortality benefit detected from those who received three infusions of ZMapp treatment [195]. Despite the outcome, recent studies have demonstrated robust efficacy of mAb treatment regimens

Table 3.2 Selected list of direct-acting EBOV antivirals.

Drug category	EBOV target	Drug ID	Cell-based assay(s) used	*In vivo* model(s) (% survival)[a)]	FDA/clinical trial status for EVD	References
Antibody	GP	KZ52	LV	GP (100%); NHP (0%)	None	[192, 193]
		ZMapp	PSE; LV	NHP (92%)	Phase 2	[128, 194, 195]
		ZMab	PSE; LV	M (100%); GP (50–100%); NHP (100%)	None	[194, 196, 197]
		MB-003	PSE: LV	NHP (43–66%)	None	[194, 198, 199]
		ch133/ch226	LV	NHP (66%)	None	[200]
		MIL77	NT	NHP (100%)	None	[201]
		mAb114	PSE	NHP (100%)	None	[202]
		mAb 6D6	PSE; LV	M (80–100%)	None	[203]
		m4B8	PSE; LV	M (80–100%)	None	[204]
	GP/NPC1	FMV09~548	PSE; LV	M (20%)	None	[205]
	$GP/GP_{1,2}$	FMV09~MR72	PSE: LV	M (70–100%)	None	[205]
Nucleic acid	VP35, L	TKM-130803	LV	GP (60%); NHP (66–100%)	None	[206–208]
	VP24	AVI-7537	LV	NHP (75%)	None	[209]
Nucleoside analog	L	Ribavirin	LV	NHP (0%)	None	[210]
		Brincidofovir	LV	NT	None	[211, 212]
		Galidesivir (BCX4430)	MG; LV	M (80–100%); NHP (0–100%)	Phase 1	[213, 214]
		Favipiravir (T-705)	LV	M (100%); NHP (0%)	Phase 2	[215–220]
		Remdesivir (GS-5734)	MG; LV	NHP (100%)	Phase 2	[80, 81, 83, 221–223]

a) Range represents data reported from independent studies that may include varying dosing schedules, challenge dose, or species of model.
Abbreviations: EVD, Ebola virus disease; LV, live Ebola virus; MG, minigenome; PSE, pseudotyped virus; M, mouse model; GP, guinea pig model; NHP, nonhuman primate model; NT, not tested. Inhibitors of viral protein interactions (18β glycerrhetic acid, licochalcone A) were not included in the list because they were neither evaluated in cell-based assays nor in animal models. Clinical trial statuses were retrieved from https://clinicaltrials.gov.

in NHPs using a simplified mAb cocktail (with only two antibodies) [201] or even using a single mAb [202]. Moreover, several groups have demonstrated the efficacy of pan-ebolavirus antibodies that target highly conserved regions of filovirus GPs [203, 204, 226]. Last but not least, recent innovations in antibody engineering have resulted in the development of a novel "Trojan horse" bispecific antibody strategy to inhibit filovirus infection [205]. Wec and colleagues coupled the variable domain from a mAb specific for the conserved surface-exposed glycan cap epitope of EBOV GP to another variable domain from mAbs that either recognize NPC1 or the NPC1 binding site on $GP_{1,2}$ [205]. Using this dual variable domain strategy allows for the bispecific antibodies to effectively enter cellar endosomes through uptake with the EBOV virion to which it is bound. Upon endosomal acidification and activation of $GP_{1,2}$, the second variable domain either directly blocks the receptor binding site on $GP_{1,2}$ (GP/$GP_{1,2}$-specific antibody) or alternatively targets NPC1 to block its interaction with $GP_{1,2}$ (GP/NPC1-specific antibody). In two different mouse models for mouse-adapted EBOV and SUDV, the GP/$GP_{1,2}$-specific antibody provided 70% and 100% protection, respectively (Table 3.2). The GP/NPC1-specific antibody did not work as well in the mouse models likely due to reduced binding to mouse NPC1, as there are significant species-dependent amino acid sequence differences compared to its human ortholog of NPC1 [205]. Future studies should evaluate the efficacy of both GP/$GP_{1,2}$-specific and GP/NPC1-specific antibodies in NHPs.

3.6.2.2 Inhibitors of Viral Protein Interactions

Just as molecular biology has greatly advanced the body of knowledge concerning EBOV genetics, biochemistry and structural biology have provided insight into interactions between EBOV proteins. NP–NP, NP–VP35, and VP35–L interactions have crucial roles in forming and securing a functional RNP complex [227–229]. NP interactions with VP30 and VP24 regulate RNA synthesis and enable genome packaging, respectively, while VP35–VP40 interaction was sufficient to allow for packaging of a minigenome into VLPs [230–233]. Recent studies indicate that destabilizing these interactions may be a viable strategy for developing antiviral therapeutics. N-terminal peptides from VP35 have been shown to regulate the oligomerization of NP, resulting in the release of RNA from NP-RNA complexes [229, 234]. The use of *in silico* screening alongside biochemistry, structural biology, and medicinal chemistry ultimately led to the development of pyrrolidinone-based small molecule compounds which block the NP–VP35 interaction [235]. On the other hand, a distinct mass spectrometry-metabolomics approach identified two compounds (18β glycerrhetic acid, licochalcone A) (Figure 3.7) isolated from licorice roots which dysregulate NP oligomerization and stability [236]; but their antiviral activities have yet to be confirmed in cell-based assays. High-throughput screens assaying the NP–VP35 interaction using fluorescence polarization may lead to future novel compounds with therapeutic potential [237].

Distinct from small-molecule and peptide inhibitors of protein interactions, RNA aptamers are nucleic acid sequences with high affinity and specificity to their target ligands [238]. While the technology to generate and select aptamers against viral proteins has been available for over two decades, only recently has

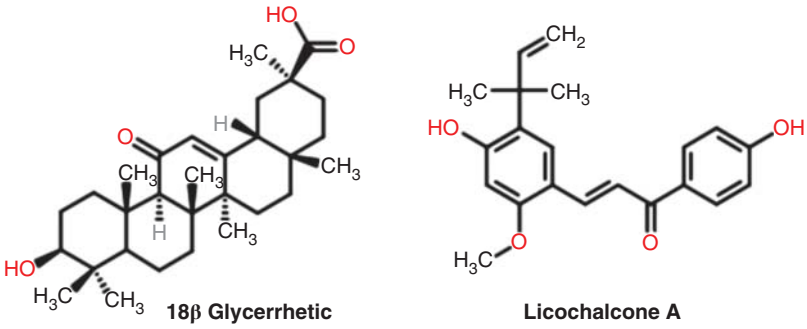

Figure 3.7 Viral protein interaction inhibitors. Source: Fu et al. 2016 [236]. Reproduced with permission of Springer.

there been interest toward developing RNA aptamers against EBOV proteins. Binning and colleagues developed RNA aptamers targeted against two basic patches within the dsRNA-binding interferon inhibitory domain of EBOV VP35 [239]. These aptamers disrupted the NP–VP35 interaction crucial to forming functional EBOV polymerase complexes, and were shown to inhibit reporter EBOV minigenome transcription and replication. Therapeutic aptamers have been developed for a number of human diseases including macular degeneration, choroidal neovascularization, intravascular thrombus, and acute coronary syndrome among many others, some of which already have FDA approval [240]. Although EBOV-targeted aptamers have not been tested *in vivo*, the significant existing body of research regarding aptamers will be valuable toward guiding future attempts to evaluate their efficacy.

3.6.2.3 Nucleic Acid Inhibitors

Another direct-acting anti-EBOV strategy is to reduce viral gene expression by either targeting viral mRNA for degradation by small interfering RNA (si-RNA) or by sterically blocking translation of viral mRNA using sequence-specific anti-sense PMOs [241]. Geisbert and colleagues initially demonstrated the postexposure prophylactic potential of lipid-encapsulated siRNAs specific to the L, VP24, and VP35 sequences from the EBOV Mayinga and Kikwit variants in both guinea pig and NHP models, with seven postexposure treatments required for complete protection of NHPs [206, 207]. A subsequent therapeutic NHP study performed during the West African EVD outbreak demonstrated 100% protective efficacy against the circulating Makona variant of EBOV when treatment with a cocktail of VP35 and L-specific siRNAs (specific to the Makona variant) commenced 3 dpi [208] (Table 3.2). Given this promising result, this particular cocktail of EBOV Makona variant–specific LNP si-RNAs (TKM-130803) was evaluated in a phase 2 clinical trial in Sierra Leone. Unfortunately, of the 14 patients who received intravenous infusion of TKM-130803 for up to 7 days post-admission, 11 died, which indicates that the treatment provided no improvement in patient survival compared to the control group [242, 243]. Since all the patients in this trial had very high viremia at the time of admission, the sheer magnitude of EBOV replication may have outweighed any antiviral effect provided by TKM-130803.

In 2006, several studies documented the *in vivo* anti-EBOV activities of individual and mixtures of PMOs specifically targeted against EBOV genes in the mouse, guinea pig, and NHP models [244, 245]. Ensuing chemical modifications of PMOs through conjugation with arginine-rich peptides (PMOplus) enhanced the efficacy of VP24-specific PMOs against EBOV infection in mice [246]. Pharmacokinetic and ascending dose studies were performed of an experimental combination of PMOplus compounds specific for EBOV VP24 and VP35 (AVI-6002), and was shown to be safe and well tolerated in humans [247]. A subsequent study determined that the VP24-specific PMOplus compound (AVI-7537) administered as a postexposure prophylactic was able to protect 75% of infected NHPs [209] (Table 3.2). These encouraging results indicate that AVI-7537 should be further developed as a potential EBOV therapeutic.

3.6.2.4 Nucleoside Analogs/Polymerase Inhibitors

Nucleoside analogs are a class of small-molecule antivirals which can directly inhibit viral transcription and replication by targeting the viral RNA-dependent RNA polymerase (RdRp). Several nucleoside analogs have been used as components of FDA-approved combination therapies against both human immunodeficiency virus (HIV) and hepatitis C virus (HCV) [248, 249]. One of the earliest described broad-spectrum antiviral nucleosides is the guanosine analog ribavirin (Figure 3.8), which has shown activity against other hemorrhagic fever viruses such as Lassa virus [250, 251], Hantaan virus [252], dengue virus

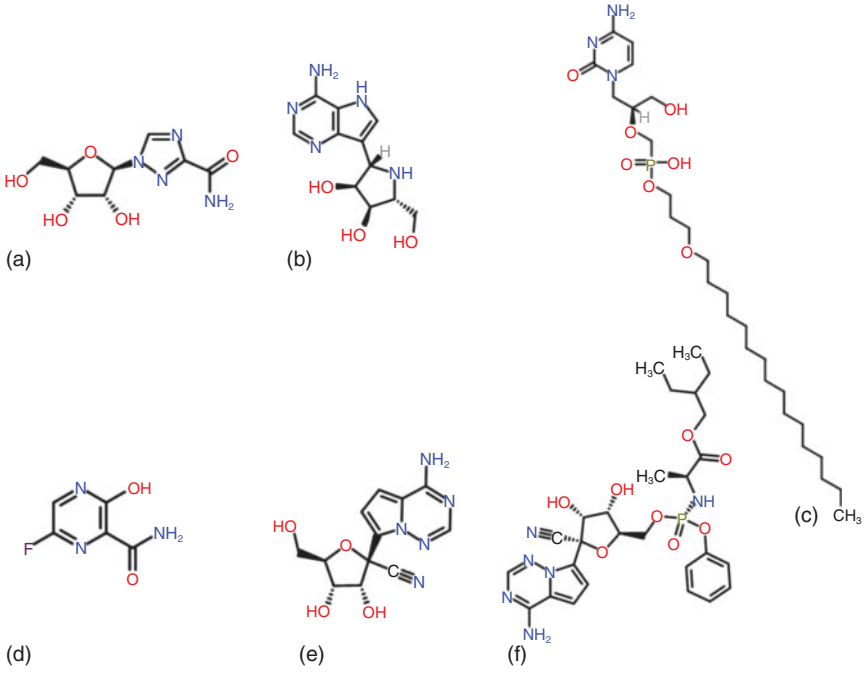

Figure 3.8 Nucleoside analog inhibitors. (a) Ribavirin. Source: Alfson et al. 2015 [210]. Reproduced with permission of ASM. (b) Galidesivir (BCX4430) [213, 214]. (c) Brincidofovir [211, 212]. (d) Favipiravir (T-705) [215–220]. (e) GS-441524 (Nuc) [80, 81, 83]. (f) Remdesivir(GS-5734) [80, 81, 83, 221–223].

[253], and yellow fever virus [254]. With its FDA-approved status, ribavirin's documented broad-spectrum activity via its multimodal mechanisms of action (lethal RNA mutagen, inhibition of cellular inosine monophosphate dehydrogenase [IMPDH], among several others) [255, 256] make it an attractive candidate for treatment of EVD. Although ribavirin has demonstrated *in vitro* antiviral activity against EBOV (EC_{50}: 27 μM) and provides partial *in vivo* protection in mice, it was only able to delay time to death in a cynomolgus macaque model [210] (Table 3.2). During the West African EVD outbreak, a clinical trial began for brincidofovir (Figure 3.8), which originally was developed as an anti-DNA virus treatment but was shown to have *in vitro* anti-EBOV activity due to its lipid moiety conjugated to the nucleotide analog [211]. However, due to low enrollment, the trial was discontinued [257]. Brincidofovir was also administered to three patients with EVD in the United States, but its contribution to survival could not be determined [212], and eventually its manufacturer withdrew its support as a EVD therapeutic [258].

Galidesivir (BCX4430) is an adenosine nucleoside analog that has shown broad-spectrum *in vitro* activity across nine RNA virus families including the filoviruses, and is currently in phase 1 clinical trials [213, 214, 259, 260]. The aza-sugar ring modification of BCX4430 enables it to act as a non-obligate RNA chain terminator against viral RdRps (Figure 3.8). BCX4430 inhibited both EBOV minigenome and live virus replication in a dose-dependent manner; and when administered orally and intramuscularly within four hours of infection with mouse-adapted EBOV, protected 80–100% of mice [214]. In NHPs, however, a dose range study (3.4–16 mg/kg BID) in the cynomolgus macaque model in which BCX4430 was administered 48 hours post-EBOV infection extended time to death at the highest dose, but failed to provide survival benefit. Only when BCX4430 was administered at a higher dose (25 mg/kg IM BID) within one hour of EBOV infection was there complete protection in a different rhesus macaque NHP model [213] (Table 3.2). While preliminary findings from phase 1 clinical trials of BCX4430 are positive, further studies are needed to address several areas regarding optimal dosage regimens, pharmacological kinetics in animal models, as well as to define maximum tolerable dose in humans.

Favipiravir (T-705) (Figure 3.8) is a modified pyrazine analog which was initially described as a potent inhibitor of influenza A virus (IAV) replication both in cell culture and in a mouse model of infection [261]. The ribosylated triphosphorylated form of T-705 was then shown to inhibit IAV polymerase nascent strand synthesis in a dose-dependent manner [262]. Since then, T-705 has shown *in vitro* and *in vivo* antiviral activity against a host of RNA viruses across multiple negative and positive strand virus families (reviewed in [263]). In 2014, two complementary studies documented the *in vitro* and *in vivo* activity of T-705 against inhalational EBOV infection using immune-deficient mouse models [215, 216]. While one study demonstrated the efficacy of T-705 as an anti-EBOV postexposure prophylactic [216], the other study determined the therapeutic window for T-705 treatment to be up to 6 dpi [215]. A subsequent study not only confirmed the prophylactic effect of T-705 against intraperitoneal EBOV infection but also investigated the pharmacokinetics of T-705 in cell culture and in mice [217]. Furthermore, they demonstrated through

dose de-escalation experiments in mice that the protective dose of T-705 was between 1.6 and 8 mg/kg/d. In contrast, this same group found that two different T-705 treatment dosage regimens against EBOV in NHP only delayed the time until disease endpoints were reached, but did not provide any survival benefit [218] (Table 3.2). The lack of survival benefit against EBOV may be attributed to the oral dosing route, as intravenous administration of T-705 in NHP against infection by a related filovirus Marburg virus (MARV) resulted in 83% protection. Since T-705 is an orally available drug that has shown a good safety profile in thousands of patients worldwide, the French drug safety agency (ANSM) approved it for compassionate use in patients with EVD [264]. In late 2014, separate studies conducted in Guinea and Sierra Leone treated EBOV-infected patients with T-705. The controlled trial conducted at the Sierra Leone-China Friendship Hospital administered T-705 to 39 patients (17–39 years old) at doses based on recommendations for use in influenza infections, along with supportive treatments [219]. Results from this study indicated a significantly lower mortality rate in the T-705 treatment group, along with significant improvement of disease symptoms and concomitant decrease in viremia [219, 243]. However, in the single-arm, historically controlled trial conducted across four treatment centers in Guinea which enrolled 111 patients (1–99 years old), despite administering higher initial doses of T-705 than in the trial in Sierra Leone, it was shown that T-705 only had a detectable antiviral effect in patients with medium to high viremia (qtr.-PCR $Ct \geq 20$), as opposed to those with very high viremia (qRT-PCR $Ct < 20$) [220, 264]. A subsequent pharmacokinetic study of 66 patients from the JIKI trial in Guinea indicated that T-705 plasma concentrations failed to reach the target exposure levels as predicted [265]. It is notable that the T-705-treated patients in the Sierra Leone study all had medium to high viremia, which may partly explain the relative success in patient outcomes observed [219]. Taken together, these two studies provide a foundation by which to design future studies to optimize T-705 dosing so as to attain maximum antiviral potency against EVD in humans.

In April 2014 amidst the West African EVD outbreak, a collaboration between Gilead Sciences, the US Centers for Disease Control and Prevention (CDC), and the US Army Medical Research Institute in Infectious Diseases (USAMRIID) resulted in the discovery of a nucleotide analog Remdesivir (GS-5734) which showed very promising therapeutic efficacy against Ebola virus in NHPs [80, 81, 83]. GS-5734 is a monophosphoramidate nucleotide prodrug of the parent adenosine nucleoside analog GS-441524 (Nuc), which was shown to inhibit viruses across *Filo-*, *Paramyxo-*, *Pneumo-,* and *Coronaviridae* families in the single-digit micromolar EC_{50} range [83, 266] (Figure 3.8). The prodrug of the parent increased the potency of GS-5734 over Nuc by 20 to 100-fold depending on the assay and cell type used. Furthermore, GS-5734 was able to reduce infectious virus titers by over 4 orders of magnitude at low nanomolar potencies in primary macrophages and a hepatic cell line, both of which represent primary cellular targets of EBOV infection [81, 83]. Due to the presence of serum carboxylesterases which inactivate the prodrug moiety of GS-5734, typical small rodent models for EBOV infection were not suitable to evaluate the efficacy of GS-5734. Pharmacokinetic studies in NHPs (cynomolgus macaques)

showed rapid conversion of GS-5734 to its active triphosphate form as well as its distribution into cells of mononuclear origin. Moreover, radiolabeled GS-5734 and its metabolites were detected in various tissues including testes, eye, and brain within 4 hours of administration. Intravenous administration of a 10 mg/kg loading dose of GS-5734 on day 3 post-EBOV infection followed by daily doses of 3 or 10 mg/kg for 12 days provided complete protection in the rhesus macaque NHP model for EBOV infection (Table 6.2). The rapid pharmacokinetics of GS-5734, along with its tissue and cellular targeting, makes it a suitable candidate for postexposure and therapeutic interventions. An ongoing phase 2 clinical trial is evaluating whether five doses of GS-5734 can accelerate eradication of EBOV RNA from the semen of convalescent survivors of EVD [221]. GS-5734 has been administered to a nurse who suffered EBOV-related relapsing meningoencephalitis, in which levels of EBOV RNA in her cerebrospinal fluid gradually decreased with commencement of treatment and was undetectable after 14 days [222]. In addition, a combination treatment of ZMapp, a buffy coat transfusion from another EBOV survivor, and a 14-day regimen of GS-5734 resulted in survival of the first neonate born to a mother diagnosed with EBOV infection [223]. While these cases of survival in response to GS-5734 treatment are encouraging, further studies are needed in regard to clinical efficacy and mechanism of action to better characterize the potency of GS-5734 against EVD in humans.

3.7 Conclusions

From the initial outbreak of EVD until the present, a significant body of EBOV research *in vitro* and *in vivo* has resulted in development of multiple therapeutic strategies to fight this highly pathogenic disease. While preclinical development of novel small-molecule therapeutics against the Ebola viruses should undoubtedly continue, future studies should provide more detailed pharmacokinetic data for the most promising therapeutics in humans to optimize future dosing regimens; and should also determine the optimal synergistic drug combinations that can be feasibly deployed in future EVD outbreaks.

Acknowledgments

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References

1 Report of an International Commission. (1978). Ebola haemorrhagic fever in Zaire, 1976. *Bull. World Health Organ.* 56: 271–293.
2 Report of a WHO/International Study Team (1978). Ebola haemorrhagic fever in Sudan, 1976. *Bull. World Health Organ.* 56: 247–270.

3 CDC. (2018). Ebola virus disease distribution map. https://www.cdc.gov/vhf/ebola/outbreaks/history/distribution-map.html (02/25).
4 CDC. (2017). Ebola (Ebola virus disease) signs and symptoms. https://www.cdc.gov/vhf/ebola/symptoms/index.html (accessed 5 March 2019).
5 Kortepeter, M.G., Bausch, D.G., and Bray, M. (2011). Basic clinical and laboratory features of filoviral hemorrhagic fever. *J. Infect. Dis.* 204 (Suppl. 3): S810–S816.
6 Towner, J.S., Rollin, P.E., Bausch, D.G. et al. (2004). Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. *J. Virol.* 78: 4330–4341.
7 Formenty, P., Leroy, E.M., Epelboin, A. et al. (2006). Detection of Ebola virus in oral fluid specimens during outbreaks of Ebola virus hemorrhagic fever in the Republic of Congo. *Clin. Infect. Dis.* 42: 1521–1526.
8 Bausch, D.G., Towner, J.S., Dowell, S.F. et al. (2007). Assessment of the risk of Ebola virus transmission from bodily fluids and fomites. *J. Infect. Dis.* 196 (Suppl. 2): S142–S147.
9 Hayman, D.T., Yu, M., Crameri, G. et al. (2012). Ebola virus antibodies in fruit bats, Ghana, West Africa. *Emerg. Infect. Dis.* 18: 1207–1209.
10 Olival, K.J., Islam, A., Yu, M. et al. (2013). Ebola virus antibodies in fruit bats, bangladesh. *Emerg. Infect. Dis.* 19: 270–273.
11 Swanepoel, R., Smit, S.B., Rollin, P.E. et al. (2007). Studies of reservoir hosts for Marburg virus. *Emerg. Infect. Dis.* 13: 1847–1851.International Scientific and Technical Committee for Marburg Hemorrhagic Fever Control in the Democratic Republic of Congo.
12 Towner, J.S., Pourrut, X., Albarino, C.G. et al. (2007). Marburg virus infection detected in a common African bat. *PLoS One* 2: e764.
13 VanSteelandt, A., Aho, J., Franklin, K. et al. (2017). Operational evaluation of rapid diagnostic testing for Ebola virus disease in Guinean laboratories. *PLoS One* 12: e0188047.
14 Phan, J.C., Pettitt, J., George, J.S. et al. (2016). Lateral flow immunoassays for Ebola virus disease detection in Liberia. *J. Infect. Dis.* 214: S222–S228.
15 Davis, K.J., Anderson, A.O., Geisbert, T.W. et al. (1997). Pathology of experimental Ebola virus infection in African green monkeys. Involvement of fibroblastic reticular cells. *Arch. Pathol. Lab. Med.* 121: 805–819.
16 Sissoko, D., Keita, M., Diallo, B. et al. (2017). Ebola virus persistence in breast milk after no reported illness: a likely source of virus transmission from mother to child. *Clin. Infect. Dis.* 64: 513–516.
17 Christie, A., Davies-Wayne, G.J., Cordier-Lasalle, T. et al. (2015, 2015). Possible sexual transmission of Ebola virus — Liberia. *Morb. Mortali. Wkly. Rep.* 64: 479–481.
18 Deen, G.F., Broutet, N., Xu, W. et al. (2017). Ebola RNA persistence in semen of Ebola virus disease survivors - final report. *N. Engl. J. Med.* 377: 1428–1437.
19 Sow, M.S., Etard, J.F., Baize, S. et al. (2016). New evidence of long-lasting persistence of Ebola virus genetic material in semen of survivors. *J. Infect .Dis.* 214: 1475–1476.

20 Uyeki, T.M., Erickson, B.R., Brown, S. et al. (2016). Ebola virus persistence in semen of male survivors. *Clin. Infect. Dis.* 62: 1552–1555.

21 Rowe, A.K., Bertolli, J., Khan, A.S. et al. (1999). Clinical, virologic, and immunologic follow-up of convalescent Ebola hemorrhagic fever patients and their household contacts, Kikwit, Democratic Republic of the Congo. *J. Infect. Dis.* 179: S28–S35.

22 Green, E., Hunt, L., Ross, J.C.G. et al. (2016). Viraemia and Ebola virus secretion in survivors of Ebola virus disease in Sierra Leone: a cross-sectional cohort study. *Lancet Infect. Dis.* 16: 1052–1056.

23 Pettitt, J., Higgs, E.S., Adams, R.D. et al. (2016). Use of existing diagnostic reverse-transcription polymerase chain reaction assays for detection of Ebola virus RNA in semen. *J. Infect. Dis.* 213: 1237–1239.

24 Erickson, B.R., Sealy, T.K., Flietstra, T. et al. (2016). Ebola virus disease diagnostics, Sierra Leone: analysis of real-time reverse transcription-polymerase chain reaction values for clinical blood and oral swab specimens. *J. Infect. Dis.* 214: S258–S262.

25 Ellerbrok, H., Jacobsen, S., Patel, P. et al. External quality assessment study for ebolavirus PCR-diagnostic promotes international preparedness during the 2014 – 2016 Ebola outbreak in West Africa. *PLoS Negl. Trop. Dis.* 2017 (11): e0005570.

26 Southern, T.R., Racsa, L.D., Albarino, C.G. et al. (2015). Comparison of filmarray and quantitative real-time reverse transcriptase PCR for detection of zaire Ebolavirus from contrived and clinical specimens. *J. Clin. Microbiol.* 53: 2956–2960.

27 Cherpillod, P., Schibler, M., Vieille, G. et al. (2016). Ebola virus disease diagnosis by real-time RT-PCR: a comparative study of 11 different procedures. *J. Clin. Virol.* 77: 9–14.

28 Rieger, T., Kerber, R., El Halas, H. et al. (2016). Evaluation of realstar reverse transcription-polymerase chain reaction kits for filovirus detection in the laboratory and field. *J. Infect. Dis.* 214: S243–S249.

29 Pinsky, B.A., Sahoo, M.K., Sandlund, J. et al. (2015). Analytical performance characteristics of the cepheid GeneXpert Ebola assay for the detection of Ebola virus. *PLoS One* 10: e0142216.

30 Towner, J.S., Sealy, T.K., Khristova, M.L. et al. (2008). Newly discovered Ebola virus associated with hemorrhagic fever outbreak in Uganda. *PLoS Pathog.* 4: e1000212.

31 Sanchez, A., Ksiazek, T.G., Rollin, P.E. et al. (1999). Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. *J. Infect. Dis.* 179 (Suppl. 1): S164–S169.

32 Ksiazek, T.G., Rollin, P.E., Jahrling, P.B. et al. (1992). Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. *J. Clin. Microbiol.* 30: 947–950.

33 Niikura, M., Ikegami, T., Saijo, M. et al. (2001). Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. *J. Clin. Microbiol.* 39: 3267–3271.

34 Lucht, A., Grunow, R., Möller, P. et al. (2003). Development, characterization and use of monoclonal VP40-antibodies for the detection of Ebola virus. *J. Virol. Met.* 111: 21–28.

35 Cross, R.W. and Ksiazek, T.G. (2017). ELISA methods for the detection of Ebolavirus infection. In: *Methods in Molecular Biology*, vol. 1628 (ed. T. Hoenen and A. Groseth), 363–372. Humana Press.

36 Emond, R.T., Evans, B., Bowen, E.T., and Lloyd, G. (1977). A case of Ebola virus infection. *Br. Med. J.* 2: 541–544.

37 Report of an International Commission. (1978). Ebola haemorrhagic fever in Zaire, 1976. *Bull. World Health Organ.* 56: 271–293.

38 van der Groen, G., Jacob, W., and Pattyn, S.R. (1979). Ebola virus virulence for newborn mice. *J. Med. Virol.* 4: 239–240.

39 Johnson, K.M., Lange, J.V., Webb, P.A., and Murphy, F.A. (1977). Isolation and partial characterisation of a new virus causing acute haemorrhagic fever in Zaire. *Lancet* 1: 569–571.

40 Bowen, E.T., Lloyd, G., Harris, W.J. et al. (1977). Viral haemorrhagic fever in southern Sudan and northern Zaire. Preliminary studies on the aetiological agent. *Lancet* 1: 571–573.

41 Pattyn, S., van der Groen, G., Jacob, W. et al. (1977). Isolation of Marburg-like virus from a case of haemorrhagic fever in Zaire. *Lancet* 1: 573–574.

42 Ellis, D.S., Simpson, I.H., Francis, D.P. et al. (1978). Ultrastructure of Ebola virus particles in human liver. *J. Clin. Pathol.* 31: 201–208.

43 Zaki, S.R., Shieh, W.J., Greer, P.W. et al. (1999). A novel immunohistochemical assay for the detection of Ebola virus in skin: implications for diagnosis, spread, and surveillance of Ebola hemorrhagic fever. Commission de Lutte contre les Epidemies a Kikwit. *J. Infect. Dis.* 179 (Suppl. 1): S36–S47.

44 Jahrling, P.B., Geisbert, T.W., Dalgard, D.W. et al. (1990). Preliminary report: isolation of Ebola virus from monkeys imported to USA. *Lancet* 335: 502–505.

45 Baize, S., Pannetier, D., Oestereich, L. et al. (2014). Emergence of Zaire Ebola virus disease in Guinea. *N. Engl. J. Med.* 371: 1418–1425.

46 Koehler, J.W., Hall, A.T., Rolfe, P.A. et al. (2014). Development and evaluation of a panel of filovirus sequence capture probes for pathogen detection by next-generation sequencing. *PLoS One* 9: e107007.

47 Maganga, G.D., Kapetshi, J., Berthet, N. et al. (2014). Ebola virus disease in the Democratic Republic of Congo. *N. Engl. J. Med.* 371: 2083–2091.

48 Matranga, C.B., Andersen, K.G., Winnicki, S. et al. (2014). Enhanced methods for unbiased deep sequencing of Lassa and Ebola RNA viruses from clinical and biological samples. *Genome Biol.* 15: 519.

49 Ksiazek, T.G., Rollin, P.E., Williams, A.J. et al. (1999). Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. *J. Infect. Dis.* 179: S177–S187.

50 Baize, S., Leroy, E.M., Georges-Courbot, M.C. et al. (1999). Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. *Nat. Med.* 5: 423–426.

51 van der Groen, G., Kurata, T., and Mets, C. (1983). Modifications to indirect immunofluorescence tests on Lassa, Marburg, and Ebola material. *Lancet* 1: 654.

52 Ksiazek, T.G., West, C.P., Rollin, P.E. et al. (1999). ELISA for the detection of antibodies to Ebola viruses. *J. Infect. Dis.* 179 (Suppl. 1): S192–S198.

53 Macneil, A., Reed, Z., and Rollin, P.E. (2011). Serologic cross-reactivity of human IgM and IgG antibodies to five species of Ebola virus. *PLoS Negl. Trop. Dis.* 5: e1175.

54 Rimoin, A.W., Lu, K., Bramble, M.S. et al. (2018). Ebola virus neutralizing antibodies detectable in survivors of the Yambuku, Zaire outbreak 40 years after infection. *J. Infect. Dis.* 217: 223–231.

55 Natesan, M., Jensen, S.M., Keasey, S.L. et al. (2016). Human survivors of disease outbreaks caused by Ebola or Marburg virus exhibit cross-reactive and long-lived antibody responses. *Clin. Vaccine Immunol.* 23: 717–724.

56 Glynn, J.R., Bower, H., Johnson, S. et al. (2017). Asymptomatic infection and unrecognised Ebola virus disease in Ebola-affected households in Sierra Leone: a cross-sectional study using a new non-invasive assay for antibodies to Ebola virus. *Lancet Infect. Dis.* 17: 645–653.

57 Spengler, J.R., McElroy, A.K., Harmon, J.R. et al. (2015). Relationship between Ebola virus real-time quantitative polymerase chain reaction-based threshold cycle value and virus isolation from human plasma. *J. Infect. Dis.* 212 (Suppl. 2): S346–S349.

58 Yoshida, R., Muramatsu, S., Akita, H. et al. (2016). Development of an immunochromatography assay (QuickNavi-Ebola) to detect multiple species of Ebolaviruses. *J. Infect. Dis.* 214: S185–S191.

59 Kurosaki, Y., Magassouba, N., Oloniniyi, O.K. et al. (2016). Development and evaluation of reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay coupled with a portable device for rapid diagnosis of Ebola virus disease in guinea. *PLoS Negl. Trop. Dis.* 10: e0004472.

60 Li, H., Wang, X., Liu, W. et al. (2015). Survey and visual detection of zaire ebolavirus in clinical samples targeting the nucleoprotein gene in sierra leone. *Front Microbiol.* 6: 1332.

61 Benzine, J.W., Brown, K.M., Agans, K.N. et al. (2016). Molecular diagnostic field test for point-of-care detection of Ebola virus directly from blood. *J. Infect. Dis.* 214: S234–S242.

62 Liu, J., Ochieng, C., Wiersma, S. et al. (2016). Development of a TaqMan array card for acute-febrile-illness outbreak investigation and surveillance of emerging pathogens, including Ebola virus. *J. Clin. Microbiol.* 54: 49–58.

63 Daaboul, G.G., Freedman, D.S., Scherr, S.M. et al. (2017). Enhanced light microscopy visualization of virus particles from Zika virus to filamentous ebolaviruses. *PLoS One* 12: e0179728.

64 Kainulainen, M.H., Nichol, S.T., Albarino, C.G., and Spiropoulou, C.F. (2017). Rapid determination of ebolavirus infectivity in clinical samples using a novel reporter cell line. *J. Infect. Dis.* 216: 1380–1385.

65 McElroy, A.K., Erickson, B.R., Flietstra, T.D. et al. (2014). Ebola hemorrhagic fever: novel biomarker correlates of clinical outcome. *J. Infect. Dis.* 210: 558–566.

66 McElroy, A.K., Erickson, B.R., Flietstra, T.D. et al. (2014). Biomarker correlates of survival in pediatric patients with Ebola virus disease. *Emerg. Infect. Dis.* 20: 1683–1690.

67 McElroy, A.K., Harmon, J.R., Flietstra, T.D. et al. (2016). Kinetic analysis of biomarkers in a cohort of US patients with Ebola virus disease. *Clin. Infect. Dis.* 63: 460–467.

68 Baize, S., Leroy, E.M., Georges, A.J. et al. (2002). Inflammatory responses in Ebola virus-infected patients. *Clin. Exp. Immunol.* 128: 163–168.

69 Liu, X., Speranza, E., Munoz-Fontela, C. et al. (2017). Transcriptomic signatures differentiate survival from fatal outcomes in humans infected with Ebola virus. *Genome Biol.* 18: 4.

70 Ruibal, P., Oestereich, L., Ludtke, A. et al. (2016). Unique human immune signature of Ebola virus disease in Guinea. *Nature* 533: 100–104.

71 Duy, J., Koehler, J.W., Honko, A.N. et al. (2016). Circulating microRNA profiles of Ebola virus infection. *Sci. Rep.* 6: 24496.

72 Watt, A., Moukambi, F., Banadyga, L. et al. (2014). A novel life cycle modeling system for Ebola virus shows a genome length-dependent role of VP24 in virus infectivity. *J. Virol.* 88: 10511–10524.

73 Groseth, A. and Hoenen, T. (1628). Forty years of Ebolavirus molecular biology: understanding a novel disease agent through the development and application of new technologies. *Methods Mol. Biol.* 2017: 15–38.

74 Hunt, C.L., Lennemann, N.J., and Maury, W. (2012). Filovirus entry: a novelty in the viral fusion world. *Viruses* 4: 258–275.

75 Quinn, K., Brindley, M.A., Weller, M.L. et al. (2009). Rho GTPases modulate entry of Ebola virus and vesicular stomatitis virus pseudotyped vectors. *J. Virol.* 83: 10176–10186.

76 Carette, J.E., Raaben, M., Wong, A.C. et al. (2011). Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* 477: 340–343.

77 Cote, M., Misasi, J., Ren, T. et al. (2011). Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. *Nature* 477: 344–348.

78 Simmons, J.A., D'Souza, R.S., Ruas, M. et al. (2016). Ebolavirus glycoprotein directs fusion through NPC1+ endolysosomes. *J. Virol.* 90: 605–610.

79 Mohr, E.L., McMullan, L.K., Lo, M.K. et al. (2015). Inhibitors of cellular kinases with broad-spectrum antiviral activity for hemorrhagic fever viruses. *Antiviral Res.* 120: 40–47.

80 Siegel, D., Hui, H.C., Doerffler, E. et al. (2017). Discovery and synthesis of a phosphoramidate prodrug of a pyrrolo[2,1-f][triazin-4-amino] adenine C-nucleoside (GS-5734) for the treatment of Ebola and emerging viruses. *J. Med. Chem.* 60: 1648–1661.

81 Warren, T.K., Jordan, R., Lo, M.K. et al. (2016). Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. *Nature* 531: 381–385.

82 Cong, Y., Dyall, J., Hart, B.J. et al. (2016). Evaluation of the activity of lamivudine and zidovudine against Ebola virus. *PLoS One* 11: e0166318.

83 Lo, M.K., Jordan, R., Arvey, A. et al. (2017). GS-5734 and its parent nucleoside analog inhibit Filo-, Pneumo-, and Paramyxoviruses. *Sci. Rep.* 7: 43395.

84 Huggins, J., Zhang, Z.X., and Bray, M. (1999). Antiviral drug therapy of filovirus infections: S-adenosylhomocysteine hydrolase inhibitors inhibit Ebola virus in vitro and in a lethal mouse model. *J. Infect. Dis.* 179 (Suppl. 1): S240–S247.

85 Mudhasani, R., Kota, K.P., Retterer, C. et al. (2015). High-content image-based screening of a signal transduction pathway inhibitor small-molecule library against highly pathogenic RNA viruses. *J. Biomol. Screen.* 20: 141–152.

86 Cheng, H., Lear-Rooney, C.M., Johansen, L. et al. (2015). Inhibition of Ebola and Marburg virus entry by G protein-coupled receptor antagonists. *J. Virol.* 89: 9932–9938.

87 Kouznetsova, J., Sun, W., Martinez-Romero, C. et al. (2014). Identification of 53 compounds that block Ebola virus-like particle entry via a repurposing screen of approved drugs. *Emerg. Microbes Infect.* 3: e84.

88 Sun, W., He, S., Martinez-Romero, C. et al. (2017). Synergistic drug combination effectively blocks Ebola virus infection. *Antiviral Res.* 137: 165–172.

89 Basu, A., Li, B., Mills, D.M. et al. (2011). Identification of a small-molecule entry inhibitor for filoviruses. *J. Virol.* 85: 3106–3119.

90 Nelson, E.A., Barnes, A.B., Wiehle, R.D. et al. (2016). Clomiphene and its isomers block Ebola virus particle entry and infection with similar potency: potential therapeutic implications. *Viruses* 8.

91 Albarino, C.G., Wiggleton Guerrero, L., Lo, M.K. et al. (2015). Development of a reverse genetics system to generate a recombinant Ebola virus Makona expressing a green fluorescent protein. *Virology* 484: 259–264.

92 Welch, S.R., Guerrero, L.W., Chakrabarti, A.K. et al. (2016). Lassa and Ebola virus inhibitors identified using minigenome and recombinant virus reporter systems. *Antiviral Res.* 136: 9–18.

93 Uebelhoer, L.S., Albarino, C.G., McMullan, L.K. et al. (2014). High-throughput, luciferase-based reverse genetics systems for identifying inhibitors of Marburg and Ebola viruses. *Antiviral Res.* 106: 86–94.

94 Halfmann, P., Kim, J.H., Ebihara, H. et al. (2008). Generation of biologically contained Ebola viruses. *Proc. Natl. Acad. Sci. U. S. A.* 105: 1129–1133.

95 Towner, J.S., Paragas, J., Dover, J.E. et al. (2005). Generation of eGFP expressing recombinant Zaire ebolavirus for analysis of early pathogenesis events and high-throughput antiviral drug screening. *Virology* 332: 20–27.

96 Hoenen, T., Groseth, A., Callison, J. et al. (2013). A novel Ebola virus expressing luciferase allows for rapid and quantitative testing of antivirals. *Antiviral Res.* 99: 207–213.

97 Cross, R.W., Mire, C.E., Borisevich, V. et al. (2016). The domestic ferret (Mustela putorius furo) as a lethal infection model for 3 species of Ebolavirus. *J. Infect. Dis.* 214: 565–569.

98 Kozak, R., He, S., Kroeker, A. et al. (2016). Ferrets infected with Bundibugyo virus or Ebola virus recapitulate important aspects of human filovirus disease. *J. Virol.* 90: 9209–9223.

99 Bray, M., Davis, K., Geisbert, T. et al. (1998). A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. *J. Infect. Dis.* 178: 651–661.

100 Connolly, B.M., Steele, K.E., Davis, K.J. et al. (1999). Pathogenesis of experimental Ebola virus infection in guinea pigs. *J. Infect. Dis.* 179 (Suppl. 1): S203–S217.

101 Spengler, J.R., Chakrabarti, A.K., Coleman-McCray, J.D. et al. (2015). Utility of oral swab sampling for Ebola virus detection in guinea pig model. *Emerg. Infect. Dis.* 21: 1816–1819.

102 Volchkov, V.E., Chepurnov, A.A., Volchkova, V.A. et al. (2000). Molecular characterization of guinea pig-adapted variants of Ebola virus. *Virology* 277: 147–155.

103 Wong, G., He, S., Wei, H. et al. (2016). Development and characterization of a guinea pig-adapted sudan virus. *J. Virol.* 90: 392–399.

104 Bray, M. (2001). The role of the type I interferon response in the resistance of mice to filovirus infection. *J. Gen. Virol.* 82: 1365–1373.

105 Brannan, J.M., Froude, J.W., Prugar, L.I. et al. (2015). Interferon alpha/beta receptor-deficient mice as a model for Ebola virus disease. *J. Infect. Dis.* 212 (Suppl. 2): S282–S294.

106 de Wit, E., Munster, V.J., Metwally, S.A., and Feldmann, H. (2011). Assessment of rodents as animal models for Reston ebolavirus. *J. Infect. Dis.* 204 (Suppl. 3): S968–S972.

107 Bird, B.H., Spengler, J.R., Chakrabarti, A.K. et al. (2016). Humanized mouse model of Ebola virus disease mimics the immune responses in human disease. *J. Infect. Dis.* 213: 703–711.

108 Ludtke, A., Oestereich, L., Ruibal, P. et al. (2015). Ebola virus disease in mice with transplanted human hematopoietic stem cells. *J. Virol.* 89: 4700–4704.

109 Spengler, J.R., Lavender, K.J., Martellaro, C. et al. (2016). Ebola virus replication and disease without immunopathology in mice expressing transgenes to support human myeloid and lymphoid cell engraftment. *J. Infect. Dis.* 214: S308–S318.

110 Spengler, J.R., Prescott, J., Feldmann, H., and Spiropoulou, C.F. (2017). Human immune system mouse models of Ebola virus infection. *Curr. Opin. Virol.* 25: 90–96.

111 Spengler, J.R., Saturday, G., Lavender, K.J. et al. (2017). Severity of disease in humanized mice infected with Ebola virus or Reston virus is associated with magnitude of early viral replication in liver. *J. Infect. Dis.* 217: 58–63.

112 Ryabchikova, E.I., Kolesnikova, L.V., and Netesov, S.V. (1999). Animal pathology of filoviral infections. *Curr. Top. Microbiol. Immunol.* 235: 145–173.

113 Bowen, E.T., Platt, G.S., Lloyd, G. et al. (1980). A comparative study of strains of Ebola virus isolated from southern Sudan and northern Zaire in 1976. *J. Med. Virol.* 6: 129–138.

114 Baskerville, A., Bowen, E.T., Platt, G.S. et al. (1978). The pathology of experimental Ebola virus infection in monkeys. *J. Pathol.* 125: 131–138.

115 Fisher-Hoch, S.P., Platt, G.S., Lloyd, G. et al. (1983). Haematological and biochemical monitoring of Ebola infection in rhesus monkeys: implications for patient management. *Lancet* 2: 1055–1058.

116 Carrion, R. Jr., Ro, Y., Hoosien, K. et al. (2011). A small nonhuman primate model for filovirus-induced disease. *Virology* 420: 117–124.
117 Chupurnov, A.A., Chernukhin, I.V., Ternovoi, V.A. et al. (1995). Attempts to develop a vaccine against Ebola fever. *Vopr. Virusol.* 40: 257–260.
118 Riabchikova, E.I., Kolesnikova, L.V., and Rassadkin, I.N. (1998). Microscopic study of species specific features of hemostatic impairment in Ebola virus infected monkeys. *Vestn. Ross. Akad. Med. Nauk.* 51–55.
119 Bradfute, S.B., Warfield, K.L., and Bray, M. (2012). Mouse models for filovirus infections. *Viruses* 4: 1477–1508.
120 Reed, D.S., Lackemeyer, M.G., Garza, N.L. et al. (2011). Aerosol exposure to Zaire ebolavirus in three nonhuman primate species: differences in disease course and clinical pathology. *Microb. Infect.* 13: 930–936.
121 Ryabchikova, E.I., Kolesnikova, L.V., and Luchko, S.V. (1999). An analysis of features of pathogenesis in two animal models of Ebola virus infection. *J. Infect. Dis.* 179 (Suppl. 1): S199–S202.
122 Bennett, R.S., Huzella, L.M., Jahrling, P.B. et al. (2017). Nonhuman primate models of Ebola virus disease. *Curr. Top. Microbiol. Immunol.* 411: 171–193.
123 Geisbert, T.W., Strong, J.E., and Feldmann, H. (2015). Considerations in the use of nonhuman primate models of Ebola virus and Marburg virus infection. *J. Infect. Dis.* 212 (Suppl. 2): S91–S97.
124 Jaax, N.K., Davis, K.J., Geisbert, T.J. et al. (1996). Lethal experimental infection of rhesus monkeys with Ebola-Zaire (Mayinga) virus by the oral and conjunctival route of exposure. *Arch. Pathol. Lab. Med.* 120: 140–155.
125 Johnson, E., Jaax, N., White, J., and Jahrling, P. (1995). Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. *Int. J. Exp. Pathol.* 76: 227–236.
126 Sullivan, N.J., Geisbert, T.W., Geisbert, J.B. et al. (2003). Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* 424: 681–684.
127 Marzi, A., Ebihara, H., Callison, J. et al. (2011). Vesicular stomatitis virus-based Ebola vaccines with improved cross-protective efficacy. *J. Infect. Dis.* 204 (Suppl. 3): S1066–S1074.
128 Qiu, X., Wong, G., Audet, J. et al. (2014). Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature* 514: 47–53.
129 Geisbert, T.W., Pushko, P., Anderson, K. et al. (2002). Evaluation in nonhuman primates of vaccines against Ebola virus. *Emerg. Infect. Dis.* 8: 503–507.
130 Mattia, J.G., Vandy, M.J., Chang, J.C. et al. (2016). Early clinical sequelae of Ebola virus disease in Sierra Leone: a cross-sectional study. *Lancet Infect. Dis.* 16: 331–338.
131 Zeng, X., Blancett, C.D., Koistinen, K.A. et al. (2017). Identification and pathological characterization of persistent asymptomatic Ebola virus infection in rhesus monkeys. *Nat. Microbiol.* 2: 17113.
132 Bray, M., Driscoll, J., and Huggins, J.W. (2000). Treatment of lethal Ebola virus infection in mice with a single dose of an *S*-adenosyl-L-homocysteine hydrolase inhibitor. *Antiviral Res.* 45: 135–147.

133 Bray, M., Raymond, J.L., Geisbert, T., and Baker, R.O. (2002). 3-Deazaneplanocin A induces massively increased interferon-alpha production in Ebola virus-infected mice. *Antiviral Res.* 55: 151–159.

134 Kolokoltsov, A.A., Adhikary, S., Garver, J. et al. (2012). Inhibition of Lassa virus and Ebola virus infection in host cells treated with the kinase inhibitors genistein and tyrphostin. *Arch. Virol.* 157: 121–127.

135 Kondratowicz, A.S., Hunt, C.L., Davey, R.A. et al. (2013). AMP-activated protein kinase is required for the macropinocytic internalization of ebolavirus. *J. Virol.* 87: 746–755.

136 Nelson, E.A., Dyall, J., Hoenen, T. et al. (2017). The phosphatidylinositol-3-phosphate 5-kinase inhibitor apilimod blocks filoviral entry and infection. *PLoS Negl. Trop. Dis.* 11: e0005540.

137 Bekerman, E., Neveu, G., Shulla, A. et al. (2017). Anticancer kinase inhibitors impair intracellular viral trafficking and exert broad-spectrum antiviral effects. *J. Clin. Invest.* 127: 1338–1352.

138 Garcia, M., Cooper, A., Shi, W. et al. (2012). Productive replication of Ebola virus is regulated by the c-Abl1 tyrosine kinase. *Sci. Transl. Med.* 4: 123ra24.

139 Ilinykh, P.A., Tigabu, B., Ivanov, A. et al. (2014). Role of protein phosphatase 1 in dephosphorylation of Ebola virus VP30 protein and its targeting for the inhibition of viral transcription. *J. Biol. Chem.* 289: 22723–22738.

140 Smith, D.R., McCarthy, S., Chrovian, A. et al. (2010). Inhibition of heat-shock protein 90 reduces Ebola virus replication. *Antiviral Res.* 87: 187–194.

141 Garcia-Dorival, I., Wu, W., Armstrong, S.D. et al. (2016). Elucidation of the cellular interactome of Ebola virus nucleoprotein and identification of therapeutic targets. *J. Proteome Res.* 15: 4290–4303.

142 Reid, S.P., Shurtleff, A.C., Costantino, J.A. et al. (2014). HSPA5 is an essential host factor for Ebola virus infection. *Antiviral Res.* 109: 171–174.

143 Chang, J., Warren, T.K., Zhao, X. et al. (2013). Small molecule inhibitors of ER alpha-glucosidases are active against multiple hemorrhagic fever viruses. *Antiviral Res.* 98: 432–440.

144 Ma, J., Zhang, X., Soloveva, V. et al. (2018). Enhancing the antiviral potency of ER alpha-glucosidase inhibitor IHVR-19029 against hemorrhagic fever viruses in vitro and in vivo. *Antiviral Res.* 150: 112–122.

145 Warfield, K.L., Warren, T.K., Qiu, X. et al. (2017). Assessment of the potential for host-targeted iminosugars UV-4 and UV-5 activity against filovirus infections in vitro and in vivo. *Antiviral Res.* 138: 22–31.

146 Elshabrawy, H.A., Fan, J., Haddad, C.S. et al. (2014). Identification of a broad-spectrum antiviral small molecule against severe acute respiratory syndrome coronavirus and Ebola, Hendra, and Nipah viruses by using a novel high-throughput screening assay. *J. Virol.* 88: 4353–4365.

147 Zhou, Y., Vedantham, P., Lu, K. et al. (2015). Protease inhibitors targeting coronavirus and filovirus entry. *Antiviral Res.* 116: 76–84.

148 Wang, Y., Cui, R., Li, G. et al. (2016). Teicoplanin inhibits Ebola pseudovirus infection in cell culture. *Antiviral Res.* 125: 1–7.

149 Zhou, N., Pan, T., Zhang, J. et al. (2016). Glycopeptide antibiotics potently inhibit cathepsin L in the late endosome/lysosome and block the entry of

Ebola virus, middle east respiratory syndrome coronavirus (MERS-CoV), and severe acute respiratory syndrome coronavirus (SARS-CoV). *J. Biol. Chem.* 291: 9218–9232.

150 Gehring, G., Rohrmann, K., Atenchong, N. et al. (2014). The clinically approved drugs amiodarone, dronedarone and verapamil inhibit filovirus cell entry. *J. Antimicrob. Chemother.* 69: 2123–2131.

151 Sakurai, Y., Kolokoltsov, A.A., Chen, C.C. et al. (2015). Ebola virus. Two-pore channels control Ebola virus host cell entry and are drug targets for disease treatment. *Science* 347: 995–998.

152 Johansen, L.M., Brannan, J.M., Delos, S.E. et al. (2013). FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. *Sci. Transl. Med.* 5: 190ra79.

153 Shoemaker, C.J., Schornberg, K.L., Delos, S.E. et al. (2013). Multiple cationic amphiphiles induce a Niemann-Pick C phenotype and inhibit Ebola virus entry and infection. *PLoS One* 8: e56265.

154 Fan, H., Du, X., Zhang, J. et al. (2017). Selective inhibition of Ebola entry with selective estrogen receptor modulators by disrupting the endolysosomal calcium. *Sci. Rep.* 7: 41226.

155 Johansen, L.M., DeWald, L.E., Shoemaker, C.J. et al. (2015). A screen of approved drugs and molecular probes identifies therapeutics with anti-Ebola virus activity. *Sci. Transl. Med.* 7: 290ra89.

156 Pattabhi, S., Wilkins, C.R., Dong, R. et al. (2015). Targeting innate immunity for antiviral therapy through small molecule agonists of the RLR pathway. *J. Virol.* 90: 2372–2387.

157 Rhein, B.A., Powers, L.S., Rogers, K. et al. (2015). Interferon-gamma Inhibits Ebola Virus Infection. *PLoS Pathog.* 11: e1005263.

158 Biedenkopf, N., Lange-Grunweller, K., Schulte, F.W. et al. (2017). The natural compound silvestrol is a potent inhibitor of Ebola virus replication. *Antiviral Res.* 137: 76–81.

159 Hill-Batorski, L., Halfmann, P., Neumann, G., and Kawaoka, Y. (2013). The cytoprotective enzyme heme oxygenase-1 suppresses Ebola virus replication. *J. Virol.* 87: 13795–13802.

160 Panchal, R.G., Reid, S.P., Tran, J.P. et al. (2012). Identification of an antioxidant small-molecule with broad-spectrum antiviral activity. *Antiviral Res.* 93: 23–29.

161 Ramanathan, A., Robb, G.B., and Chan, S.H. (2016). mRNA capping: biological functions and applications. *Nucleic Acids Res.* 44: 7511–7526.

162 De Clercq, E. (1987). *S*-adenosylhomocysteine hydrolase inhibitors as broad-spectrum antiviral agents. *Biochem. pharmacol.* 36: 2567–2575.

163 Nygren, P.J. and Scott, J.D. (2015). Therapeutic strategies for anchored kinases and phosphatases: exploiting short linear motifs and intrinsic disorder. *Front. Pharmacol.* 6: 158.

164 Saeed, M.F., Kolokoltsov, A.A., Freiberg, A.N. et al. (2008). Phosphoinositide-3 kinase-Akt pathway controls cellular entry of Ebola virus. *PLoS Pathog.* 4: e1000141.

165 Shimojima, M., Takada, A., Ebihara, H. et al. (2006). Tyro3 family-mediated cell entry of Ebola and Marburg viruses. *J. Virol.* 80: 10109–10116.

166 Kruse, T., Biedenkopf, N., Hertz, E.P.T. et al. (2018). The Ebola virus nucleoprotein recruits the host PP2A-B56 phosphatase to activate transcriptional support activity of VP30. *Mol. Cell.* 69: 136–145. e6.

167 Smith, D.A., Carland, C.R., Guo, Y., and Bernstein, S.I. (2014). Getting folded: chaperone proteins in muscle development, maintenance and disease. *Anat. Rec. (Hoboken)* 297: 1637–1649.

168 Hadden, M.K., Lubbers, D.J., and Blagg, B.S. (2006). Geldanamycin, radicicol, and chimeric inhibitors of the Hsp90 N-terminal ATP binding site. *Curr. Top. Med. Chem.* 6: 1173–1182.

169 Chang, J., Block, T.M., and Guo, J.T. (2013). Antiviral therapies targeting host ER alpha-glucosidases: current status and future directions. *Antiviral Res.* 99: 251–260.

170 Chandran, K., Sullivan, N.J., Felbor, U. et al. (2005). Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. *Science* 308: 1643–1645.

171 Wang, H., Shi, Y., Song, J. et al. (2016). Ebola viral glycoprotein bound to its endosomal receptor niemann-pick C1. *Cell* 164: 258–268.

172 Barr, S.C., Warner, K.L., Kornreic, B.G. et al. (2005). A cysteine protease inhibitor protects dogs from cardiac damage during infection by *Trypanosoma cruzi*. *Antimicrob. Agents Chemother.* 49: 5160–5161.

173 Abdulla, M.H., Lim, K.C., Sajid, M. et al. (2007). Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. *PLoS Med.* 4: e14.

174 Renslo, A.R. and McKerrow, J.H. (2006). Drug discovery and development for neglected parasitic diseases. *Nat. Chem. Biol.* 2: 701–710.

175 Basu, A., Mills, D.M., Mitchell, D. et al. (2015). Novel small molecule entry inhibitors of Ebola virus. *J. Infect. Dis.* 212 (Suppl. 2): S425–S434.

176 Kuroda, M., Fujikura, D., Nanbo, A. et al. (2015). Interaction between TIM-1 and NPC1 is important for cellular entry of Ebola virus. *J. Virol.* 89: 6481–6493.

177 Scott, C.C. and Gruenberg, J. (2011). Ion flux and the function of endosomes and lysosomes: pH is just the start: the flux of ions across endosomal membranes influences endosome function not only through regulation of the luminal pH. *Bioessays* 33: 103–110.

178 Mingo, R.M., Simmons, J.A., Shoemaker, C.J. et al. (2015). Ebola virus and severe acute respiratory syndrome coronavirus display late cell entry kinetics: evidence that transport to NPC1+ endolysosomes is a rate-defining step. *J. Virol.* 89: 2931–2943.

179 Miller, M.E., Adhikary, S., Kolokoltsov, A.A., and Davey, R.A. (2012). Ebolavirus requires acid sphingomyelinase activity and plasma membrane sphingomyelin for infection. *J. Virol.* 86: 7473–7483.

180 Yoneyama, M., Onomoto, K., Jogi, M. et al. (2015). Viral RNA detection by RIG-I-like receptors. *Curr. Opin. Immunol.* 32: 48–53.

181 Chung, D.H., Golden, J.E., Adcock, R.S. et al. (2016). Discovery of a broad-spectrum antiviral compound that inhibits pyrimidine biosynthesis and establishes a type 1 interferon-independent antiviral state. *Antimicrob. Agents Chemother.* 60: 4552–4562.

182 Pryke, K.M., Abraham, J., Sali, T.M. et al. (2017). A novel agonist of the TRIF pathway induces a cellular state refractory to replication of zika, chikungunya, and dengue viruses. *mBio* 8.

183 Goulet, M.L., Olagnier, D., Xu, Z. et al. (2013). Systems analysis of a RIG-I agonist inducing broad spectrum inhibition of virus infectivity. *PLoS Pathog.* 9: e1003298.

184 Smith, L.M., Hensley, L.E., Geisbert, T.W. et al. (2013). Interferon-beta therapy prolongs survival in rhesus macaque models of Ebola and Marburg hemorrhagic fever. *J. Infect. Dis.* 208: 310–318.

185 Cohen, J. and Enserink, M. (2016). Infectious disease. As Ebola epidemic draws to a close, a thin scientific harvest. *Science* 351: 12–13.

186 Prescott, J.B., Marzi, A., Safronetz, D. et al. (2017). Immunobiology of Ebola and Lassa virus infections. *Nat. Rev. Immunol.* 17: 195–207.

187 Olsen, M.E., Filone, C.M., Rozelle, D. et al. (2016). Polyamines and hypusination are required for Ebolavirus gene expression and replication. *mBio* 7.

188 Kogure, T., Kinghorn, A.D., Yan, I. et al. (2013). Therapeutic potential of the translation inhibitor silvestrol in hepatocellular cancer. *PLoS One* 8: e76136.

189 Patton, J.T., Lustberg, M.E., Lozanski, G. et al. (2015). The translation inhibitor silvestrol exhibits direct anti-tumor activity while preserving innate and adaptive immunity against EBV-driven lymphoproliferative disease. *Oncotarget* 6: 2693–2708.

190 Maruyama, T., Rodriguez, L.L., Jahrling, P.B. et al. (1999). Ebola virus can be effectively neutralized by antibody produced in natural human infection. *J. Virol.* 73: 6024–6030.

191 Qiu, X. and Kobinger, G.P. (2014). Antibody therapy for Ebola: is the tide turning around? *Hum. Vaccin. Immunother.* 10: 964–967.

192 Oswald, W.B., Geisbert, T.W., Davis, K.J. et al. (2007). Neutralizing antibody fails to impact the course of Ebola virus infection in monkeys. *PLoS Pathog.* 3: e9.

193 Parren, P.W., Geisbert, T.W., Maruyama, T. et al. (2002). Pre- and postexposure prophylaxis of Ebola virus infection in an animal model by passive transfer of a neutralizing human antibody. *J. Virol.* 76: 6408–6412.

194 Davidson, E., Bryan, C., Fong, R.H. et al. (2015). Mechanism of binding to Ebola virus glycoprotein by the ZMapp, ZMAb, and MB-003 cocktail antibodies. *J. Virol.* 89: 10982–10992.

195 PREVAIL II Writing Group; Multi-National PREVAIL II Study Team, Davey, R.T. Jr., Dodd, L. et al. (2016). A randomized, controlled trial of ZMapp for Ebola virus infection. *N. Engl. J. Med.* 375: 1448–1456.

196 Qiu, X., Audet, J., Wong, G. et al. (2012). Successful treatment of Ebola virus-infected cynomolgus macaques with monoclonal antibodies. *Sci. Transl. Med.* 4: 138ra81.

197 Qiu, X., Audet, J., Wong, G. et al. (2013). Sustained protection against Ebola virus infection following treatment of infected nonhuman primates with ZMAb. *Sci. Rep.* 3: 3365.

198 Olinger, G.G. Jr., Pettitt, J., Kim, D. et al. (2012). Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques. *Proc. Natl. Acad. Sci. U. S. A.* 109: 18030–18035.

199 Pettitt, J., Zeitlin, L., Kim, D.H. et al. (2013). Therapeutic intervention of Ebola virus infection in rhesus macaques with the MB-003 monoclonal antibody cocktail. *Sci. Transl. Med.* 5: 199ra113.

200 Marzi, A., Yoshida, R., Miyamoto, H. et al. (2012). Protective efficacy of neutralizing monoclonal antibodies in a nonhuman primate model of Ebola hemorrhagic fever. *PLoS One* 7: e36192.

201 Qiu, X., Audet, J., Lv, M. et al. (2016). Two-mAb cocktail protects macaques against the Makona variant of Ebola virus. *Sci. Transl. Med.* 8: 329ra33.

202 Corti, D., Misasi, J., Mulangu, S. et al. (2016). Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody. *Science* 351: 1339–1342.

203 Furuyama, W., Marzi, A., Nanbo, A. et al. (2016). Discovery of an antibody for pan-ebolavirus therapy. *Sci Rep* 6: 20514.

204 Holtsberg, F.W., Shulenin, S., Vu, H. et al. (2016). Pan-ebolavirus and pan-filovirus mouse monoclonal antibodies: protection against Ebola and Sudan viruses. *J. Virol.* 90: 266–278.

205 Wec, A.Z., Nyakatura, E.K., Herbert, A.S. et al. (2016). A "Trojan horse" bispecific-antibody strategy for broad protection against ebolaviruses. *Science* 354: 350–354.

206 Geisbert, T.W., Hensley, L.E., Kagan, E. et al. (2006). Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J. Infect. Dis.* 193: 1650–1657.

207 Geisbert, T.W., Lee, A.C., Robbins, M. et al. (2010). Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet* 375: 1896–1905.

208 Thi, E.P., Mire, C.E., Lee, A.C. et al. (2015). Lipid nanoparticle siRNA treatment of Ebola-virus-Makona-infected nonhuman primates. *Nature* 521: 362–365.

209 Warren, T.K., Whitehouse, C.A., Wells, J. et al. (2015). A single phosphorodiamidate morpholino oligomer targeting VP24 protects rhesus monkeys against lethal Ebola virus infection. *mBio* 6.

210 Alfson, K.J., Worwa, G., Carrion, R. Jr., and Griffiths, A. (2015). Determination and therapeutic exploitation of Ebola virus spontaneous mutation frequency. *J. Virol.* 90: 2345–2355.

211 McMullan, L.K., Flint, M., Dyall, J. et al. (2016). The lipid moiety of brincidofovir is required for in vitro antiviral activity against Ebola virus. *Antiviral Res.* 125: 71–78.

212 Florescu, D.F., Kalil, A.C., Hewlett, A.L. et al. (2015). Administration of brincidofovir and convalescent plasma in a patient with Ebola virus disease. *Clin. Infect. Dis.* 61: 969–973.

213 Taylor, R., Kotian, P., Warren, T. et al. (2016). BCX4430 – a broad-spectrum antiviral adenosine nucleoside analog under development for the treatment of Ebola virus disease. *J. Infect. Public Health* 9: 220–226.

214 Warren, T.K., Wells, J., Panchal, R.G. et al. (2014). Protection against filovirus diseases by a novel broad-spectrum nucleoside analogue BCX4430. *Nature* 508: 402–405.

215 Oestereich, L., Ludtke, A., Wurr, S. et al. (2014). Successful treatment of advanced Ebola virus infection with T-705 (favipiravir) in a small animal model. *Antiviral Res.* 105: 17–21.

216 Smither, S.J., Eastaugh, L.S., Steward, J.A. et al. (2014). Post-exposure efficacy of oral T-705 (Favipiravir) against inhalational Ebola virus infection in a mouse model. *Antiviral Res.* 104: 153–155.

217 Bixler, S.L., Bocan, T.M., Wells, J. et al. (2018). Intracellular conversion and in vivo dose response of favipiravir (T-705) in rodents infected with Ebola virus. *Antiviral Res.* 151: 50–54.

218 Bixler, S.L., Bocan, T.M., Wells, J. et al. (2018). Efficacy of favipiravir (T-705) in nonhuman primates infected with Ebola virus or Marburg virus. *Antiviral Res.* 151: 97–104.

219 Bai, C.Q., Mu, J.S., Kargbo, D. et al. (2016). Clinical and virological characteristics of Ebola virus disease patients treated with favipiravir (T-705)-sierra leone, 2014. *Clin. Infect. Dis.* 63: 1288–1294.

220 Sissoko, D., Laouenan, C., Folkesson, E. et al.; JIKI Study Group (2016). Experimental treatment with favipiravir for Ebola virus disease (the JIKI trial): a historically controlled, single-arm proof-of-concept trial in guinea. *PLoS Med.* 13: e1001967.

221 ClinicalTrials.gov. GS-5734 to assess the antiviral activity, longer-term clearance of Ebola virus, and safety in male Ebola survivors with evidence of Ebola virus persistence in semen. https://clinicaltrials.gov/ct2/show/NCT02818582?cond=Ebola+virus+disease&rank=33 (accessed 22 February 2018).

222 Jacobs, M., Rodger, A., Bell, D.J. et al. (2016). Late Ebola virus relapse causing meningoencephalitis: a case report. *Lancet* 388: 498–503.

223 Dornemann, J., Burzio, C., Ronsse, A. et al. (2017). First newborn baby to receive experimental therapies survives Ebola virus disease. *J. Infect. Dis.* 215: 171–174.

224 Dye, J.M., Herbert, A.S., Kuehne, A.I. et al. (2012). Postexposure antibody prophylaxis protects nonhuman primates from filovirus disease. *Proc. Natl. Acad. Sci. U. S. A.* 109: 5034–5039.

225 Takada, A., Ebihara, H., Jones, S. et al. (2007). Protective efficacy of neutralizing antibodies against Ebola virus infection. *Vaccine* 25: 993–999.

226 Hernandez, H., Marceau, C., Halliday, H. et al. (2015). Development and characterization of broadly cross-reactive monoclonal antibodies against all known Ebolavirus species. *J. Infect. Dis.* 212 (Suppl. 2): S410–S413.

227 Trunschke, M., Conrad, D., Enterlein, S. et al. (2013). The L-VP35 and L-L interaction domains reside in the amino terminus of the Ebola virus L protein and are potential targets for antivirals. *Virology* 441: 135–145.

228 Watanabe, S., Noda, T., and Kawaoka, Y. (2006). Functional mapping of the nucleoprotein of Ebola virus. *J. Virol.* 80: 3743–3751.

229 Kirchdoerfer, R.N., Abelson, D.M., Li, S. et al. (2015). Assembly of the Ebola virus nucleoprotein from a chaperoned VP35 complex. *Cell Rep.* 12: 140–149.

230 Banadyga, L., Hoenen, T., Ambroggio, X. et al. (2017). Ebola virus VP24 interacts with NP to facilitate nucleocapsid assembly and genome packaging. *Sci. Rep.* 7: 7698.

231 Kirchdoerfer, R.N., Moyer, C.L., Abelson, D.M., and Saphire, E.O. (2016). The Ebola Virus VP30-NP interaction is a regulator of viral RNA synthesis. *PLoS Pathog.* 12: e1005937.

232 Xu, W., Luthra, P., Wu, C. et al. (2017). Ebola virus VP30 and nucleoprotein interactions modulate viral RNA synthesis. *Nat. Commun.* 8: 15576.

233 Johnson, R.F., McCarthy, S.E., Godlewski, P.J., and Harty, R.N. (2006). Ebola virus VP35-VP40 interaction is sufficient for packaging 3E-5E minigenome RNA into virus-like particles. *J. Virol.* 80: 5135–5144.

234 Leung, D.W., Borek, D., Luthra, P. et al. (2015). An Intrinsically Disordered Peptide from Ebola Virus VP35 Controls Viral RNA Synthesis by Modulating Nucleoprotein-RNA Interactions. *Cell Rep.* 11: 376–389.

235 Brown, C.S., Lee, M.S., Leung, D.W. et al. (2014). In silico derived small molecules bind the filovirus VP35 protein and inhibit its polymerase cofactor activity. *J. Mol. Biol.* 426: 2045–2058.

236 Fu, X., Wang, Z., Li, L. et al. (2016). Novel chemical ligands to Ebola virus and Marburg virus nucleoproteins identified by combining affinity mass spectrometry and metabolomics approaches. *Sci. Rep.* 6: 29680.

237 Liu, G., Nash, P.J., Johnson, B. et al. (2017). A sensitive in vitro high-throughput screen to identify pan-filoviral replication inhibitors targeting the VP35-NP interface. *ACS Infect. Dis.* 3: 190–198.

238 Kang, K.N. and Lee, Y.S. (2013). RNA aptamers: a review of recent trends and applications. *Adv. Biochem. Eng. Biotechnol.* 131: 153–169.

239 Binning, J.M., Wang, T., Luthra, P. et al. (2013). Development of RNA aptamers targeting Ebola virus VP35. *Biochemistry* 52: 8406–8419.

240 Sundaram, P., Kurniawan, H., Byrne, M.E., and Wower, J. (2013). Therapeutic RNA aptamers in clinical trials. *Eur. J. Pharm. Sci.* 48: 259–271.

241 Kole, R., Krainer, A.R., and Altman, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nat. Rev. Drug Discov.* 11: 125–140.

242 Dunning, J., Sahr, F., Rojek, A. et al. (2016). Experimental treatment of Ebola virus disease with TKM-130803: a single-arm phase 2 clinical trial. *PLoS Med.* 13: e1001997.RAPIDE-TKM trial team.

243 Liu, G., Wong, G., Su, S. et al. (2017). Clinical evaluation of Ebola virus disease therapeutics. *Trends Mol. Med.* 23: 820–830.

244 Enterlein, S., Warfield, K.L., Swenson, D.L. et al. (2006). VP35 knockdown inhibits Ebola virus amplification and protects against lethal infection in mice. *Antimicrob. Agents Chemother.* 50: 984–993.

245 Warfield, K.L., Swenson, D.L., Olinger, G.G. et al. (2006). Gene-specific countermeasures against Ebola virus based on antisense phosphorodiamidate morpholino oligomers. *PLoS Pathog.* 2: e1.

246 Swenson, D.L., Warfield, K.L., Warren, T.K. et al. (2009). Chemical modifications of antisense morpholino oligomers enhance their efficacy against Ebola virus infection. *Antimicrob. Agents Chemother.* 53: 2089–2099.

247 Heald, A.E., Iversen, P.L., Saoud, J.B. et al. (2014). Safety and pharmacokinetic profiles of phosphorodiamidate morpholino oligomers with activity against ebola virus and marburg virus: results of two single-ascending-dose studies. *Antimicrob. Agents Chemother.* 58: 6639–6647.

248 Cihlar, T. and Ray, A.S. (2010). Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antiviral Res.* 85: 39–58.

249 Eltahla, A.A., Luciani, F., White, P.A. et al. (2015). Inhibitors of the hepatitis C virus polymerase; mode of action and resistance. *Viruses* 7: 5206–5224.

250 McCormick, J.B., King, I.J., Webb, P.A. et al. (1986). Lassa fever. Effective therapy with ribavirin. *N. Engl. J. Med.* 314: 20–26.

251 Stephen, E.L. and Jahrling, P.B. (1979). Experimental Lassa fever virus infection successfully treated with ribavirin. *Lancet* 1: 268–269.

252 Sun, Y., Chung, D.H., Chu, Y.K. et al. (2007). Activity of ribavirin against Hantaan virus correlates with production of ribavirin-5′-triphosphate, not with inhibition of IMP dehydrogenase. *Antimicrob. Agents Chemother.* 51: 84–88.

253 Takhampunya, R., Ubol, S., Houng, H.S. et al. (2006). Inhibition of dengue virus replication by mycophenolic acid and ribavirin. *J. Gen. Virol.* 87: 1947–1952.

254 Leyssen, P., De Clercq, E., and Neyts, J. (2006). The anti-yellow fever virus activity of ribavirin is independent of error-prone replication. *Mol. Pharmacol.* 69: 1461–1467.

255 Graci, J.D. and Cameron, C.E. (2006). Mechanisms of action of ribavirin against distinct viruses. *Rev. Med. Virol.* 16: 37–48.

256 Crotty, S., Maag, D., Arnold, J.J. et al. (2000). The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. *Nat. Med.* 6: 1375–1379.

257 Dunning, J., Kennedy, S.B., Antierens, A. et al. (2016). Experimental treatment of Ebola virus disease with brincidofovir. *PLoS One* 11: e0162199.RAPIDE-BCV trial team.

258 Chimerix, I. Chimerix focusing efforts on CMV and adenovirus pivotal trials. http://ir.chimerix.com/news-releases/news-release-details/chimerix-focusing-efforts-cmv-and-adenovirus-pivotal-trials (02/22).

259 Julander, J.G., Bantia, S., Taubenheim, B.R. et al. (2014). BCX4430, a novel nucleoside analog, effectively treats yellow fever in a Hamster model. *Antimicrob. Agents Chemother.* 58: 6607–6614.

260 Julander, J.G., Siddharthan, V., Evans, J. et al. (2017). Efficacy of the broad-spectrum antiviral compound BCX4430 against Zika virus in cell culture and in a mouse model. *Antiviral Res.* 137: 14–22.

261 Furuta, Y., Takahashi, K., Fukuda, Y. et al. (2002). In vitro and in vivo activities of anti-influenza virus compound T-705. *Antimicrob. Agents Chemother.* 46: 977–981.

262 Furuta, Y., Takahashi, K., Kuno-Maekawa, M. et al. (2005). Mechanism of action of T-705 against influenza virus. *Antimicrob. Agents Chemother.* 49: 981–986.

263 Furuta, Y., Komeno, T., and Nakamura, T. (2017). Favipiravir (T-705), a broad spectrum inhibitor of viral RNA polymerase. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.* 93: 449–463.

264 Mentre, F., Taburet, A.M., Guedj, J. et al. (2015). Dose regimen of favipiravir for Ebola virus disease. *Lancet Infect. Dis.* 15: 150–151.

265 Nguyen, T.H., Guedj, J., Anglaret, X. et al.; JIKI STudy Group (2017). Favipiravir pharmacokinetics in Ebola-infected patients of the JIKI trial reveals concentrations lower than targeted. *PLoS Negl. Trop. Dis.* 11: e0005389.

266 Sheahan, T.P., Sims, A.C., Graham, R.L. et al. (2017). Broad-spectrum antiviral GS-5734 inhibits both epidemic and zoonotic coronaviruses. *Sci. Transl. Med.* 9.

Part II

Kinetoplastids

4

Designing Drugs to Target *Trypanosoma cruzi*, the Etiological Agent of Chagas Disease: When Chemistry needs Biology

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4.1 Introduction

Researchers in the field know there are precious few clinical compounds and late-stage preclinical candidates available for Chagas disease therapy. Despite more than a decade of contemporary drug discovery for this disease, including drug repurposing, screening campaigns, and lead optimization programs, very few new chemical entities have moved forward to clinical trials. The scope of this review is not to present an overview of the compounds and chemical scaffolds in various stages of development as potential new chemical entities to treat Chagas disease, as this has been extensively described in recent years and can be found elsewhere (see, for example, [1, 2]). Rather, we consider how current screening paradigms can be used to support drug discovery and lead optimization programs for Chagas disease and identify the potential gaps and challenges to be addressed in the future. Indeed, we believe that a better understanding of the complex pathology of *Trypanosoma cruzi* is a *sine qua non* condition to aid drug design by driving the development of new *in vitro* and *in vivo* assays and testing paradigms relevant to assessing all aspects of compound efficacy. These data can then be used to better inform structure optimization, compound evaluation, and triage for improved pharmacodynamic outcomes and to guide medicinal chemistry beyond *in vitro* potency optimization for Chagas disease drug discovery.

4.2 Chagas Disease Overview

American trypanosomiasis, better known as Chagas disease (CD), was first described by Carlos Chagas [3] more than a century ago. Since then, CD epidemiology has changed: while still endemic in Latin America, the disease has become a global public health issue following its spread in non-endemic countries due to population migration [4–7]. CD is the major cause of infectious cardiomyopathy worldwide [8]. Globally, around 6–7 million people are infected and 10 000 die annually [9, 10]. The disease is the result of human infection by

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the protozoan parasite *T. cruzi*. Its clinical presentation can be separated into two main phases. The acute phase is asymptomatic and typically undetected, lasting for a couple of months during which the parasite is readily identified through blood examination. The chronic phase can last for decades; the infection is controlled by the immune system and the parasite is hardly detectable. While most infected patients in the chronic phase will remain asymptomatic, a certain proportion – between 10% and 40% will eventually develop symptoms, mainly cardiomyopathies and, in certain cases, digestive tract megasyndromes, or both [11]. Progressive heart failure and sudden death remain the major causes of mortality in these patients [12].

Current treatment relies on two old nitro heterocyclic trypanocidal drugs, benznidazole (Abarax/ELEA and Rochagan/LAFEPE) and nifurtimox (Lampit/Bayer). Although these drugs have been shown to be efficacious in both phases of the disease, particularly in children, their use is limited due to side effects occurring during treatment. Access to the medication can also be an issue [13, 14]. New and safer drugs are clearly needed for CD, and various initiatives have emerged to address this need as well as public–private partnerships (PPPs) with the involvement of multiple stakeholders, and incentives such as the FDA priority review voucher to promote the involvement of other institutions in R&D for neglected diseases [15]. This has led to a radical change in the Chagas R&D landscape during the past decade with the introduction of new tools and collaborative approaches and, consequently, to novel ways of identifying and assessing chemical entities as potential inhibitors of *T. cruzi* [16, 17]. However, many challenges remain in both clinical and preclinical settings [18]. A major hurdle for the clinical development of new drugs for CD is the absence of an adequate test that can assess successful treatment in a timely manner. Treatment efficacy relies on serological tests showing the disappearance of *T. cruzi* antibodies (seroreversion), which can take decades to occur. The need for surrogate markers of cure is further underlined by the approval by the FDA of benznidazole monotherapy only for the treatment of chagasic children between 2 and 12 years of age [19, 20]. Although there is no absolute proof in patients that parasitological cure is synonymous with clinical cure, i.e. stopping progression of the disease toward cardiac or gastrointestinal symptoms, there is a consensus that parasite persistence is needed for the development of CD. All current CD drug discovery and development efforts are therefore focusing on strategies to eliminate *T. cruzi* from the human body (drugs and therapeutic vaccines). How to model this outcome in a preclinical setting is still a major challenge to the field.

4.3 Toward Sterile Cure in a Chagas Disease Mouse Model: Which Way Forward?

The suitability of mouse models of infection for predicting human disease pathology is an area of active interest and can only be validated with new drugs and clinical trials to provide feedback into the optimization cycle (back translation). Following drug treatment, achieving a 100% curative outcome for

all *T. cruzi*-infected mice in an *in vivo* efficacy experiment with a new chemical entity – so-called sterile cure – is a clearly defined goal for researchers in the Chagas field [21], although it is still debated [22, 23]. On the one hand, it represents an unambiguous endpoint in a gatekeeper assay to preclinical development. On the other, it is a narrowly defined decision point in one highly specific assay. Moreover, the ability to measure this endpoint beyond reasonable doubt has been a challenge in both laboratory and clinical settings. The bioluminescent imaging (BLI) mouse model is a recent addition to the CD screening cascade and has the potential to bridge the translational gap [24].

Real-time bioluminescence imaging of mice inoculated with transgenic *T. cruzi* parasites gives a fascinating insight into the highly dynamic nature of the infection. In acutely infected mice, parasites are visualized in almost every tissue, the major hotspots (highest luminescence intensities) being predominant in cardiac tissue and smooth and skeletal muscle. In chronically infected mice, the GI tract appears to be a privilege site for *T. cruzi* persistence. During that phase, it is postulated that foci of parasites seemingly appearing and disappearing from detection are due to the trafficking of parasites or infected cells from the gut to other sites, possibly through the lymphatic route. Even after drug treatment, images can reveal persistent, low levels of infection in immune-privileged sites (reservoir sites) such as the lower GI tract, skin, and adipose tissue [25].

Interestingly, the BLI model successfully differentiated the sterilizing efficacy of nitroimidazole drugs (benznidazole and fexinidazole) from the nonsterilizing, suppressive action of *T. cruzi* CYP51 inhibitor posaconazole [26]. This result reflected the outcome of two independent clinical trials [27, 28], which showed *T. cruzi* parasite recrudescence as assessed by PCR during follow-up in up to 100% of patients treated with posaconazole, while this phenomenon was observed in only 10–20% of benznidazole-treated patients. This provides preliminary evidence of the ability to translate an efficacy outcome in a preclinical animal model into a clinical setting. The result needs to be further validated with other compounds acting through different biochemical mechanisms. A potential front-runner is the benzoxaborole AN6759 that was shown to achieve sterile cure in another Chagas murine model [29]. The mode of action of this compound is yet to be fully elucidated.

In the meantime, sterile cure is a very high bar for new compounds to overcome in order to progress out of the discovery phase, creating a concern that the criterion is too stringent and will lead to the rejection of good compounds. Getting the screening cascade correct for a smooth translation of *in vitro* activity into *in vivo* efficacy, and ultimately clinical efficacy, is a goal for all drug discovery efforts. The Chagas field has matured considerably in this respect over the past 10 years thanks to the input of a growing research community and funding support. In the sections to follow, we consider how good medicinal chemistry design and newly developed biological assays (issued from a better understanding of host and *T. cruzi* parasite interactions) need to merge in order to deconvolute aspects of sterile cure and provide new treatments for this global health problem.

4.3.1 Feeding the Chagas Disease Pipeline: Compound Selection and Identification of Potential Hits/Starting Points

Using target-based or phenotypic screens to find compounds to feed CD discovery programs is a constant activity. Fortunately, the move to medium- and high-throughput automated screening, using either colorimetry [30] and/or imaging (high content) as readouts [31, 32], has facilitated the evaluation in recent years of a high volume of compounds that have been made available by academic laboratories, research institutes, and pharmaceutical companies [33, 34]. The data generated from several whole-cell phenotypic screening campaigns measuring intracellular amastigote inhibition has been published, injecting compound diversity and new opportunities into the field. The European Union has created a target-based screening initiative, in which targets submitted can be tested against a collection of compounds issued from various pharmaceutical companies [35, 36], while a consortium of industry partners has engaged with DND*i* – a PPP research & development organization – in a neglected tropical disease drug-booster initiative allowing partners to evaluate and optimize seed compounds in the absence of committed biology and chemistry resources [37].

There is certainly room to accommodate a variety of approaches to generating new hits in this area, with both target-based screens and whole-cell assays taking their place in the lineup. The pros and cons of driving research efforts with either one of these approaches have been discussed elsewhere [38]. Simplistically, it can be seen as a matter of timing. Target-based assays facilitate rational compound design, mechanistic understanding, and optimization of on-target selectivity and hence safety. The well-recognized drawback is a possible lack of translation between target activity and potency in an infected whole cell. While poor compound permeability is often cited as a reason for this disconnect, protein binding (extra- and intracellular) is a common culprit. Whole-cell assays (phenotypic screens) fast track compounds with cellular penetration, a very important checkpoint for compounds targeting an intracellular pathogen, but sacrifice the opportunity to use structure-based design in the first instance. Any mechanistic information that could guide compound evaluation is also left unused. For example, designing into the active space of a human homolog of the parasite target can be avoided to improve selectivity and safety margins, and potentially undesirable off-target effects that are flagged by knowing the mechanism of action can be investigated at an early stage.

4.3.2 Choosing the "Right" Starting Points

The treatment of intracellular infections is a complex and challenging task, due to the many biological barriers and elimination mechanisms that drugs must overcome to reach the site of infection in sufficient concentration to illicit the desired pharmacodynamic response. Designing compounds to overcome these hurdles is an interesting challenge for medicinal chemists. The mobile distribution of *T. cruzi* and its ability to penetrate almost any nucleated mammalian cell type in any tissue suggests that close attention should be paid to

the physicochemical and pharmacokinetic attributes of molecules during hit assessment and optimization, rather than to potency alone. Achieving a high intracellular compound to pathogen concentration depends on molecules lasting long enough in the body to achieve critical levels inside the cell. This is aided by unimpeded movement through biological barriers and water, which contributes up to 70% of the content of the cytosol (where the parasite resides). We postulate that *T. cruzi* may be targeted most effectively by low-molecular-weight, polar molecules (with a weakly basic center) that are highly stable toward metabolic degradation. These simple molecular descriptors positively impact common aspects of translational failure in the *T. cruzi* discovery pipeline, such as (i) protein target to whole-cell assay disconnect due to low permeability or high protein binding; (ii) unsuitable *in vitro* absorption/distribution/metabolism/excretion (ADME) profiles, e.g. low-solubility and high-oxidative metabolism, predictive of poor systemic exposure after oral dosing; and (iii) insufficient systemic exposure or free-drug levels to demonstrate parasite knockdown *in vivo* or curative activity. The proposed molecular template is consistent with drug design for other intracellular pathogens, e.g. bacteria and *Leishmania* and is a characteristic of benznidazole and fexinidazole, two drugs with 100% curative efficacy in the BLI mouse model of *T. cruzi* infection [39].

Ligand efficiency metrics are a relatively recent medicinal chemistry evaluation tool that targets potency and physicochemical properties of molecules to define how effectively structural features are used to interact with biochemical targets *in vitro* [40]. These metrics have been applied to guide hit evaluation and progression using data from whole-cell assays, although not strictly designed for this purpose. Efficiency metric LELP (ligand-efficiency-dependent lipophilicity) incorporates a molecular weight descriptor into the analysis, reducing the emphasis on potency [41]. A comparative LELP/ lipophilic ligand efficiency (LLE) analysis for compounds pertinent to the CD field (Table 4.1 and Figure 4.1) shows that

Table 4.1 A ligand efficiency analysis for compounds of interest for the treatment of Chagas disease and visceral leishmaniasis.

Code	IC_{50} Am.	pIC_{50}	MW	*g* Log *D*	Sol pH 6.5	LELP	LLE
Benznidazole	4.648	5.3	260.2	1.2	100	3.121	4.133
Fexinidazole	9.207	5.0	279.3	2.25	100	6.196	2.790
Posaconazole	0.002	8.7	700.8	3.6	6.3	15.410	5.099
EPL-BS1246	0.007	8.2	459.9	4.5	1.6	11.280	3.655
GNF6702	0.12	6.9	429.4	3.5	NA	11.810	3.421
AN6759	7.769	5.1	339.1	2.57	100	9.178	2.540
VL-6148	*Undisclosed*					5.244	4.494
VL-2098	0.03	7.5	359.3	2.45	2	5.943	5.073

IC_{50} Am. µM – IC_{50} amastigotes killing in primary *in vitro* assay; pIC_{50}; MW – molecular weight; *g* Log *D* – distribution coefficient estimation using chromatography at pH 7.4; Sol pH 6.5 – kinetic solubility estimation using nephelometry (pH 6.5 phosphate buffer);
LELP – ligand-efficiency-dependent lipophilicity; LLE – lipophilic ligand efficiency.

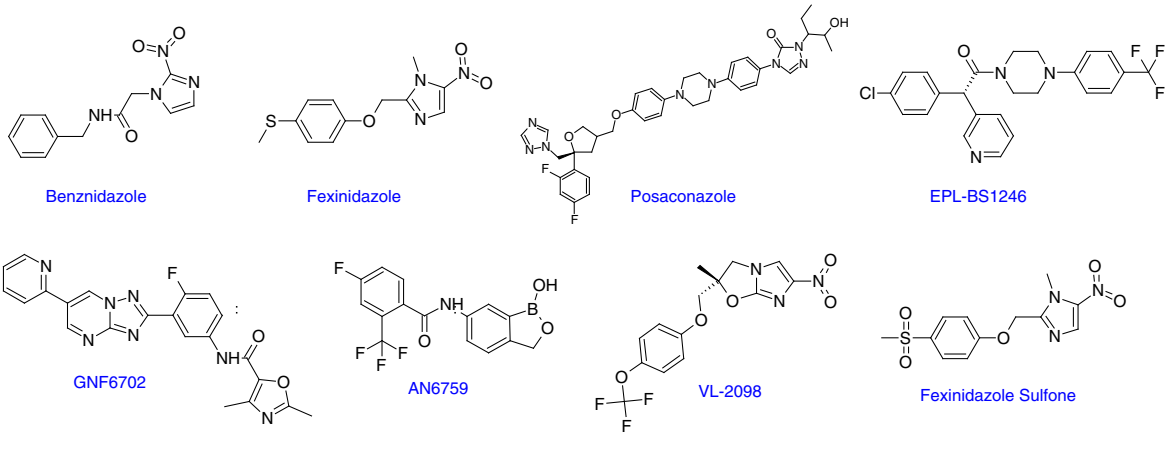


Figure 4.1 Compounds featured in Table 4.1.

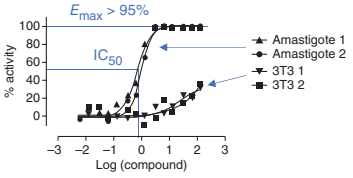
benznidazole and fexinidazole score well (LELP = 3.1 and 5.2, respectively), while higher molecular weight *T. cruzi* CYP51 inhibitors posaconazole and fenarimol analog EPL-BS1246 [42] (as representative examples of this compound class) are out of the optimized zone for oral drugs ($-10 < \text{LELP} < 10$). Oxaborole AN6759 and GNF6702 are representative of two other compound series with impressive pan kinetoplastid activity (human African trypanosomiasis, CD, visceral leishmaniasis) [43, 44]. VL-2098, a reference compound, preclinical candidate for leishmaniasis stopped for safety reasons, is included for comparison given that it also targets an intracellular kinetoplastid pathogen. Although this is a simplistic analysis for a complicated problem with many caveats, calculating a value for LELP and considering that the best hits may actually be fragment-like, could eliminate a bias toward choosing the most potent compounds as the best starting points.

Alongside putting potency into perspective, the value of starting with a chemical scaffold highly resistant to metabolic degradation cannot be underestimated. A significant amount of time can be spent tweaking structures to slow down degradation via oxidative metabolism when a fundamental flaw exists in the chemical scaffold itself. The level of compound coverage required to treat infectious diseases demands molecules that have high AUCs and long half-lives in order to achieve high free-drug concentrations for an extended time. Liabilities with the scaffold should be identified and fixed early to guide structural optimization in a productive direction.

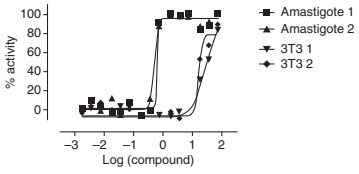
4.3.3 Using *In Vitro* Assays to Guide Structural Optimization

Selecting for compounds able to kill *all* parasites in a host cell over compounds that leave a few amastigotes remaining may lead to better outcomes *in vivo* with respect to achieving sterile cure in animal models and hence treatment of human infection [45, 46]. Along with potency, additional parameters that can be determined from a digitally imaged high-content screening assay using amastigotes as the relevant intracellular multiplying life stage include host cell toxicity, efficacy (E_{max}), and speed of kill [47]. Figure 4.2a shows a typical dose–response from a high-content screening assay that is used to rank compound activity via measurement of the lowest compound concentration required to kill 50%, 90%, or 99% of the amastigotes present, the comparison parameter being determined by what the team decides is the most important or relevant criterion. The assay format concomitantly assesses compound toxicity toward the host cell. Calculating an IC_{50} value toward the host cell prevents over interpretation of compound potency if the selectivity index (SI) is low (Figure 4.2b). Maximal efficacy (E_{max}), the maximum response achievable from an applied compound to a biological system, refers in this context to the complete elimination of parasites from infected host cells (Figure 4.2c). Ensuring compounds are fully efficacious *in vitro* is indicative of a cidal mode of action, as recovery of the amastigote population is prevented. Accounting for assay variability, a practical cutoff value would be c. $E_{max} \geq 95\%$.

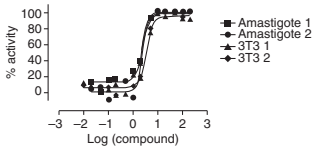
T. cruzi CYP51 inhibitors are a compound class with lower efficacy in this assay format ($E_{max} \sim 85\%$) (Figure 4.2d). Exemplar compounds posaconazole and ravuconazole were shown to be very potent in *in vitro* phenotypic assays (low nanomolar IC_{50}), but did not achieve an $E_{max} \geq 95\%$ [45]. Clinical studies with



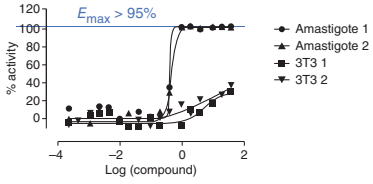
(a) Amastigote activity (n = 2) and host cell toxicity 3T3 mouse fibroblasts (n = 2) for nifurtimox IC_{50} 0.843 μM, E_{max} >95%



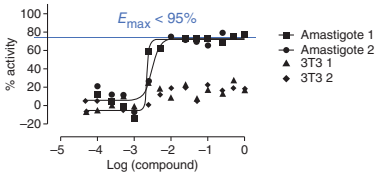
(b) Novel analog series of *T. cruzi* inhibitors Some analogues show a low SI which interferes with determination of the true IC_{50}



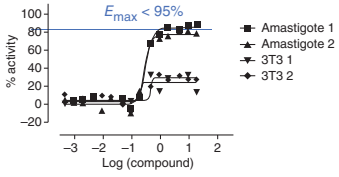
(b) Puromicin as an extreme example of host cell toxicity



(c) Mean % activity EC100 for novel *T. cruzi* inhibitor



(d) Mean % activity <95% for posaconazole typical example profile of a *T. cruzi* CYP51 inhibitor



(d) Mean % activity <95% for novel *T. cruzi* analog containing a pendent pyridazine group that was introduced during the SAR Typical example profile of a *T. cruzi* CYP51 inhibitor

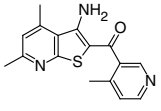
Figure 4.2 Extracting all the information from the raw data available from a primary high-content screen: (a) example dose–response curve; (b) if host cell and IC_{50} curves overlap, compound potency cannot be uncoupled from host cell toxicity; (c) profile of a fully efficacious compound (E_{max} > 95%) versus (d) a *T. cruzi* CYP51 inhibitor that is not (E_{max} > 85%).

these azole compounds were unsuccessful. Indeed, these drugs did not lead to parasitological cure as parasite rebound, as assessed by PCR in blood, occurred within six months to one year of follow-up in 80–100% of the patients treated, demonstrating suppressive rather than curative efficacy [28, 48]. Unfortunately, *T. cruzi* CYP51 is an extremely promiscuous enzyme and structural changes typically made during the medicinal chemistry optimization process can lead compounds into this pharmacophoric space by accident (Figure 4.3). This can happen after introduction of a sterically unencumbered basic nitrogen heterocycle such as pyridine, imidazole, pyridazine, etc. or even a pendent nitrile or primary amine, for example. In the absence of a specific CYP51 counter assay [49], looking at the *in vitro* data for an E_{max} value <95% coupled with a surprising boost in activity is an indication that this might have happened.

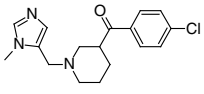
Speculation about a dormant or metabolically quiescent form of *T. cruzi* is becoming more relevant as discovery groups strive to optimize compounds toward achieving 100% sterile cure *in vivo*. Reactivation of a dormant *T. cruzi* infection in patients coinfected with HIV, for example, or following transplantation is a clear indication that the parasite can survive in a latent form [50]. Cellular quiescence is a common mechanism used by pathogens to survive following stresses such as drug pressure or immune surveillance, and dormancy very often underlies chronic infection [51]. This phenomenon can be observed in liver stages of relapsing malaria parasite *Plasmodium*, for example [52], and is now a well-understood mechanism of *Mycobacterium tuberculosis* contributing to symptom-free latent TB [53]. It has also been described in *Salmonella* and *Leishmania*, among others [54, 55]. Rather than exhibiting genetic resistance (mutations in specific genes following drug pressure), the quiescent physiological state of pathogens exhibits phenotypic drug resistance, allowing these pathogens to survive for an extended period of time without causing any symptoms. This was a challenge for TB drug discovery programs until the development of specific assays targeting the nonreplicating form of *M. tuberculosis* [56, 57]. It is very tempting to postulate a similar hypothesis for CD that could explain the current challenges encountered in CD discovery programs and to encourage the CD community to develop an *in vitro* screening assay for drugs targeting the nonreplicating *T. cruzi* parasite.

4.3.4 Getting Compounds to the Site of Action

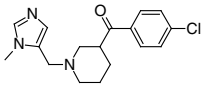
Strategies to optimize *in vivo* efficacy require an understanding of the relationships between drug concentrations and effect, and are expressed as the pharmacokinetic–pharmacodynamic (PK/PD) relationship. These are deduced from pharmacokinetic studies measuring compound concentration over time and the outcome of *in vivo* efficacy studies. Time, minimum inhibitory concentration (MIC), and maximum plasma concentration (C_{max}) are key parameters that have been used to correlate efficacy with drug dosage for antibiotic use [58], and it may be possible to learn from and apply drug treatment strategies developed in this field, particularly with respect to intracellular bacterial infections, to successfully optimize activity against *T. cruzi*.



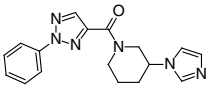
E_{max}: 99.49
IC_{50}: 1.144
Tc CYP51: 0.28



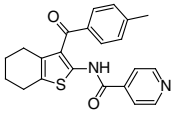
E_{max}: 96.5
IC_{50}: 10.86
Tc CYP51: 0.31



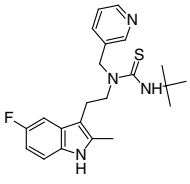
E_{max}: 89.99
IC_{50}: 1.415
Tc CYP51: 0.14



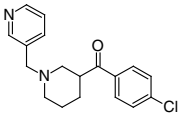
E_{max}: 87.5
IC_{50}: 0.1283
Tc CYP51: 0.0078



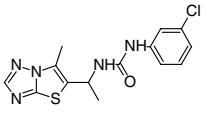
E_{max}: 81.46
IC_{50}: 3.562
Tc CYP51: 0.02



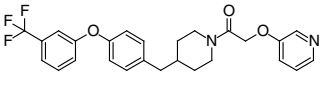
E_{max}: 77.99
IC_{50}: 1.585
Tc CYP51: 0.01



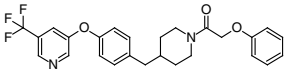
E_{max}: 77.5
IC_{50}: 9.785
Tc CYP51: 0.5



E_{max}: 76.46
IC_{50}: 3.616
Tc CYP51: 0.1



E_{max}: 72.36
IC_{50}: 12.77
Tc CYP51: 0.25



E_{max}: 71.2
IC_{50}: 7.705
Tc CYP51: 0.31

Figure 4.3 Example compounds that have been confirmed as *T. cruzi* CYP51 inhibitors.

Individual PK parameters may drive the PK/PD relationship: (i) time – corresponding to the length of time compound concentration is above the MIC – is critically dependent on the half-life of the compound, dosage, and frequency of administration over a given time period; (ii) peak plasma concentration (C_{max}), which relates efficacy to drug concentration and depends on the dose given and the volume of distribution of the drug; and (iii) area under the concentration–time curve (AUC)/MIC, which combines both types of effect since it corresponds to the total amount of drug to which the infectious agent is exposed over a time period and is inversely proportional to drug clearance [58]. The *in vivo* free-drug concentration in the plasma is an additional important PK/PD parameter, as only free drug is available to engage in target interaction [59]. Optimizing compounds to influence PK/PD parameters is a tricky business. Several excellent reviews highlight the pitfalls and caveats of adopting medicinal chemistry design strategies to target volume of distribution [60], plasma protein and tissue binding [61], and half-life [62]. Selecting the correct dosing paradigm is also a critical factor for success [63].

A few examples of PK/PD relationships in the kinetoplastid field have been reported – fexinidazole [64] for Chagas disease and oxaboroles for stage II HAT [65]. Work on an early *T. cruzi* CYP51 series, derived from an agricultural herbicide fenarimol, established a useful PK/PD criterion for the triage of compounds into lengthy efficacy studies. Compounds were found to be able to reduce parasitemia to undetectable levels in a mouse model of acute infection after 20 days of dosing when compound concentration was greater than the unbound *in vitro* IC_{90} at 24 hours following oral dosing. The PK/PD relationship was an indicator of efficacy rather than curative activity [66]. Significant progress has been made in *in vivo* parasite detection since this work was done, and no curative activity was observed in the BLI mouse model with these series analogs. This begs the question as to whether it is realistic to expect a PK/PD relationship based on systemic PK parameters to predict cure for a *T. cruzi* intracellular infection.

Other important issues to consider when designing compounds to target *T. cruzi* at their site of action are the infection dynamics, and the presence of potential reservoir sites allowing long-term parasite persistence and escape from the host immune response and/or drug pressure. The presence of reservoirs in various tissues and organs of the human body is a common feature of pathogens; adipose tissue, for example, has been shown to serve as a reservoir for HIV, *M. tuberculosis*, and various parasites, including *T. cruzi* [67–70]. This raises additional challenges not only for the medicinal chemist but also for the bioengineer and drug delivery expert.

Structure–kinetic relationships, which describe the kinetics of drug binding and residence time, provide growing importance for better understanding drug effects and improving clinical efficacy [71–74]. While general pharmacokinetics relates to the absorption, distribution, metabolism, and elimination of drugs in the body, cellular pharmacokinetics is centered on evaluation of the penetration, distribution, degradation, and efflux of drugs in individual cells. Knowledge of the biochemical target is obviously critical to being able to explore the possible impact of the drug–target half-life on *in vivo* efficacy. In antibiotic research, the measurement of the intracellular concentration of antibiotics over time has

been shown to provide useful correlations with pharmacodynamic effects; this may provide an interesting perspective on strategies to optimize *in vivo* efficacy against *T. cruzi*, i.e. sterile cure versus parasite suppression [63].

In order to avoid the technical challenge of measuring intracellular drug concentrations, a separate study showed how modeling the chemical kinetics of drug–target binding could help predict optimum dosing regimens for antibiotics [63]. Of the four factors controlling drug effects identified (i.e. the half-life of the antibiotic–target complex, the diffusion barrier between the extracellular antibiotic and its target, the threshold of bound target required to suppress bacterial growth [target occupancy at MIC], and drug effects when the antibiotic is present only at sub-MIC levels), it was concluded that the rate of cell penetration and half-life of the drug–target complex were sufficient to identify the most effective antibiotic treatment strategy. Modeling potential PK–PD scenarios avoids lengthy testing cycles that rely on empirical optimization strategies to drive progress. Would it be possible to apply this modeling strategy to optimize the dosing paradigm for promising *T. cruzi* drugs to achieve sterile cure?

4.3.5 Mechanism of Action: Is There a Need for Target Deconvolution before Starting a Lead Optimization Program?

One parameter the medicinal chemist cannot control is, of course, the mode of action, especially if the hit set under assessment has been generated using a phenotypic screen. Efforts have been made recently to deconvolute hits from these screens in an attempt to quickly identify *T. cruzi* targets of potential interest. This has led to the addition of a few new targets to the already long list of putative targets for the parasite, in particular *T. cruzi* proteasome, cytochrome b, PEX14–PEX5 interaction disruption essential for the glycosomal/peroxisomal import and amino-acyl-tRNA synthetases, to name a few [2, 44, 73–76]. History has shown, however, that there is a long way from the *in vitro* and *in vivo* validation of a target to its proof of potential in clinical settings, the latest examples of the CYP51 inhibitors that failed in proof-of-concept clinical trials for CD, raising the issue of target validation for CD. The question arises then: are all targets equally good, and, if not, what is a good target for Chagas disease? If one believes that anti-chagasic compounds should aim at getting rid of all forms of the *T. cruzi* parasite, then one should ensure that their target is present and expressed not only in all life stages of the parasite but possibly also in the parasite's quiescent state. One could therefore challenge the methodology for parasite target identification; indeed, very often and, for good reason (practicability and speed), quickly replicating parasites (epimastigotes) are being used to generate resistant mutants before whole-genome sequencing and characterization of changes leading to the identification of the putative target. Even the cross-check in a growth inhibition assay of intracellular amastigotes is not necessarily a guarantee of the right target, as the standard assays might not be able to differentiate replicative from non-replicative intracellular parasites.

This raises the potential issue of the effectiveness of the mechanism of action (MoA) of identified compounds currently in lead optimization for CD. Benznidazole, a compound with a modest systemic exposure after oral dosing,

has demonstrated a 100% cure rate in mice models following a five-day treatment; therefore, there is no obvious reason why compounds that have a very good PK profile and high volume of distribution should not have a 100% cure as well, unless their MoA or target(s) are inadequate or possibly not targeting quiescent forms of *T. cruzi* (see Section 4.3.1), or they are not reaching putative reservoirs sites.

Metabolic plasticity of *T. cruzi* as it switches from one stage to another, i.e. from replicating to nonreplicating phases during its life cycle in the different hosts, is crucial to its survival in different environmental conditions. This phenomenon implies changes in the expression of specific proteins and a rigorous regulated adaptive metabolic mechanism. A switch from glucose to consumption of amino acids, for example, was shown to occur in epimastigotes; amino acid consumption was more abundant in the induced stationary phase of parasites in that stage as shown through metabolomics profiling [77]. It remains to be determined which pathways – energetic metabolism, replication pathways, protein synthesis and translation, or specific enzymes – and how many are required to be targeted by lead compounds to achieve a sterile cure. The development of new assays using quiescent forms of *T. cruzi* or different phenotypes of the parasite – if judged essential and if at all possible – could become important before investing resources in a long lead optimization program on a potentially inadequate starting point.

4.4 Conclusion

There is a growing momentum in the Chagas drug discovery community. Rapid progress in the field in the past decade is evident, particularly in increasing knowledge of the required properties of compounds to make good anti-*T. cruzi* entities. Recent identification of novel biochemical targets of the parasite may lead to other focused medicinal chemistry design efforts. Together with a greater understanding of *T. cruzi* infection dynamics and tissue tropism, and the confidence that current mice models translate well into efficacy against human infection (with further data expected from ongoing clinical trials of different doses and regimens that include benznidazole), there is hope that future drug discovery efforts will ultimately lead to drug candidates that meet the criteria detailed in the target product profile for chronic and acute forms of CD.

However, there is still a significant gap in our knowledge of host/parasite interactions and disease progression [78]. This gap has prevented medicinal chemists from integrating biological knowledge into the design of new compounds; the answer to the question, "How can we optimize a compound that will lead to sterile cure?" is an ongoing question in Chagas drug discovery teams.

So far, aside from the standard improvement of ADME properties for compounds of interest, the focus has often been on the optimization of *in vitro* potency – a very small part of the puzzle – coupled recently with efforts not to fall back into the CYP51 inhibitor chemical space. Efforts not to target TcCYP51 inhibitors followed new knowledge issuing from CD clinical trials and nonclinical studies with azoles as well as the development of assays able to filter out such compounds.

All drug discovery programs rely on the quality of hit molecules selected as starting points; recent developments have shown, however, that even when taking these points into account and selecting starting points based on the newest knowledge in the CD field, lead optimization programs have not, thus far, led to new chemical entities as potential CD drug candidates. One might argue that designing compounds that will lead to sterile cure in animal models may be a too stringent pass criterion; on the other hand, one could emphasize the utmost importance of the relevance of the mechanism of action and targets of new hit molecules. It is emerging that when designing compounds, we may need to target different phenotypic forms of *T. cruzi*, with the dormant or quiescent form becoming increasingly more important. In that respect, the application of knowledge gained from other fields such as tuberculosis, HIV, and malaria could be useful (e.g. assays using nonreplicative forms of the parasite, the targeting of reservoir sites, or a combination treatment). The generation of data on the MoA and its integration into optimization programs will also be important, not only to predict potential safety issues (e.g. the need for a higher safety margin considering the risk/benefit ratio in asymptomatic Chagas patients) but to assess, if possible, the adequacy of a compound series MoA and/or target identified to start a medicinal chemistry program. In the latter case, the identification of new targets could open up new avenues for CD drug discovery currently not being pursued, such as target-based screens, structure-based drug design, and fragment-based drug design, to name but a few.

In summary, designing drugs and treatment strategies to cure *T. cruzi* infections is clearly a complex process requiring the optimization of multiple parameters, many of which remain unknown or are not possible to measure. More biological understanding is required in order for medicinal chemists to design appropriate molecules that are able to reach highly adaptive, phenotypically varied, and promiscuous intracellular parasites.

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References

1 Keenan, M. and Chaplin, J.H. (2015). A new era for chagas disease drug discovery? *Prog. Med. Chem.* 54: 185–230.
2 Duschak, V.G. (2016). Targets and patented drugs for chemotherapy of chagas disease in the last 15 years-period. *Recent. Pat. Antiinfect. Drug Discov.* 11: 74–173.
3 Chagas, C. (1909). Nova tripanozomiase humana: estudos sobre a morfolojia e o ciclo evolutivo do Schizotrypanum cruzi n. gen., n. sp., ajente etiolojico de nova entidade morbida do homem. *Mem. Inst. Oswaldo Cruz* 1: 159–218.
4 Schmunis, G.A. and Yadon, Z.E. (2015). Chagas disease: a Latin American health problem becoming a world health problem. *PLoS Negl. Trop. Dis.* 9: e0003540.
5 Meymandi, S.K., Hernandez, S., and Forsyth, C.J. (2017). A community-based screening program for Chagas disease in the USA. *Trends Parasitol.* 33: 828–831.
6 Requena-Méndez, A., Aldasoro, E., de Lazzari, E. et al. (2015). Prevalence of Chagas disease in Latin-American migrants living in Europe: a systematic review and meta-analysis. *PLoS Negl. Trop. Dis.* 9: e0003540.
7 Jackson, Y., Pinto, A., and Pett, S. (2014). Chagas disease in Australia and New Zealand: risks and needs for public health interventions. *Trop. Med. Int. Health* 19: 212–218.
8 Moolani, Y., Bukhman, G., and Hotez, P.J. (2012). Neglected tropical diseases as hidden causes of cardiovascular disease. *PLoS Negl. Trop. Dis.* 6: e1499.
9 WHO Fact Sheet No 340. http://www.who.int/mediacentre/factsheets/fs340/en/ (accessed January 2018).
10 Cucunubá, Z.M., Okuwoga, O., Basáñez, M.G., and Nouvellet, P. (2016). Increased mortality attributed to Chagas disease: a systematic review and meta-analysis. *Parasit. Vectors* 9: 42.
11 Pérez-Molina, J.A. and Molina, I. (2018). Chagas disease. *Lancet* 391: 82–94.
12 Ayub-Ferreira, S.M., Mangini, S., Issa, V.S. et al. (2013). Mode of death on Chagas heart disease: comparison with other etiologies. a subanalysis of the REMADHE prospective trial. *PLoS Negl. Trop. Dis.* 7: e2176.
13 Brum-Soares, L., Cubides, J.C., Burgos, I. et al. (2016). High seroconversion rates in *Trypanosoma cruzi* chronic infection treated with benznidazole in people under 16 years in Guatemala. *Rev. Soc. Bras. Med. Trop.* 49: 721–727.
14 Olivera, M.J., Cucunubá, Z.M., Álvarez, C.A., and Nicholls, R.S. (2015). Safety profile of nifurtimox and treatment interruption for chronic Chagas disease in Colombian adults. *Am. J. Trop. Med. Hyg.* 93: 1224–1230.
15 Berman, J. and Radhakrishna, T. (2017). The tropical disease priority review voucher: a game-changer for tropical disease products. *Am. J. Trop. Med. Hyg.* 96: 11–13.
16 Chatelain, E. (2015). Chagas disease drug discovery: toward a new era. *J. Biomol. Screening* 20: 22–35.

17 De Rycker, M., Thomas, J., Riley, J. et al. (2016). Identification of trypanocidal activity for known clinical compounds using a new trypanosoma cruzi hit-discovery screening cascade. *PLoS Negl. Trop. Dis.* 10: e0004584.
18 Chatelain, E. (2016). Chagas disease research and development: is there light at the end of the tunnel? *Comput. Struct. Biotechnol. J.* 15: 98–103.
19 Balouz, V., Agüero, F., and Buscaglia, C.A. (2017). Chagas disease diagnostic applications: present knowledge and future steps. *Adv. Parasitol.* 97: 1–45.
20 https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm573942.htm (accessed 16 February 2018).
21 Katsuno, K., Burrows, J.N., Duncan, K. et al. (2015). Hit and lead criteria in drug discovery for infectious diseases of the developing world. *Nat. Rev. Drug Discovery* 14: 751–758.
22 Urbina, J.A. (1999). Parasitological cure of Chagas disease: is it possible? Is it relevant? *Mem. Inst. Oswaldo Cruz* 94 (Suppl. 1): 349–355.
23 Urbina, J.A. and McKerrow, J.H. (2015). Drug susceptibility of genetically engineered *Trypanosoma cruzi* strains and sterile cure in animal models as a criterion for potential clinical efficacy of anti-T. cruzi drugs. *Antimicrob. Agents Chemother.* 59: 7923–7924.
24 Lewis, M.D., Francisco, A.F., Taylor, M.C., and Kelly, J.M. (2015). A new experimental model for assessing drug efficacy against *Trypanosoma cruzi* infection based on highly sensitive in vivo imaging. *J. Biomol. Screening* 20: 36–43.
25 Lewis, M.D. and Kelly, J.M. (2016). Putting infection dynamics at the heart of Chagas disease. *Trends Parasitol.* 32: 899–911.
26 Francisco, A.F., Lewis, M.D., Jayawardhana, S. et al. (2015). Limited ability of posaconazole to cure both acute and chronic *Trypanosoma cruzi* infections revealed by highly sensitive in vivo imaging. *Antimicrob. Agents Chemother.* 59: 4653–4661.
27 Morillo, C.A., Waskin, H., Sosa-Estani, S. et al. (2017). Benznidazole and posaconazole in eliminating parasites in asymptomatic *T. cruzi* carriers: the stop-Chagas trial. *J. Am. Coll. Cardiol.* 69: 939–947.
28 Molina, I., Gómez i Prat, J., Salvador, F. et al. (2014). Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. *N. Engl. J. Med.* 370: 1899–1908.
29 Bustamante, J.M., Craft, J.M., Crowe, B.D. et al. (2014). New, combined, and reduced dosing treatment protocols cure *Trypanosoma cruzi* infection in mice. *J. Infect. Dis.* 209: 150–162.
30 Buckner, F.S., Verlinde, C.L., La Flamme, A.C., and Van Voorhis, W.C. (1996). Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. *Antimicrob. Agents Chemother.* 40: 2592–2597.
31 Moon, S., Siqueira-Neto, J.L., Moraes, C.B. et al. (2014). An image-based algorithm for precise and accurate high throughput assessment of drug activity against the human parasite *Trypanosoma cruzi*. *PLoS ONE* 9 (2): e87188.
32 Sykes, M.L. and Avery, V.M. (2015). Development and application of a sensitive, phenotypic, high-throughput image-based assay to identify compound

activity against *Trypanosoma cruzi* amastigotes. *Int. J. Parasitol. Drugs Drug Resist.* 5: 215–228.

33 Carmody, L.C., Germain, A., Barker, D. et al. (2010). *Identification of Small-Molecule Inhibitors of Trypanosoma cruzi Infection. Probe Reports from the NIH Molecular Libraries Program [Internet]*. Bethesda (MD): National Center for Biotechnology Information (US) (updated 2011 Feb 10).

34 Peña, I., Pilar Manzano, M., Cantizani, J. et al. (2015). New compound sets identified from high throughput phenotypic screening against three kinetoplastid parasites: an open resource. *Sci. Rep.* 5: 8771.

35 Besnard, J., Jones, P.S., Hopkins, A.L., and Pannifer, A.D. (2015). The Joint European Compound Library: boosting precompetitive research. *Drug Discovery Today* 20: 181–186.

36 Karawajczyk, A., Giordanetto, F., Benningshof, J. et al. (2015). Expansion of chemical space for collaborative lead generation and drug discovery: the European lead factory perspective. *Drug Discovery Today* 20: 1310–1316.

37 https://www.dndi.org/diseases-projects/open-innovation/drug-discovery-booster/ (accessed 16 February 2018).

38 Swinney, D.C. (2013). Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clin. Pharmacol. Ther.* 93: 299–301.

39 Francisco, A.F., Jayawardhana, S., Lewis, M.D. et al. (2016). Nitroheterocyclic drugs cure experimental *Trypanosoma cruzi* infections more effectively in the chronic stage than in the acute stage. *Sci. Rep.* 6: 35351.

40 Hopkins, A.L., Keserü, G.M., Leeson, P.D. et al. (2014). The role of ligand efficiency metrics in drug discovery. *Nature Rev. Drug Disc.* 13: 105–121.

41 Tarcsay, A., Nyiri, K., and Keseru, G.M. (2012). Impact of lipophilic efficiency on compound quality. *J. Med. Chem.* 55: 1252–1260.

42 Keenan, M., Chaplin, J.H., Alexander, P.W. et al. (2013). Two analogues of fenarimol show curative activity in an experimental model of chagas disease. *J. Med. Chem.* 56: 10158–10170.

43 Jacobs, R.T., Plattner, J.J., and Keenan, M. (2011). Boron-based drugs as antiprotozoals. *Curr. Opin. Infect. Dis.* 24: 586–592.

44 Khare, S., Nagle, A.S., Biggart, A. et al. (2016). Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature* 537: 229–233.

45 Moraes, C.B., Giardini, M.A., Kim, H. et al. (2014). Nitroheterocyclic compounds are more efficacious than CYP51 inhibitors against *Trypanosoma cruzi*: implications for Chagas disease drug discovery and development. *Sci. Rep.* 4: 4703.

46 Cal, M., Ioset, J.R., Fügi, M.A. et al. (2016). Assessing anti-*T. cruzi* candidates in vitro for sterile cidality. Int. *J. Parasitol. Drugs Drug Resist.* 6: 165–170.

47 Sykes, M.L. and Avery, V.M. (2018). 3-pyridyl inhibitors with novel activity against Trypanosoma cruzi reveal in vitro profiles can aid prediction of putative cytochrome P450 inhibition. *Sci. Rep.* 8: 4901.

48 Torrico, F., Gascon, J., Ortiz, L. et al., E1224 Study Group (2018). Treatment of adult chronic indeterminate Chagas disease with benznidazole and three E1224 dosing regimens: a proof-of-concept, randomised, placebo-controlled

trial. *Lancet Infect. Dis.* pii: S1473-3099 (17): 30538–30538. (Epub ahead of print).

49 Riley, J., Brand, S., Voice, M. et al. (2015). Development of a fluorescence-based *Trypanosoma cruzi* CYP51 inhibition assay for effective compound triaging in drug discovery programmes for Chagas disease. *PLoS Negl. Trop. Dis.* 9: e0004014.

50 Pacheco, R.S., Ferreira, M.S., Machado, M.I. et al. (1998). Chagas' disease and HIV co-infection: genotypic characterization of the *Trypanosoma cruzi* strain. *Mem. Inst. Oswaldo Cruz* 93: 165–169.

51 Rittershaus, E.S., Baek, S.H., and Sassetti, C.M. (2013). The normalcy of dormancy: common themes in microbial quiescence. *Cell Host Microbe* 13: 643–651.

52 Voorberg-van der Wel, A., Roma, G., Gupta, D.K. et al. (2017). A comparative transcriptomic analysis of replicating and dormant liver stages of the relapsing malaria parasite *Plasmodium cynomolgi*. *Elife* 6: pii: e29605.

53 Boon, C. and Dick, T. (2012). How Mycobacterium tuberculosis goes to sleep: the dormancy survival regulator DosR a decade later. *Future Microbiol.* 7: 513–518.

54 Helaine, S., Cheverton, A.M., Watson, K.G. et al. (2014). Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. *Science* 343: 204–208.

55 Kloehn, J., Saunders, E.C., O'Callaghan, S. et al. (2015). Characterization of metabolically quiescent Leishmania parasites in murine lesions using heavy water labeling. *PLoS Pathog.* 11: e1004683.

56 Gold, B., Roberts, J., Ling, Y. et al. (2015). Rapid, semiquantitative assay to discriminate among compounds with activity against replicating or non-replicating *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 59: 6521–6538.

57 Vocat, A., Hartkoorn, R.C., Lechartier, B. et al. (2015). Bioluminescence for assessing drug potency against nonreplicating Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 59: 4012–4019.

58 Van Bambeke, F., Barcia-Macay, M., Lemaire, S., and Tulkens, P.M. (2006). Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. *Curr. Opin. Drug Discovery Dev.* 9: 218–230.

59 Smith, D.A., Di, L., and Kerns, E.H. (2010). The effect of plasma-protein binding on in vivo efficacy: misconceptions in drug discovery. *Nat. Rev. Drug Discovery* 9: 929–939.

60 Smith, D.S., Beaumont, K., Maurer, T.S., and Di, L. (2015). Volume of distribution in drug design. *J. Med. Chem.* 58: 5691–5698.

61 Liu, X., Wright, M., and Hop, C.E. (2014). Rational use of plasma protein and tissue binding data in drug design. *J. Med. Chem.* 57: 8238–8248.

62 Smith, D.A., Beaumont, K., Maurer, T.S., and Di, L. (2017). Relevance of half-life in drug design. *J. Med. Chem.* 61 (10): 4273–4282.

63 Abel zur Wiesch, P., Clarelli, F., Cohen, T. (2017). Using chemical reaction kinetics to predict optimal antibiotic treatment strategies. *PLOS Computat. Biol.* 13: e1005321.

64 Bahia, M.T., Nascimento, A.F., Mazzeti, A.L. et al. (2014). Antitrypanosomal activity of fexinidazole metabolites, potential new drug candidates for Chagas disease. *Antimicrob. Agents Chemother.* 58: 4362–4370.

65 Wring, S., Gaukel, E., Nare, B. et al. (2014). Pharmacokinetics and pharmacodynamics utilizing unbound target tissue exposure as part of a disposition-based rationale for lead optimization of benzoxaboroles in the treatment of Stage 2 Human African Trypanosomiasis. *Parasitology* 141: 104–118.

66 Keenan, M., Abbott, M.J., Alexander, P.W. et al. (2012). Analogues of fenarimol are potent inhibitors of Trypanosoma cruzi and are efficacious in a murine model of Chagas disease. *J. Med. Chem.* 55: 4189–4204.

67 Couturier, J. and Lewis, D.E. (2018). HIV persistence in adipose tissue reservoirs. *Curr. HIV/AIDS Rep.* 15: 60–71.

68 Beigier-Bompadre, M., Montagna, G.N., Kühl, A.A. et al. (2017). Mycobacterium tuberculosis infection modulates adipose tissue biology. *PLoS Pathog.* 13: e1006676.

69 Tanowitz, H.B., Scherer, P.E., Mota, M.M., and Figueiredo, L.M. (2017). Adipose tissue: a safe haven for parasites? *Trends Parasitol.* 33: 276–284.

70 Ferreira, A.V., Segatto, M., Menezes, Z. et al. (2011). Evidence for *Trypanosoma cruzi* in adipose tissue in human chronic Chagas disease. *Microbes Infect.* 13: 1002–1005.

71 Vauquelin, G. (2016). Effects of target binding kinetics on in vivo drug efficacy: k_{off}, k_{on} and rebinding. *Br. J. Pharmacol.* 173: 2319–2334.

72 Dahl, G. and Akerud, T. (2013). Pharmacokinetics and the drug-target residence time concept. *Drug Discovery Today* 18: 697–707.

73 Walkup, G.K., You, Z., Ross, P.L. et al. (2015). Translating slow-binding inhibition kinetics into cellular and in vivo effects. *Nat. Chem. Biol.* 11: 416–423.

74 Khare, S., Roach, S.L., Barnes, S.W. et al. (2015). Utilizing chemical genomics to identify cytochrome b as a novel drug target for Chagas disease. *PLoS Pathog.* 11: e1005058.

75 Dawidowski, M., Emmanouilidis, L., Kalel, V.C. et al. (2017). Inhibitors of PEX14 disrupt protein import into glycosomes and kill Trypanosoma parasites. *Science* 355: 1416–1420.

76 Pham, J.S., Dawson, K.L., Jackson, K.E. et al. (2013). Aminoacyl-tRNA synthetases as drug targets in eukaryotic parasites. *Int. J. Parasitol. Drugs Drug Resist.* 4: 1–13.

77 Barisón, M.J., Rapado, L.N., Merino, E.F. et al. (2017). Metabolomic profiling reveals a finely tuned, starvation-induced metabolic switch in *Trypanosoma cruzi* epimastigotes. *J. Biol. Chem.* 292: 8964–8977.

78 Francisco, A.F., Jayawardhana, S., Lewis, M.D. et al. (2017). Biological factors that impinge on Chagas disease drug development. *Parasitology* 144: 1871–1880.

5

Drug Discovery and Development for Human African Trypanosomiasis

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5.1 Overview of Disease

Human African trypanosomiasis (HAT) is a parasitic disease caused by *Trypanosoma brucei* (Figure 5.1) and is one of 20 neglected tropical diseases (NTDs), as designated by the World Health Organization (WHO). HAT is confined to sub-Saharan Africa, and around 50 million people are estimated to live in the affected area. However, due to recent efforts to eradicate HAT, the number of infected people has been reduced to an estimated 15 000 cases worldwide; but due to a variety of sociopolitical and economic factors facing the region, that number can vary wildly [1]. This is contrasted by the 2184 new cases reported in 2016 [2], although this number may be misrepresented by a factor of 4 or 5 [3].

T. brucei is transmitted through the bite of an infected tsetse fly, which is a blood-feeding insect that preys on mammalian hosts. *T. b. gambiense* and *T. b. rhodesiense* are both human infective, but differ in their geographical distribution as well as in the time frame for disease progression. *T. b. brucei* only affects certain nonhuman animal types, but can still have further economic impacts on these poorer regions [4].

As evidenced by the declining numbers of new HAT cases, the large-scale implementation of control tools is having a positive impact on the effort to eliminate HAT. Despite this, HAT continues to be a risk for a severe epidemic and this has been seen numerous times. Current treatments for this disease are aging and are associated with complex treatment regimens and often result in the development of long-term health issues; in addition, reports of resistance are becoming more frequent. Combined, these factors contribute to the need for new lead compounds to fill the drug discovery pipeline for HAT.

5.2 Etiology and Epidemiology

The *T. brucei* life cycle has two distinct stages, which are denoted by its current host (Figure 5.2). Infection with HAT occurs through the bite of an infected tsetse

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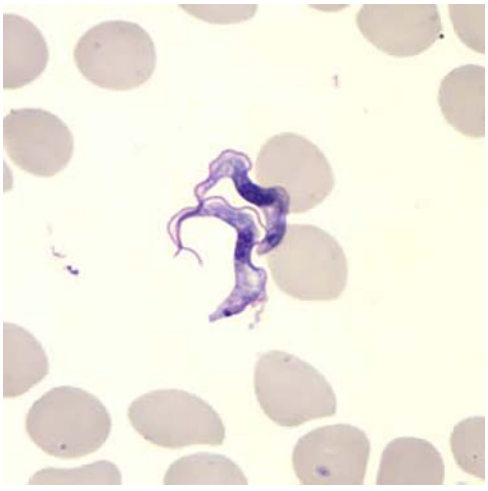


Figure 5.1 Giemsa-stained light photomicrograph of a blood smear contains two *Trypanosoma brucei* parasites. Source: https://phil.cdc.gov/Details.aspx?pid=11821 [83].

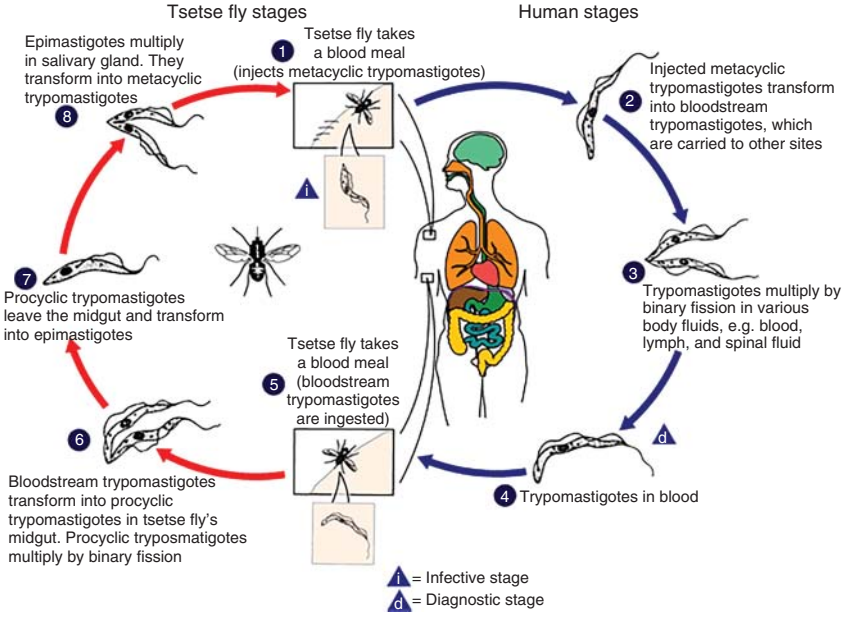


Figure 5.2 Life cycle of *Trypanosoma brucei* in both the tsetse fly and human. Source: https://www.cdc.gov/parasites/sleepingsickness/biology.html [5].

fly and can lead to the formation of a chancre at the bite site. Once the parasite is in the bloodstream, it converts itself from a metacyclic trypomastigote to a bloodstream trypomastigote, and begins to multiply by binary fission in several different types of body fluid. The parasites in the bloodstream are then transferred to a new tsetse fly the next time that human is bitten. The bloodstream trypomastigote changes into a procyclic trypomastigote in the tsetse fly's midgut, and the parasite undergoes binary fission to replicate. The procyclic trypomastigote changes

itself into an epimastigote, travels to the salivary gland, and finally changes into a metacyclic trypomastigote. The newly infected tsetse fly bites another human, and the cycle begins again.

HAT is a focal disease with 84% of all reported cases due to *T. b. gambiense* in 2012 coming from the Democratic Republic of Congo [6]. *T. b. gambiense* accounts for 98% of all cases and is found almost exclusively in Central and West Africa, whereas *T. b. rhodesiense* is found almost exclusively in East Africa and these two parasites currently share no overlap in endemic areas (Figure 5.3) [9, 10]. Beyond geographical differences, there are also differences in how the disease presents between the two subspecies. HAT progresses in two

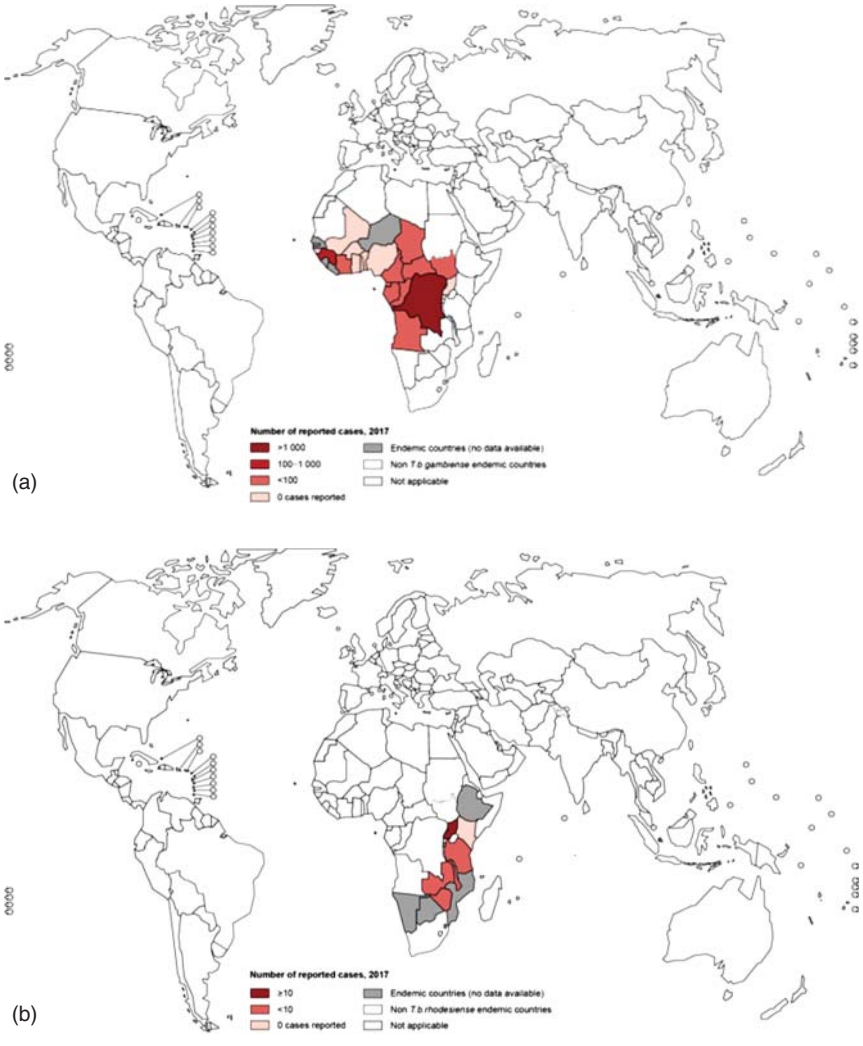


Figure 5.3 Distribution of human African trypanosomiasis worldwide as seen in 2017 (a) depiction of the spread of HAT caused by *T. b. gambiense* [7], and (b) depiction of cases caused by *T. b. rhodesiense* [8]. Source: Reproduced with permission of World Health Organization.

stages, the first is referred to as the hemolymphatic stage, where the parasites reside in the blood and lymphatic systems, the second stage (also known as the meningoencephalitic stage) occurs once the parasite crosses the blood–brain barrier (BBB) and affects the central nervous system (CNS). Infection with *T. b. gambiense* leads to a chronic infection whereby patients experience a longer Stage 1 phase, lasting months or even years before the parasite crosses the BBB. *T. b. rhodesiense* has a much shorter disease course, and can cause death within 3–12 months of initial infection [11].

Initially, symptoms include joint pain, headaches, fever, drowsiness, and swelling of the lymph nodes. However, none of these alone are sufficient to diagnose HAT, especially because there are various other tropic and subtropic diseases that can mimic or coexist with HAT, such as HIV infection, meningitis, malaria, enteric fever, tuberculous meningitis, or even the common cold. Once the parasite enters the CNS, the Stage 2 symptoms clinically manifest and there are a host of neurological features including motor, psychiatric, sensory, and sleep abnormalities [12]. Psychiatric manifestations include lassitude, irritability, personality changes, and psychosis [13]. One of the primary neurological conditions associated with HAT is the reversal of the sleep–wake cycle, with the patient experiencing nocturnal insomnia and daytime somnolence. Patients can die from complications associated with their immunocompromised state, which causes an increased risk of opportunistic infections such as pneumonia or meningitis. Otherwise, HAT leads to seizures, coma, and eventual death. However, patients with Stage 2 *T. b. rhodesiense* infections present with suppressed clinical symptoms, with many experiencing only drowsiness or tremors [14]. This is not to say that it is less fatal, with the majority of deaths from *T. b. rhodesiense* infection occurring within six months of the onset of the illness due to congestive heart failure, arrhythmia, and pericardial effusion [12].

Over the past century *T. b. rhodesiense* and *T. b. gambiense* have been responsible for a number of epidemics [5]. The first started in 1896 and lasted for 20 years, and was located in Uganda and the Congo Basin [11]. During this 20-year span, an estimated 700 000–800 000 people were killed. The next major epidemic began in Uganda in 1901 and made its way to Sudan, Tanzania, Zambia, and Malawi over the next seven years [11]. By 1905, one-third of the population had died from HAT and it is thought to have been exacerbated by the spreading of trade and cultural contact due to colonialism. Using modern disease control techniques, the epidemic was finally ended in 1960. However, due to the ban of dichlorodiphenyltrichloroethane, a potent mosquito toxin, and the relaxation of regulations after 1960, another epidemic began in 1970. This epidemic mainly affected Angola, Congo, Sudan, and Uganda and lasted until 1990 and ended with the introduction of eflornithine [15].

As mentioned previously, *T. b. brucei* infects mammals other than humans; this is called African animal trypanosomiasis (AAT or nagana), which is an infection in livestock with *T. congolense*, *T. vivax,* or *T. b. brucei*. AAT is also transmitted through infected tsetse flies and is responsible for infections in a variety of domestic animals; however, humans are immune to the AAT form of trypanosomiasis [15]. Across sub-Saharan Africa, 65% of the labor force and 32% of the GDP comes from agriculture, and current estimates have shown that cattle density is reduced

by 37–70% and the yearly offtake rate is reduced by 50% because of the effects of AAT [3]. In addition, these livestock are also one of the primary food sources for many villages in the region [9]. AAT has significant economic consequences in areas of the world that are already struggling.

5.3 Current Treatments

The current treatments for HAT consist of suramin and pentamidine for Stage 1 HAT infections, and melarsoprol, eflornithine, or nifurtimox-eflornithine combination therapy (NECT) for Stage 2 infections (Figure 5.4). They are summarized in Table 5.1, and described in detail later.

5.3.1 Stage 1 Treatments

Suramin was the first treatment identified for Stage 1 HAT and came about after it was discovered that various naphthalene dyes had notable trypanocidal activity. It is a symmetrical drug that is readily soluble in water, and through extensive research into the structure–activity relationship (SAR) around this molecule, it has been noted that any changes to the structure significantly hamper the efficacy against trypanosomes [16]. Due to suramin's large size and polar structure, it is unable to cross the BBB, which renders it only viable as a Stage 1 treatment. Suramin is also known to bind a variety of serum proteins, including low-density lipoproteins (LDLs). Typically, suramin will bind to an LDL and enter the parasite through receptor-mediated endocytosis. Apoprotein B uses its positively charged amino acids to bind to the negatively charged residues on LDL. This process is inhibited when LDL is bound to suramin, inhibiting the cholesterol uptake the parasite needs for growth. In addition,

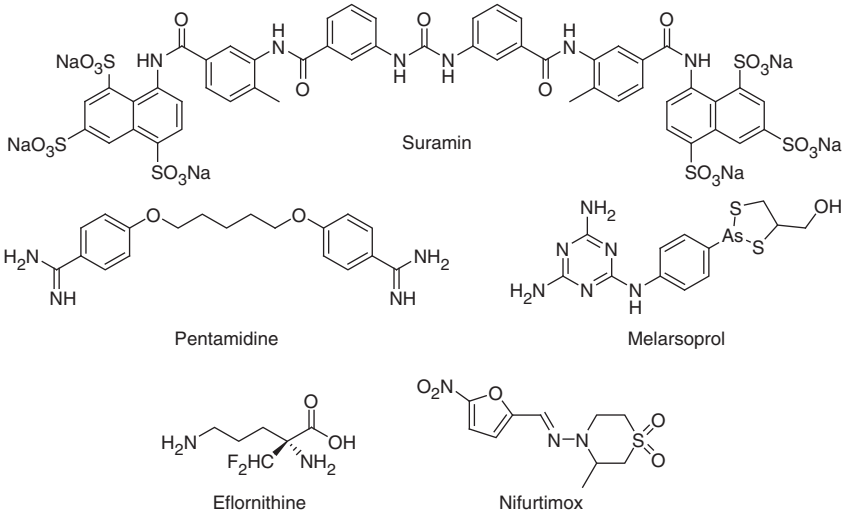


Figure 5.4 Current chemotherapeutics for HAT.

Table 5.1 A summary of the current treatments for HAT including the mechanism of action, dosing regimen, and known problems associated with its use.

Indication	Drug	Mechanism of action	Dosing regimen	Associated problems
Stage 1	Pentamidine (1940)	Inhibits P2-mediated adenosine uptake; thought to have a variety of nonspecific interactions which contribute to the potency	4 mg/kg/d intramuscularly or intravenously (diluted in saline, in 2-h infusions) × 7 d[a)]	Preferred treatment for *T. b. gambiense* infections Has been used as a prophylactic treatment; highly plasma protein bound – ~70% which leads to a long elimination half-life (~several weeks)
	Suramin (1920s)	Binds a large variety of serum proteins, including low-density lipoproteins; also inhibits many positively charged glycolytic proteins	Test dose of 4–5 mg/kg intravenously, then 20 mg/kg intravenously once per week × 5 wk (maximum 1 g/injection)	Used primarily for Stage 1 *T. b. rhodesiense* HAT Associated with toxicity issues
Stage 2	Melarsoprol (1947)	Thought to be related to its ability to bind purine transporters	Series of four injections given over 4 d. Ten-day treatment hiatus to recover and the injection procedure is repeated. After another 10 d, the final four doses are given.[b)] Treatment is extremely painful	Due to poor aqueous solubility, it is stored and injected into the body while solubilized in propylene glycol Highly toxic, with ~5% treatment-related mortality. Increasing number of treatment failures (up to 30% in some regions); particularly in patients with mutations of the purine transporters
	Eflornithine (1981)	Irreversibly inhibits ornithine decarboxylase, which is important in the biosynthesis of polyamines	Administer 400 mg/kg/d intravenously in four 2-h infusions (each dose diluted in 100 ml of water for injection)[c)] × 14 d[b)]	Resistance is easy to develop in the laboratory through loss of amino acid transporter Not recommended for *T. b. rhodesiense* infections

Table 5.1 (Continued)

Indication	Drug	Mechanism of action	Dosing regimen	Associated problems
	Nifurtimox–eflornithine combination therapy – NECT (2009)		Nifurtimox 15 mg/kg/d orally in three doses × 10 d; Eflornithine 400 mg/kg/d intravenously in two 2-h infusions (each dose diluted in 250 ml of water for injection)[c] × 7 d[b]	Favored over eflornithine monotherapy as it simplifies the treatment regimen Nifurtimox trialed as a monotherapy, although it failed due to variable cure rates and toxicity with high doses and prolonged treatment Resistance is a problem

a) Pentamidine can be given after the first trimester of pregnancy.
b) Nifurtimox, eflornithine, and melarsoprol, all of which are theoretically contraindicated, in practice are given when the mother is in advanced second-stage disease and her condition does not permit waiting. If postponement of treatment until childbirth is judged possible, a full course of pentamidine should be administered, principally to prevent vertical transmission.
c) Children weighing <10 kg: dilute in 50 ml of water for injection. Children weighing 10–25 kg: dilute in 100 ml of water for injection. If water for injection is unavailable, eflornithine can be diluted in 5% dextrose or saline.

suramin binds to and inhibits many of the positively charged glycolytic proteins in trypanosomes [17]. Side effects for this treatment are extremely common, and they include nephrotoxicity, peripheral neuropathy, and bone marrow toxicity with agranulocytosis and thrombocytopenia. Rarely, it is possible for acute and late hypersensitivity to occur [17]. Currently, suramin has not seen resistance in either *T. b. gambiense* or *T. b. rhodesiense*, but that is not to say it is not possible. Research into *T. evansi*, a different trypanosome species that infects large mammals, but not humans, has shown that trypanosome resistance is possible and genetically stable. With enough selective pressure, it seems likely that resistance would occur in the relevant strains [18].

Pentamidine is the second treatment for Stage 1 HAT. It was introduced in 1941 as a trypanocide, and is still the treatment of choice for Stage 1 *T. b. gambiense* infection. Typically, pentamidine is given intramuscularly over the course of a week. However, if there are facilities available for an IV infusion, then that is the method of choice, as it only takes two hours [18]. After injection, pentamidine will stay in the body for a significant amount of time as the elimination half-life can be several weeks, depending on the patient's age, sex, and general health. While pentamidine is most effective against Stage 1 HAT due to its poor penetration of the BBB, it has shown promising effectiveness in early Stage 2 patients.

Once in the body, around 70% of the drug is bound to plasma proteins which are then brought into the parasite using membrane transporters [18]. Pentamidine has been shown to inhibit P2-mediated adenosine uptake, and is effective at adenosine levels increased 100-fold over natural *T. b. brucei*. Pentamidine does not rely solely on any one transporter, making it unlikely for transport-related pentamidine resistance to occur. This could be one of the reasons that resistance to this drug has been seen only a few times clinically, even when it was being used as a mass prophylactic [17]. Pentamidine is a very common form of treatment for HAT even though the mechanism of action is not very well understood. Due to the high accumulation levels in the body, it is thought that there are a variety of nonspecific actions which contribute to potency. The first is due to pentamidine's ability to bind nucleic acids and form complexes with various types of double-stranded DNA, excluding human DNA. It has also been shown to be an effective agonist for the *N*-methyl-D-aspartate glutamate receptor and the delta2 receptor, both of which are necessary for a trypanosome's survival [18]. Pentamidine has relatively mild side effects compared to the other treatments, and, when given intramuscularly, the most common side effects include site pain and transient swelling, abdominal pain and gastrointestinal problems, and hypoglycemia. Other side effects frequently seen when pentamidine is used to treat other diseases are leucopenia, thrombocytopenia, hyperkalemia, and QT prolongation [4].

5.3.2 Stage 2 Treatments

Melarsoprol was first used to treat HAT in 1947, and continues to be the treatment of choice for Stage 2 *T. b. gambiense* or *T. b. rhodesiense* infections. This is mainly because very few drugs have shown the ability to both pass through the BBB and have strong activity against the parasite. The dosing schedule typically starts with a series of four injections over four days. The patient is then given 10 days to recover before the injection procedure is repeated. After another 10 days, the patient is given the final four doses. Due to the poor aqueous solubility, it is stored and injected into the body while solubilized in propylene glycol [17]. This is extremely painful for the patient, and commonly causes thrombophlebitis and severe necrosis at the injection site. Furthermore, melarsoprol itself is extremely toxic due to the trivalent arsenic serving as the active moiety. Dimercaprol is used to moderate this toxicity, although this has also been shown to induce convulsions and coma [18]. This combination therapy has side effects which include encephalopathy in 10% of patients and mortality in 5% of patients. The mechanism of action for melarsoprol is thought to be related to its ability to bind purine transporters. Trypanosomes are not able to synthesize their own nucleic acids and must take them from their host, making the purine transporters an important step in replication. Providing evidence for this theory, melarsoprol has shown failures in treating patients with a mutation of the purine transporter. In some places, this resistance has reached levels as high as 30% [17].

Eflornithine was first synthesized in 1978 as a possible cancer chemotherapeutic. Around the same time, it was independently discovered to have strong trypanocidal properties. However, due to differences in trypanosome subspecies, it is only effective against *T. b. gambiense*, and not *T. b. rhodesiense*

infections. The dosing regimen typically consists of 100 mg/kg injections every six hours for two weeks. In addition to this being an extremely time-consuming regimen, the sheer amount of material used per person can make distribution a challenge. After two weeks, the average patient will have been dosed with 300 g of eflornithine [18]. The side effects of this intense regimen are very similar to the side effects of most cytotoxic cancer chemotherapeutics, including convulsions, gastrointestinal disturbances, bone marrow toxicity, hearing impairment, and alopecia. The bone marrow toxicity frequently causes anemia, leucopenia, and thrombocytopenia. In mice, rats, and rabbits, eflornithine has also been shown to halt fetal development. The mechanism of action comes from eflornithine's ability to selectively and irreversibly inhibit ornithine decarboxylase (ODC) [19]. ODC acts as a catalyst for the transformation of ornithine to putrescine, the rate-limiting step in the synthesis of the polyamines spermidine and spermine. These polyamines assist in nucleic acid production, regulate protein synthesis, and are vital for the growth of all eukaryotic cells. However, eflornithine is much more effective in trypanosomes than in human cells, likely due to the slower turnover of the enzyme, and as a result halts the growth of the parasite. As such, this allows the host's immune system to respond to the infection; however, it is imperative that the host has a strong immune system [11]. Eflornithine is relatively new as the first-line treatment for Stage 2 HAT, so there have been no clinical reports of resistance. However, it was shown that the deletion of an amino acid transporter gene, TbAAT6, decreased the efficacy of eflornithine 40-fold when compared to the wild type. Two independently derived lines have shown the ability to produce this mutation [20].

To overcome the complex treatment regimen of eflornithine, a combination therapy known as NECT was developed by DND*i*. NECT is a combination of nifurtimox, which is currently used to treat American trypanosomiasis, and eflornithine [21]. While eflornithine alone has high efficacy and reversible side effects, it has a logistically challenging administration method. The combination therapy has a much easier regimen to follow. Eflornithine is given intravenously every 12 hours for 7 days, while nifurtimox is given orally every 8 hours for 10 days [20, 21]. Testing has shown that NECT has a higher cure rate (96.5–97.9% versus 91.6–92.3%) as well as a 10% non-inferiority margin when compared to eflornithine monotherapy [21]. NECT also had half the major adverse event reports compared to eflornithine and other adverse events were also reduced due to the shorter eflornithine treatment. As with eflornithine, NECT is only effective when used to treat *T. b. gambiense* infections. Patients with *T. b. rhodesiense* must still rely on melarsoprol for their treatment [22]. Given the increasing levels of resistance and the severity of the observed side effects with respect to current treatments, there is a significant need to develop novel treatments for HAT which target both stages of the diseases and all parasite subspecies.

5.4 Diagnostics

Due to the lethality of HAT, as well as the clinical symptoms that are indiscernible from other tropical diseases, there is a dire need to create a rapid diagnostic

test. A rapid diagnostic is defined as a test that *"yields results during the same clinic visit, and can also be used in health centers with minimal infrastructure or trained personnel, particularly without the use of electricity"* [23]. One of the primary challenges for early HAT detection is that there is no easy way to determine which stage of the disease the patient has without the use of a lumbar puncture to identify the parasites and white blood count within the cerebrospinal fluid (CSF) [23]. This is critical for selecting the necessary treatment as the CNS-penetrant drugs melarsoprol and eflornithine would be necessary for Stage 2. Due to this, the most efficient method of diagnosis is to focus on serological tests for parasite confirmation, and to identify which individuals require a lumbar puncture to stage the infection.

According to the WHO, HAT is considered to be in the second stage if the CSF contains one or more of the following: trypanosomes, raised white blood cell count (>5 cells/μl), or increased protein content (>370 mg/l) [24]. If patients are found to have a white blood cell count in the range of 6–20 cells/μl in the CSF, they are often referred to as being in the intermediate stage as they often do not have symptoms of neuroinflammation [24]. These patients are treated immediately with pentamidine as it may still be effective; however, if the white blood cell count is in the range of 11–20 cells/μl in the CSF, the effectiveness of pentamidine is greatly diminished and these patients are treated with melarsoprol or eflornithine [25]. If trypanosomes are detected in the CSF, the infection is immediately categorized as Stage 2, and melarsoprol or eflornithine treatment begins. Finally, protein content can be greatly elevated in patients with HAT, ranging from 100 to 2000 mg/l [25]. However, the protein content in the CSF can be artificially high even in the first stage of HAT due to the diffusion of IgG into the CSF. To confirm that the trypanosome has reached the CSF, a protein elevation level of 750 mg/l is often used. White blood cell count, and trypanosome identification in the CSF are the two primary methods for staging HAT following a lumbar puncture; this is because there is no standard procedure for measuring protein content elevation.

One of the cornerstones of serological tests is the card agglutination test for trypanosomiasis (CATT) developed in the late 1970s [26]. The CATT is a direct agglutination assay for the detection of *T. b. gambiense*-specific antibodies in the blood, plasma, or serum of HAT patients; this is always followed up by parasitological confirmation tests in detected individuals. Some of the drawbacks of the CATT are that it is designed for mass screening programs, and therefore is manufactured in vials of 50 test units which must be discarded once the vial has been reconstituted. In certain endemic areas, this can lead to unnecessary waste as they may not see 50 patients in a day. Furthermore, the CATT has poor thermal stability and degrades within one month of exposure to high temperatures, which greatly limits its storage in health facilities that lack access to proper refrigeration. The Institute of Tropical Medicine in Antwerp, Belgium, has developed a new form of the CATT that is based on a thermostable lyophilization medium and is manufactured in 10-unit vials called the CATT-D10 [27]. This test has been shown to be thermally stable after exposure to very high or alternating temperatures, ranging from 4 to 45 °C, over a period as long as 18 months. While having only 10 doses increases the shipping and production costs, it is intended

for peripheral health care facilities that often see less than 20 patients per day, thus resulting in a net improvement in cost efficiency [27]. The CATT is also specific for infections of *T. b. gambiense* and does not detect *T. b. rhodesiense* infections.

Another field diagnostic option for HAT is an immunochromatographic lateral flow device which is capable of testing one person at a time [28, 29]. It is more sensitive than CATT as it can detect nanogram amounts of antibodies to target antigens in biological fluids, primarily through a finger prick. The antibodies tested are the *T. b. gambiense*-specific LiTat 1, 3, and 5, which can be detected as early as 3–4 weeks postinfection; however, care must be taken in patients who have previously been treated for HAT as these antibodies can persist for up to three years after cure [30].

As mentioned earlier, there is currently no *T. b. rhodesiense* version of the CATT that is widely available, although there have been promising results for trypanosomiasis agglutination tests [31]. There are immunofluorescence- and ELISA-based serological tests; however, the sensitivity is variable and it requires highly trained technicians, which greatly hinders ease of access for endemic areas. Due to this, the primary method of diagnosing infection due to *T. b. rhodesiense* is through the identification of clinical symptoms. After HAT has been staged, it is generally a straightforward process to determine which parasite has infected the host due to the geographical distribution of *T. b. gambiense* (being found in Central and Western Africa) and *T. b. rhodesiense* (found in Eastern Africa), with the parasites currently sharing no overlap in endemic areas [9]. The distribution of bloodstream trypanosomes of *T. b. rhodesiense* are also much more numerous than that of *T. b. gambiense*, making a stained thick or thin blood smear a viable option for parasite confirmation as well [19].

The diagnosis of HAT remains difficult due to the lack of reliable access to rapid field diagnostics in endemic areas, as well as less invasive staging techniques. Serological tests currently only exist for the *T. b. gambiense* infection, while infections due to *T. b. rhodesiense* rely heavily on trypanosome discovery. The only way to properly stage either form of HAT is through a lumbar puncture, followed by white blood cell count and parasite detection. Although small advances have been made in the past few decades on HAT diagnosis, it is getting easier to identify clinical signs of infections, and there has been a push to create more accessible and specific diagnostic instrumentation for endemic areas.

5.5 Medicinal Chemistry

While increasing the ease of diagnosis would certainly benefit patients, this alone is not able to cure HAT. Out of 1556 approved drugs from 1975 to 2004, only 1.3% were specifically developed for NTDs, even though NTDs account for 11.4% of the global disease burden [32]. Due to this, there has been an emergence of organizations focusing on drug discovery for NTDs such as the Bill & Melinda Gates Foundation, Drug & Diagnostics for Tropical Diseases, and the DND*i*, which specifically focuses on the development of drugs for the treatment of trypanosomiasis and leishmaniasis, among others [32]. The DND*i* target product profile

Table 5.2 Target product profile for HAT as determined by DND*i*.

Effective against	***T. b. gambiense T. b. rhodesiense***
Effective in melarsoprol-refractory patients	Yes
Clinical efficacy	>95% at 18 mo follow-up
Safe for	Pregnancy Breastfeeding Children
Formulations	Children Adults
Monitoring of adverse effects needed	No
Stability	Zone 4
Shelf life	>3 yr
Treatment timetable	<7 d
Dose administration	Oral Intravenous
Trypanocidal or static	Cidal
Spectrum	Multi-target
Cost	<30€ per course

for a new chemotherapeutic is presented in Table 5.2. Ideally, new chemotherapeutics for HAT will be orally (p.o.) administered, treat Stage 1 and 2 infections (thereby eliminating the need to stage disease progression), and will be effective against HAT infections caused by both *T. b. gambiense* and *T. b. rhodesiense* [33].

There have been several authoritative reviews highlighting the current approaches used in HAT drug discovery efforts [34–37]. The two primary methods for hit identification are phenotypic- or target-based screening, of which both have advantages and disadvantages. In phenotypic screening, a small molecule is screened against an organism's phenotype in a whole-organism-based assay. The advantage of this is that the molecule is tested against a fully functioning biological system; cellular systems often lack the complexity of whole biological systems and thus are unable to adequately provide a sufficient model for drug discovery. In whole-organism-based assays, the only compounds that appear as hits are ones that have biological activity and cell penetration. However, in phenotypic-based drug discovery, the molecular mechanism of action (MMOA) is often unknown. In target-based screening just the opposite is done, the small molecule is tested against a specific receptor or receptor family, often with the aid of computational modeling. The advantage of this is that the MMOA is known, and can therefore provide a greater understanding of what attributes of the drug are contributing to its overall efficacy. However, because target-based drug discovery is performed on a single target, it does not account for polypharmacology or hits with multiple MMOA. One of the reasons why target-based screening is taking a back seat to the phenotypic version is that many drugs that are effective in target-based studies show a lack of efficacy in whole-cell analysis, often due to permeability issues [38].

While both methods are routinely used in drug discovery, the data shows that phenotypic screening is superior for the discovery of first-in-class drugs [39]. However, for follower drugs, target-based screening is vastly superior

[39]. These results are intuitive, as using the whole biological system as a model would be fruitful for finding a drug, but knowing the exact MMOA would be beneficial for optimizing a drug. This is not to say that target-based high-throughput screening (HTS) is ineffective for first-in-class drug discovery; valuable lead anti-trypanosomal drug candidates have come from target-based HTS campaigns such as a recent hexokinase small-molecule inhibitor as well as a trypanothione reductase inhibitor, both of which show promise [40, 41].

One of the largest open-source phenotypic HTS campaigns was performed by GlaxoSmithKline (GSK), wherein they screened 1.8 million compounds against three kinetoplastids, *Leishmania donovani* (*L. donovani*), *Trypanosoma cruzi* (*T. cruzi*), and *T. b. brucei*, over the course of two years [42]. From this herculean effort, 27 000 compounds showed >40% inhibition of *T. b. brucei* at 4.2 μM; subsequent confirmation of activity left 15 200 compounds. From these, 4200 showed >80% inhibition at 4.2 μM as well as various "drug-like" criteria: calculated properties forecast index (cPFI) <8, <5 aromatic rings, MW < 500 Da, and positive predicted CNS penetration. These were then screened against *T. b. brucei* with an orthogonal ATP-based luminescence assay and cytotoxicity was assessed in HepG2 cells. Seven hundred noncytotoxic anti-*T. b. brucei* compounds were identified, 88% of which were not previously published and the remaining 12% were published with uses unrelated to the activity against *T. b. brucei* [42]. After this, MMOA hypotheses were created on the basis of historical screening data of similar inhibitors. By far the highest target hit were kinases, with roughly 58% of all active hits most likely being kinase inhibitors. This is not surprising as kinases are a known target when pursing anti-trypanosomal drug discovery; the trypanosoma genome contains less than 200 kinases, and at least 43 of those are essential in cell cycle and differentiation based on *in vitro* RNAi screenings [43]. Also identified in the potential MMOA hypotheses analysis were phosphodiesterases (PDE), cysteine peptidases, and cytochromes (CYPs). There are five PDEs present in *T. brucei*, but PDEs B1 and B2 (*Tbr*PDEB1 and *Tbr*PDEB2) are known drug targets as they regulate cyclic AMP, which is crucial for the control of cell growth, differentiation, and osmoregulation in trypanosomatoids and are currently under active investigation [44]. The cysteine peptidases of kinetoplastids are also a known target as *T. brucei* deactivates the protective action of human apolipoprotein LI via an active cysteine [45, 46]. And the primary mechanism of iron uptake in *T. brucei* is through ferric reductase, which has a homology sequence to cytochrome P450, and recent advances have been made on exploiting that similarity [47–49]. All of these potential targets present an interesting opportunity for the identification of a lead in the drug discovery process, and provides a prime example of how the discovery models can work together in an HTS campaign.

A rational medicinal chemistry strategy can be an excellent starting point for hit identification through repurposing known chemical matter for further optimization [50]. The advantage of this is that the drug, candidate, or lead compound has a well-established safety profile and published SAR that can be exploited [51]. A known drug can also be directly repurposed without further optimization, a strategy known as drug repurposing. The majority of drugs fail due to a lack of safety or efficacy; and because a safety profile is already established, the drug can

piggyback off of the original drug. This may lead to faster approval, requiring less upfront cost, and in some cases the drug can progress without phase I and II clinical trials [51]. One of the most successful cases of drug repurposing for HAT was the discovery of eflornithine. Eflornithine was initially developed as a potential antineoplastic agent, but was shown to have activity against Stage 2 *T. b. gambiense* infection as previously discussed [52]. In a further example of drug repurposing, nifurtimox, which was previously registered to treat Chagas disease, was tested against Stage 2 HAT as a monotherapy but showed limited efficacy. Further evaluation as a combination therapy led to the identification of NECT, which yielded improved safety and efficacy.

In a similar approach to drug repurposing, a library of biased leads can be screened against a disease-causing agent; this is known as lead repurposing [50]. As highlighted previously, trypanosomes express multiple essential kinases and have been shown to be a fruitful focus point in the discovery of anti-trypanosomal drugs [35, 53–57]. In 2012, a number of hydroxamic acid derivatives which had shown human histone deacetylase (HDAC) activity were screened against *T. b. brucei* [58]. Histone modifications are well described in *T. brucei* as being involved in transcription, chromatin assembly, replication, and DNA repair of chromosome biology, and inhibition of these processes has been shown to suppress bloodstream form (BSF) *T. brucei* [59–61]. A large library of HDAC inhibitors was screened with the intention of identifying anticancer activity, resulting in the identification of Belinostat. This was later repurposed against BSF *T. brucei* with the intention of decoupling the activity against human HDAC; activity levels were achieved in the sub-micromolar range [59]. The result was three different hydroxamic acid scaffolds that presented opportunistic leads for a drug candidate against BSF *T. brucei* [59–61].

The Pollastri Laboratory is currently pursuing a number of repurposing strategies in efforts to identify *T. brucei* inhibitors [62–65]. A whole-organism high-throughput screen against *T. b. brucei* using a library of known human kinases led to the identification of various scaffolds as inhibitors of trypanosomal proliferation, a number of which are currently being explored via medicinal chemistry optimization [66]. In a separate project, a number of late-stage clinical candidates and compounds already approved for use in the clinic were screened against *T. b. brucei* [67, 68]. This led to the identification of lapatinib, a known tyrosine kinase inhibitor which was approved with capecitabine to treat breast cancer in 2007 as a combination therapy, as a trypanosomal proliferation inhibitor [55, 67, 68]. In addition, the laboratory is also currently working to repurpose a series of human PDE4 inhibitors and a carbazole-derived drug for HAT [62, 64, 65, 69].

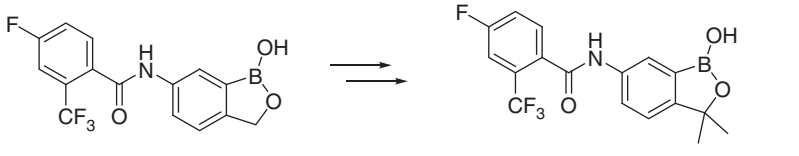
A third approach that has been gaining more traction is the use of *in silico* HTS. Screening done *in silico* uses computational models and docking algorithms to predict molecular scaffolds and conformations that could lead to a hit. The primary advantage of *in silico* HTS is the reduced financial burden when compared to traditional HTS methods, having a physical library of compounds for phenotypic- and target-based HTS, as well as the robotic assembly to test them is costly. For *in silico* HTS, a physical library of both compounds and biological targets is unnecessary; only a computer-generated library of compounds and a

crystal structure or homology model of the intended target are necessary. The hit percentage of *in silico* modeling is often also higher than that of phenotypic- and target-based screening campaigns, which is generally around 1%; *in silico* modeling has been shown to get hits as high as 50%, mostly due to the fact that the molecular scaffolds chosen can be tailored to fit into a particular active site of an enzyme [70, 71]. While *in silico* modeling has been successful in the past for diseases like cancer, Alzheimer's, and diabetes, little success has come in the way of anti-trypanosomal drug discovery [72–75]. In addition, *in silico* modeling is often used to complement phenotypic- or target-based screening as a means of confirming an MMOA or optimizing a drug candidate.

5.6 Future Drug Candidates

While the NTD drug discovery pipeline for HAT has seen a significant reduction, it has not stopped. There are currently three drugs with promising results that are in different stages of the clinical trial process. Acoziborole (formerly known as SCYX-7158) is currently in phase IIb/III trials, fexinidazole is currently being developed by Sanofi and the DND*i* and is in phase IIIb clinical trials, and GNF6702 was discovered in 2013 by Novartis and is currently undergoing preclinical toxicity studies [76, 77].

Acoziborole (Figure 5.5) was discovered in 2009 by Anacor Pharmaceuticals through a screening effort that discovered benzoxaborole 6-carboxamides as lead candidates for the treatment of HAT [78]. Benzoxaborole 6-carboxamides exhibited good *in vitro* as well as *in vivo* pharmacokinetic properties, and eventually resulted in SCYX-6759, which was the first compound with acceptable potency, pharmacokinetic properties, and BBB permeability to provide a complete cure in the Stage 2 murine model of HAT. However, SCYX-6759 was only active as a twice-daily, oral dose; and drug concentrations fell below the required minimum inhibitor concentration (MIC) in the brain after roughly 12 hours. To address this issue, SCYX-6759 was further optimized to acoziborole with two additional methyl groups at the benzylic methylene, which greatly reduced the metabolism and enabled administration as a once-daily, oral dose [76]. Acoziborole exhibited a 100% cure rate for Stage 2 HAT infection in mice when administered as a 25 mg/kg oral dose over seven days starting on day 21 postinfection. However, the



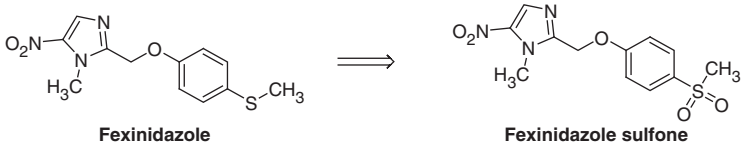
SCYX-6759
T. b. brucei 427 EC_{50}: 0.070 μg/ml
L929 EC_{50}: 9.5 μg/ml

Acoziborole (SCYX-7158)
T. b. brucei 427 EC_{50}: 0.29 ± 0.019 μg/ml
L929 EC_{50}: >50 μg/ml

Figure 5.5 Identification of acoziborole, formerly SCYX-7158, from SCYX-6759 which shows significantly improved metabolic stability, enabling once-daily dosing in mice.

mechanism by which it acts is currently unknown. Potential biochemical targets were assessed to elucidate the MMOA, but none exhibited significant binding or inhibition with the addition of acoziborole [78]. This does, however, support the toxicity profile of acoziborole, which presents a very low risk of mechanism-based toxicity. Acoziborole has passed phase I human trials and is currently in phase IIb/III trials in the Democratic Republic of Congo, where it presents a very promising drug candidate for the cure of Stage 2 HAT caused by *T. b. gambiense*.

Fexinidazole, a nitroimidazole, was identified as a promising drug candidate from a diverse set of over 700 nitroheterocyclic compounds that were assessed for antiparasitic activity and toxicity [77]. Fexinidazole had previously been in preclinical development in the 1970s as a broad-spectrum antibiotic by Sanofi-Aventis, and had been tested as an anti-trypanosomal drug in 1983, but the development was not pursued further due to a lack of commercial viability [79, 80]. Fexinidazole has shown potent inhibition in *in vitro* laboratory strains of *T. b. rhodesiense* (IC_{50}: 0.48–0.82 μg/ml) and *T. b. gambiense* (IC_{50}: 0.30–0.93 μg/ml); however, fexinidazole exhibited little to no nonspecific cytotoxicity, making it a much more attractive option than current chemotherapeutics. For Stage 1 *in vivo*, it has been effective against *T. b. rhodesiense* and *T. b. gambiense* models of infection with an oral dose of 100 mg/kg/d for four days. It is even effective against the Stage 2 variant with a dose of 200 mg/kg/d over five days, exhibiting an 88% cure rate against *T. b. brucei* GVR35 in a group of eight mice. Fexinidazole is well absorbed upon oral administration and has two primary metabolites, fexinidazole sulfoxide and fexinidazole sulfone, shown in Figure 5.6, that have *in vitro* trypanocidal activity similar to that of the parent [81]. The parent compound exhibits a half-life between one and three hours and up to 24 hours for the metabolites. While fexinidazole is rapidly metabolized *in vivo* to the sulfoxide and sulfone derivatives, its metabolism is not expected to

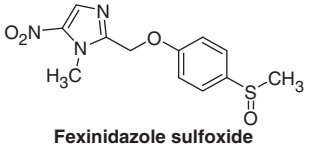


Fexinidazole

T. b. brucei BS221 IC_{50}: 2.38 ± 0.88 μM
T. b. rhodesiense STIB900 IC_{50}: 2.17 ± 0.29 μM
T. b. gambiense STIB930 IC_{50}: 1.84 ± 1.1 μM

Fexinidazole sulfone

T. b. brucei EC_{50}: 1.63 ± 0.92 μM
T. b. rhodesiense STIB900 IC_{50}: 1.44 ± 0.22 μM
T. b. gambiense STIB930 IC_{50}: 0.91 ± 0.27 μM



Fexinidazole sulfoxide

T. b. brucei EC_{50}: 1.49 ± 0.61 μM
T. b. rhodesiense STIB900 IC_{50}: 1.64 ± 0.36 μM
T.b. gambiense STIB930 IC_{50}: 0.94 ± 0.39 μM

Figure 5.6 Fexinidazole and the sulfone and sulfoxide metabolites show potent trypanocidal activity.

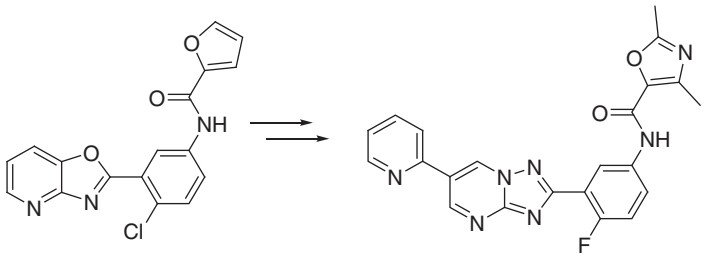


Figure 5.7 Progression from the initial hit (GNF5343) to GNF6702 which shows potent pan-kinetoplastid activity against *L. donovani*, *T. cruzi*, and *T. b. brucei*.

be significantly affected by other drugs because it is metabolized extensively by multiple CYP450 isoforms. The clinical trial design allowed a window of 13% inferiority for fexinidazole versus NECT based on regional health care providers' experiences given that oral dosing could be achieved. When compared with NECT, fexinidazole showed 91.2% positive outcomes (97.6% positive outcomes for NECT), a difference of 6.4% which led to the clinical trial being labeled a success. Fexinidazole is currently in phase IIIb clinical trials to test the feasibility of outpatient treatment.

The most recent new drug in the pipeline for HAT is known as GNF6702 (Figure 5.7) and was identified by Novartis in 2013. It has shown potent inhibition of three kinetoplastids: *L. donovani*, *T. cruzi*, and *T. b. brucei* [82]. A three million compound library was screened, and through a triage of active compounds with an $EC_{50} < 10\,\mu M$ and a >five-fold preference in selectivity over mammalian cells, GNF5343, an azabenzoxazole, was identified. After the synthesis of roughly 3000 additional compounds to improve potency and bioavailability, GNF6702 was identified which exhibited a 400-fold increase in potency against *L. donovani* compared to GNF5343, and showed desirable results against both *T. cruzi* and *T. b. brucei*. Efficacy against Stage 2 HAT was assessed by administration of GNF6702 to infected mice at 100 mg/kg once daily. The higher dosage is due to the limited exposure of the drug into the brain, with roughly 10% partitioned to the brain versus the plasma. After seven days of treatment, a sustained clearance of parasitemia was observed and mice treated with GNF6702 had no detectable parasitemia in the brain at the termination of the experiment [82].

Due to this drug showing pan-kinetoplastid activity, MMOA studies were performed and the primary mechanism of parasite growth inhibition is through the inhibition of proteasome activity. Further studies showed that GNF6702 blocked the chemotrypsin-like activity within the beta-5 subunit without competing with substrate binding. Resistance against GNF6702 is due to mutations in the beta-4 subunit, a subunit in direct physical contact with the beta-5 subunit. GNF6702 is currently being tested preclinically for toxicity issues; there is some concern that increased toxicity may be a risk due to the pan-kinetoplastid activity [82]. However, this remains a promising lead and the ability to tailor the activity toward different kinetoplastids is currently being explored and the

value of the identification of a target that is common to three of the most deadly kinetoplastids cannot be understated.

5.7 Conclusion

HAT has long suffered from lack of investment due to the lack of financial incentive for profit-driven companies making it a typical NTD. With the expected addition of novel, safe, oral drugs to the treatment arsenal, as well as the development of more cost-effective diagnostic tools, we are making progress toward the goal of eliminating HAT as a public health problem by 2020, and to have zero incidences of HAT by 2030. Historically, a rapid resurgence in the number of cases of HAT has occurred from similar levels, making continued diligence essential for complete control and elimination to be realized.

References

1 World Health Organization and Expert Committee on the Control of human African trypanosomiasis (2014). Control and surveillance of human African trypanosomiasis: WHO TRS N°984. In: WHO Technical Report Series, 237. Geneva, Switzerland: World Health Organization.
2 World Health Organization (2017). Eliminating sleeping sickness as a public health problem is on track. [cited 2017 29th November]. http://www.who.int/trypanosomiasis_african/news/HAT_elimination_on_track/en/ (accessed 27 February 2019).
3 Chappuis, F., Lima, M.A., Flevaud, L., and Ritmeijer, K. (2010). Human African trypanosomiasis in areas without surveillance. *Emerg. Infect. Dis.* 16 (2): 354–356.
4 Brun, R., Blum, J., Chappuis, F., and Burri, C. (2010). Human African trypanosomiasis. *Lancet* 375 (9709): 148–159.
5 Centers for Disease Control and Prevention (2015). Prevention, C.f.D.C.a. Parasites – African Trypanosomiasis (also known as Sleeping Sickness). [cited 2017 29th November]. https://www.cdc.gov/parasites/sleepingsickness/biology.html (accessed 29 November 2019).
6 Lumbala, C., Simarro, P.P., Cecchi, G. et al. (2015). Human African trypanosomiasis in the Democratic Republic of the Congo: disease distribution and risk. *Int. J. Health Geographics* 14 (1): 20.
7 World Health Organization. World : Distribution of Human African Trypanosomiasis (T.B. gambiense) (2016). 2016 September 27th 2017 [cited 2017 December 1st]. http://gamapserver.who.int/mapLibrary/Files/Maps/HAT_ga_2016.png (accessed 27 February 2019).
8 World Health Organization. World : Distribution of Human African Trypanosomiasis (T.B. rhodesiense), 2016. 2016 September 27th 2017 [cited 2017 December 1st]. http://gamapserver.who.int/mapLibrary/Files/Maps/HAT_rh_2016.png (accessed 27 February 2019).

9 Fevre, E.M., Coleman, P.G., Odiit, M. et al. (2001). The origins of a new *Trypanosoma brucei* rhodesiense sleeping sickness outbreak in eastern Uganda. *Lancet* 358 (9282): 625–628.

10 Babokhov, P., Sanyaolu, A.O., Oyibo, W.A. et al. (2013). A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathog. Glob. Health* 107 (5): 242–252.

11 Headrick, D.R. (2014). Sleeping sickness epidemics and colonial responses in East and Central Africa, 1900-1940. *PLoS Negl. Trop. Dis.* 8 (4): e2772.

12 Stich, A., Abel, P.M., and Krishna, S. (2002). Human African trypanosomiasis. *BMJ* 325 (7357): 203–206.

13 Chappuis, F., Loutan, L., Simarro, P. et al. (2005). Options for field diagnosis of human African trypanosomiasis. *Clin. Microbiol. Rev.* 18 (1): 133.

14 Greenwood, B.M. and Whittle, H.C. (1980). The pathogenesis of sleeping sickness. *Trans. R. Soc. Trop. Med. Hyg.* 74 (6): 716–725.

15 Steverding, D. (2008). The history of African trypanosomiasis. *Parasit. Vectors* 1 (1): 3.

16 Voogd, T.E., Vansterkenburg, E.L., Wilting, J., and Janssen, L.H. (1993). Recent research on the biological activity of suramin. *Pharmacol. Rev.* 45 (2): 177–203.

17 Nok, A.J. (2003). Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human African trypanosomiasis. *Parasitol. Res.* 90: 71–79.

18 Seebeck, T. and Maser, P. (2009). Drug resistance in African trypanosomiasis. In: *Antimicrobial Drug Resistance* (ed. D.L. Mayers), 589–604. Humana Press.

19 Duggan, A.J. and Hutchinson, M.P. (1966). Sleeping sickness in Europeans: a review of 109 cases. *J. Trop. Med. Hyg.* 69 (6): 124–131.

20 Vincent, I.M., Creek, D., Watson, D.G. et al. (2010). A molecular mechanism for eflornithine resistance in African trypanosomes. *PLoS Pathog.* 6 (11): e1001204.

21 Priotto, G., Fogg, C., Balasegaram, M. et al. (2006). Three drug combinations for late-stage *Trypanosoma brucei* gambiense sleeping sickness: a randomized clinical trial in Uganda. *PLoS Clin. Trials* 1 (8): e39.

22 Priotto, G., Kasparian, S., Mutombo, W. et al. (2009). Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei* gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet* 374 (9683): 56–64.

23 Yansouni, C.P., Bottieau, E., Lutumba, P. et al. (2013). Rapid diagnostic tests for neurological infections in central Africa. *Lancet Infect. Dis.* 13 (6): 546–558.

24 Doua, F., Miezan, T.W., Sanon Singaro, J.R. et al. (1996). The efficacy of pentamidine in the treatment of early-late stage *Trypanosoma brucei* gambiense trypanosomiasis. *Am. J. Trop. Med. Hyg.* 55 (6): 586–588.

25 Lejon, V., Legros, D., Savignoni, A. et al. (2003). Neuro-inflammatory risk factors for treatment failure in "early second stage" sleeping sickness patients treated with pentamidine. *J. Neuroimmunol.* 144 (1-2): 132–138.

26 Magnus, E., Vervoort, T., and Van Meirvenne, N. (1978). A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of T.B. gambiense trypanosomiasis. *Ann. Soc. Belg. Med. Trop.* 58 (3): 169–176.

27 Hasker, E., Mitashi, P., Baelmans, R. et al. (2010). A new format of the CATT test for the detection of human African Trypanosomiasis, designed for use in peripheral health facilities. *Trop. Med. Int. Health* 15 (2): 263–267.

28 Posthuma-Trumpie, G.A., Korf, J., and van Amerongen, A. (2009). Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Anal. Bioanal.Chem.* 393 (2): 569–582.

29 Fleming, J.R., Sastry, L., Wall, S.J. et al. (2016). Proteomic identification of immunodiagnostic antigens for Trypanosoma vivax infections in cattle and generation of a proof-of-concept lateral flow test diagnostic device. *PLoS Negl.Trop. Dis.* 10 (9): e0004977.

30 Paquet, C., Ancelle, T., Gastellu-Etchegorry, M. et al. (1992). Persistence of antibodies to *Trypanosoma brucei* gambiense after treatment of human trypanosomiasis in Uganda. *Lancet* 340 (8813): 250.

31 Akol, M.N., Olaho-Mukani, W., Odiit, M. et al. (1999). Trypanosomosis agglutination card test for *Trypanosoma brucei* rhodesiense sleeping sickness. *East Afr. Med. J.* 76 (1): 38–41.

32 Chatelain, E. and Ioset, J.-R. (2011). Drug discovery and development for neglected diseases: the DNDi model. *Drug Des. Devel. Ther.* 5: 175–181.

33 Gilbert, I.H. (2014). Target-based drug discovery for human African trypanosomiasis: selection of molecular target and chemical matter. *Parasitology* 141 (1): 28–36.

34 Njoroge, M., Njuguna, N.M., Mutai, P. et al. (2014). Recent approaches to chemical discovery and development against malaria and the neglected tropical diseases human African Trypanosomiasis and schistosomiasis. *Chem. Rev.* 114 (22): 11138–11163.

35 Dichiara, M., Marrazzo, A., Prezzavento, O. et al. (2017). Repurposing of human kinase inhibitors in neglected protozoan diseases. *ChemMedChem* 12 (16): 1235–1253.

36 Field, M.C., Horn, D., Fairlamb, A.H. et al. (2017). Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. *Nat. Rev. Microbiol.* 15: 217.

37 Ferrins, L., Rahmani, R., and Baell, J.B. (2013). Drug discovery and human African trypanosomiasis: a disease less neglected? *Future Med. Chem.* 5 (15): 1801–1841.

38 Mäser, P., Wittlin, S., Rottmann, M. et al. (2012). Antiparasitic agents: new drugs on the horizon. *Curr. Opin. Pharmacol.* 12 (5): 562–566.

39 Swinney, D.C. (2013). Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clin. Pharmacol. Ther.* 93 (4): 299–301.

40 Sharlow, E.R., Lyda, T.A., Dodson, H.C. et al. (2010). A target-based high throughput screen yields *Trypanosoma brucei* hexokinase small molecule inhibitors with antiparasitic activity. *PLoS Negl.Trop. Dis.* 4 (4): e659.

41 Holloway, G.A., Charman, W.N., Fairlamb, A.H. et al. (2009). Trypanothione reductase high-throughput screening campaign identifies novel classes of inhibitors with antiparasitic activity. *Antimicrob. Agents Chemother.* 53 (7): 2824–2833.

42 Pena, I., Pilar Manzano, M., Cantizani, J. et al. (2015). New compound sets identified from high throughput phenotypic screening against three kinetoplastid parasites: an open resource. *Sci. Rep.* 5: 8771.

43 Jones, N.G., Thomas, E.B., Brown, E. et al. (2014). Regulators of *Trypanosoma brucei* cell cycle progression and differentiation identified using a kinome-wide RNAi screen. *PLoS Pathog.* 10 (1): e1003886.

44 Bland, N.D., Wang, C., Tallman, C. et al. (2011). Pharmacological validation of *Trypanosoma brucei* phosphodiesterases B1 and B2 as druggable targets for African sleeping sickness. *J. Med. Chem.* 54 (23): 8188–8194.

45 Caffrey, C.R. and Steverding, D. (2009). Kinetoplastid papain-like cysteine peptidases. *Mol. Biochem. Parasitol.* 167 (1): p. 12-9.

46 Pérez-Morga, D., Vanhollebeke, B., Paturiaux-Hanocq, F. et al. (2005). Apolipoprotein L-I promotes Trypanosome lysis by forming pores in lysosomal membranes. *Science* 309 (5733): 469–472.

47 Mach, J., Tachezy, J., and Sutak, R. (2013). Efficient iron uptake via a reductive mechanism in procyclic *Trypanosoma brucei*. *J. Parasitol.* 99 (2): 363–364.

48 Andriani, G., Amata, E., Beatty, J. et al. (2013). Antitrypanosomal lead discovery: identification of a ligand-efficient inhibitor of Trypanosoma cruzi CYP51 and parasite growth. *J. Med. Chem.* 56 (6): 2556–2567.

49 Dauchy, F.-A., Bonhivers, M., Landrein, N. et al. (2016). *Trypanosoma brucei* CYP51: essentiality and targeting therapy in an experimental model. *PLoS Negl.Trop. Dis.* 10 (11): e0005125.

50 Klug, D.M., Gelb, M.H., and Pollastri, M.P. (2016). Repurposing strategies for tropical disease drug discovery. *Bioorg. Med. Chem. Lett.* 26 (11): 2569–2576.

51 Oprea, T.I. and Mestres, J. (2012). Drug repurposing: far beyond new targets for old drugs. *AAPS J.* 14 (4): 759–763.

52 Barrett, M.P., Boykin, D.W., Brun, R., and Tidwell, R.R. (2007). Human African trypanosomiasis: pharmacological re-engagement with a neglected disease. *Br. J. Pharmacol.* 152 (8): 1155–1171.

53 Merritt, C., Silva, L.E., Tanner, A.L. et al. (2014). Kinases as druggable targets in trypanosomatid protozoan parasites. *Chem. Rev.* 114 (22): 11280–11304.

54 Jensen, B.C., Booster, N., Vidadala, R.S.R. et al. (2016). A novel protein kinase is essential in bloodstream *Trypanosoma brucei*. *Int. J. Parasitol.* 46 (8): 479–483.

55 Amata, E., Xi, H., Colmenarejo, G. et al. (2016). Identification of "preferred" human kinase inhibitors for sleeping sickness lead discovery. Are some kinases better than others for inhibitor repurposing. *ACS Infect. Dis.* 2 (3): 180–186.

56 Urbaniak, M.D., Mathieson, T., Bantscheff, M. et al. (2012). Chemical proteomic analysis reveals the drugability of the kinome of *Trypanosoma brucei*. *ACS Chem. Biol.* 7 (11): 1858–1865.

57 Ochiana, S.O., Pandarinath, V., Wang, Z. et al. (2013). The human Aurora kinase inhibitor danusertib is a lead compound for anti-trypanosomal drug discovery via target repurposing. *Eur. J. Med. Chem.* 62: 777–784.

58 Kelly, J.M., Taylor, M.C., Horn, D. et al. (2012). Inhibitors of human histone deacetylase with potent activity against the African trypanosome *Trypanosoma brucei*. *Bioorg. Med. Chem. Lett.* 22 (5): 1886–1890.

59 Shakespear, M.R., Halili, M.A., Irvine, K.M. et al. (2011). Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol.* 32 (7): 335–343.

60 Guerrant, W., Mwakwari, S.C., Chen, P.C. et al. (2010). A structure-activity relationship study of the antimalarial and antileishmanial activities of non-peptide macrocyclic histone deacetylase inhibitors. *ChemMedChem* 5 (8): 1232–1235.

61 Sheader, K., te Vruchte, D., and Rudenko, G. (2004). Bloodstream form-specific up-regulation of silent vsg expression sites and procyclin in *Trypanosoma brucei* after inhibition of DNA synthesis or DNA damage. *J. Biol. Chem.* 279 (14): 13363–13374.

62 Ochiana, S.O., Bland, N.D., Settimo, L. et al. (2015). Repurposing human PDE4 inhibitors for neglected tropical diseases. Evaluation of analogs of the human PDE4 inhibitor GSK-256066 as inhibitors of PDEB1 of *Trypanosoma brucei*. *Chem. Biol. Drug Des.* 85 (5): 549–564.

63 Devine, W., Thomas, S.M., Erath, J. et al. (2017). Antiparasitic lead discovery: toward optimization of a chemotype with activity against multiple protozoan parasites. *ACS Med. Chem. Lett.* 8 (3): 350–354.

64 Thomas, S.M., Purmal, A., Pollastri, M., and Mensa-Wilmot, K. (2016). Discovery of a carbazole-derived lead drug for human African trypanosomiasis. *Sci. Rep.* 6: 32083.

65 Seixas, J.D., Luengo-Arratta, S.A., Diaz, R. et al. (2014). Establishment of a structure–activity relationship of 1H-imidazo[4,5-c]quinoline-based kinase inhibitor NVP-BEZ235 as a lead for African sleeping sickness. *J. Med. Chem.* 57 (11): 4834–4848.

66 Devine, W.G., Diaz-Gonzalez, R., Ceballos-Perez, G. et al. (2017). From cells to mice to target: characterization of NEU-1053 (SB-443342) and its analogues for treatment of human African trypanosomiasis. *ACS Infect. Dis.* 3 (3): 225–236.

67 Ryan, Q., Ibrahim, A., Cohen, M.H. et al. (2008). FDA drug approval summary: lapatinib in combination with capecitabine for previously treated metastatic breast cancer that overexpresses HER-2. *Oncologist* 13 (10): 1114–1119.

68 Katiyar, S., Kufareva, I., Behera, R. et al. (2013). Lapatinib-binding protein kinases in the African trypanosome: identification of cellular targets for kinase-directed chemical scaffolds. *PLoS One* 8 (2): e56150.

69 Woodring, J.L., Patel, G., Erath, J. et al. (2015). Evaluation of aromatic 6-substituted thienopyrimidines as scaffolds against parasites that cause trypanosomiasis, leishmaniasis, and malaria. *MedChemComm* 6 (2): 339–346.

70 Röhrig, U.F., Awad, L., Grosdidier, A. et al. (2010). Rational design of indoleamine 2,3-dioxygenase inhibitors. *J. Med. Chem.* 53 (3): 1172–1189.

71 Silva, L.A., Vinaud, M.C., Castro, A.M. et al. (2015). In silico search of energy metabolism inhibitors for alternative leishmaniasis treatments. *BioMed Res. Int.* 2015: 965725.

72 Phatak, S.S., Stephan, C.C., and Cavasotto, C.N. (2009). High-throughput and in silico screenings in drug discovery. *Expert Opin. Drug Discovery* 4 (9): 947–959.

73 Cavasotto, C.N., Ortiz, M.A., Abagyan, R.A., and Piedrafita, F.J. (2006). In silico identification of novel EGFR inhibitors with antiproliferative activity against cancer cells. *Bioorg. Med. Chem. Lett.* 16 (7): 1969–1974.

74 Burden, R.E., Gormley, J.A., Jaquin, T.J. et al. (2009). Antibody-mediated inhibition of cathepsin S blocks colorectal tumor invasion and angiogenesis. *Clin. Cancer Res.* 15 (19): 6042–6051.

75 Miduturu, C.V., Deng, X., Kwiatkowski, N. et al. (2011). High-throughput kinase profiling: a more efficient approach towards the discovery of new kinase inhibitors. *Chem. Biol.* 18 (7): 868–879.

76 Jacobs, R.T., Nare, B., Wring, S.A. et al. (2011). SCYX-7158, an orally-active benzoxaborole for the treatment of stage 2 human African trypanosomiasis. *PLoS Negl.Trop. Dis.* 5 (6): e1151.

77 Torreele, E., Bourdin Trunz, B., Tweats, D. et al. (2010). Fexinidazole – a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. *PLoS Negl.Trop. Dis.* 4 (12): e923.

78 Nare, B., Wring, S., Bacchi, C. et al. (2010). Discovery of novel orally bioavailable oxaborole 6-carboxamides that demonstrate cure in a murine model of late-stage central nervous system african trypanosomiasis. *Antimicrob. Agents Chemother.* 54 (10): 4379–4388.

79 Raether, W. and Hänel, H. (2003). Nitroheterocyclic drugs with broad spectrum activity. *Parasitol. Res.* 90 (1): S19–S39.

80 Jennings, F.W. and Urquhart, G.M. (1983). The use of the 2 substituted 5-nitroimidazole, Fexinidazole (Hoe 239) in the treatment of chronic *T. brucei* infections in mice. *Z Parasitenkd* 69 (5): 577–581.

81 Kaiser, M., Bray, M.A., Cal, M. et al. (2011). Antitrypanosomal activity of fexinidazole, a new oral nitroimidazole drug candidate for treatment of sleeping sickness. *Antimicrob. Agents Chemother.* 55 (12): 5602–5608.

82 Khare, S., Nagle, A.S., Biggart, A. et al. (2016). Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature* 537 (7619): 229–233.

83 CDC and Mathison, B. (2017). This Giemsa-stained light photomicrograph revealed the presence of two *Trypanosoma brucei* parasites, which were found in a blood smear. [cited 2017 29th November]. https://phil.cdc.gov/Details.aspx?pid=11821 (accessed 27 February 2019).

6

Discovery of Drugs for Leishmaniases: A Progress Report

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Leishmaniasis is caused by over 20 species of the protozoan *Leishmania* and occurs in five different continents. It follows that the disease manifests in a variety of clinical forms, the main ones being visceral, cutaneous, and mucocutaneous leishmaniasis. These manifestations result from different tissue tropisms that are primarily determined by the *Leishmania* species, although host factors (such as immunosuppression) can play a role, too. Understanding these different clinical syndromes is important for scientists working on antileishmanial drug development because the requirements of an effective drug (the target product profile [TPP]) will vary depending on the targeted syndrome.

6.1 Visceral Leishmaniasis (VL)

Two species are principally responsible for VL, *Leishmania donovani* and *Leishmania infantum*. The former occurs in South Asia (India, Bangladesh, and Nepal) and East Africa (Sudan, Ethiopia, Somalia, and Kenya). In South Asia, *L. donovani* is anthroponitic (humans are the only proven reservoir host). In East Africa, humans and animals (rodents and domestic dogs) serve as reservoirs. *L. infantum* primarily occurs in the Mediterranean (including Spain, France, and Greece), Iran, Afghanistan, Pakistan, and Brazil. Visceral leishmaniasis in Brazil (and sporadically in other South American countries) was formerly attributed to *Leishmania chagasi*; however, genomic sequencing indicates that it is the same species as *L. infantum*, which was apparently introduced to the Americas from the Old World by human migration in recent centuries [1]. Approximately, 200 000–400 000 new VL cases and 20 000–40 000 deaths from VL occur each year [2].

The primary mode of transmission for VL (and all leishmaniases) is via the bite of female sand fly vectors. Less important modes of transmission include shared needles by injection drug users, blood transfusion, and congenital infections [3, 4]. After inoculation in the skin by the sand fly, promastigote forms are taken up in dermal macrophages and transform to round-shaped amastigotes.

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They multiply within a phagolysosome compartment, rupture the host cell, and spread to other cells of the reticuloendothelial system. With *L. donovani*, ulcerative skin lesions may not occur. Instead, the organisms surreptitiously migrate to the liver, spleen, and bone marrow where they may replicate to high densities. Depending on the parasite strain, host genetics, and nutritional status, the infection can lead to the syndrome known as kala-azar ("black fever"). Fortunately, the majority of patients infected with *L. donovani* remain asymptomatic [5]. Symptoms of kala-azar come on slowly after a typical incubation period of two to six months with progressive malaise, weight loss, fever, and enlargement of the spleen and/or liver. Due to infiltration of the bone marrow and splenic involvement, patients develop severe anemia and thrombocytopenia. Definitive diagnosis is made with an aspirate of bone marrow or spleen, and demonstration of *Leishmania* amastigotes on smears, culture, or polymerase chain reaction (PCR). With advanced kala-azar, patients experience cachexia, hepatic dysfunction, bleeding, and secondary infections. Without treatment, kala-azar is almost always fatal. Coinfection with HIV is associated with increased mortality [6].

6.1.1 Current Treatment Regimens for VL

Because the infection involves internal organs of the body, treatment for VL needs to be administered systemically. Currently used drugs include amphotericin B, pentavalent antimonial drugs, paromomycin, and miltefosine. Liposomal amphotericin B is the preferred drug due to high therapeutic efficacy and a comparatively favorable side effect profile. It requires intravenous administration given in daily or intermittent doses. Cure rates are reported to be 88–100% [7, 8] in Africa and 96–100% in India [9–11]. The liposomal formulation is preferred over amphotericin B deoxycholate due to the lower toxicity. However, toxicity from liposomal amphotericin B (infusion-related fevers/chills and nephrotoxicity) is not negligible. Furthermore, the cost of liposomal amphotericin B is a substantial barrier in resource-limited settings or for uninsured patients. The pentavalent antimonial drugs, sodium stibogluconate and meglumine antimoniate, have historically been the most widely used treatments for VL, but are falling out of favor due to their comparatively worse side effect profiles and high rates of resistance in parts of India and Nepal. The drugs are either given by intravenous or intramuscular injection over 28 days, and can cause serious toxicity (cardiac and pancreatic) usually related to the cumulative dose. Monitoring for toxicity with electrocardiograms and blood tests adds to the cost of providing treatment with antimonial drugs. Miltefosine was first approved for clinical use (in India) in 2002, and later by the FDA in 2014. It is the only oral drug available for treating VL, administered once daily for 28 days. Cure rates in India are in the 90–95% range [12–14]. In recent years, failure rates have been rising, presumably due to spreading drug resistance [15, 16]. Miltefosine is not without side effects, causing vomiting in up to 65% of patients, diarrhea (5–20%), and transaminase elevation (15–30%). Importantly, miltefosine is teratogenic in animal models and should be avoided during pregnancy and used with extreme caution in women of child-bearing age. Paromomycin is a parenterally administered aminoglycoside with efficacy of

88–95% for VL in clinical trials done in India [17–19]. Efficacy of paromomycin (monotherapy) against VL in East Africa appears to be lower [20, 21]. Side effects are similar to other aminoglycosides, including nephrotoxicity and ototoxicity. The drug pentamidine has been used historically for treating VL, but it is out of favor due to suboptimal efficacy and well-known side effects. There is a growing consensus that use of combination therapy is desirable in endemic regions to help stave off resistance and enable shorter treatments, although guidelines for specific regimens have not been established.

6.2 Cutaneous Leishmaniasis (CL)

This summary only covers localized cutaneous leishmaniasis, although other cutaneous syndromes exist, including diffuse cutaneous leishmaniasis and leishmaniasis recidivans. Localized cutaneous leishmaniasis occurs on exposed skin at the site of the sand fly bites. It typically begins with a pink-colored papule that enlarges and leads to a painless ulceration with a firm border [22]. The lesions can be single or multiple, and can take on a variety of appearances (plaques, verrucous, etc.). Small, satellite lesions may develop near the central lesion and sometimes nodular lesions develop along draining lymphatics (so-called sporotrichoid spread). Healing takes place slowly over months or even years, the time depending on the *Leishmania* species and, presumably, the host factors. The most common species responsible for CL are listed later. It is useful to categorize them by the geographic regions from which they derive: Old World (Asia, Africa, Europe) versus New World (Americas).

- Old World CL:
  - *Leishmania major:* Incubation period of weeks; often multiple lesions; usually self-heals within six months. Treatment may be optional, particularly if lesions do not involve the face and are few in number.
  - *Leishmania tropica*: More chronic than *L. major,* evolving over months to years. Treatment is often recommended due to the slow rate of spontaneous resolution.
  - *Leishmania aethiopica:* Usually consists of a solitary facial lesion of long chronicity.
  - *L. infantum:* Although known to cause VL, this species can cause slow-growing cutaneous lesions that may persist for months or years. Skin manifestations do not necessarily imply visceral disease, but treatment is usually recommended.

- New World CL:
  - *Leishmania mexicana* complex (*L. mexicana, Leishmania amazonensis*): Typically produce small-sized lesions, sometimes on the ears (known as "Chiclero's ulcer"). *L. mexicana* ulcers usually heal within three to four months [23]; therefore, treatment may not be warranted.
  - *Leishmania viannia* complex (*Leishmania braziliensis, Leishmania guyanensis, Leishmania panamensis*): Lesions from these species are frequently

large and may be associated with lymphangitic spread. Furthermore, these species have the potential to cause mucosal leishmaniasis, and therefore treatment is usually recommended.

Since other diseases (fungal, mycobacteria, neoplasms, etc.) can resemble CL, a biopsy is required for definitive diagnosis. Needle aspirates or punch biopsies are tested by (preferably) three methods: histopathology (looking for intracellular amastigotes), culture, and PCR. A culture and/or PCR are highly desirable since species identification helps direct treatment decisions.

6.2.1 Current Treatment Regimens for CL

As indicated earlier, some *Leishmania* species are associated with a self-resolving clinical course and may be managed without antiparasitic chemotherapy. A topical ointment containing paromomycin has been shown to hasten time to healing for cases caused by *L. major* and perhaps non-Viannia species of New World leishmaniasis [24]; however, it is not FDA approved. Direct injection of leishmanial lesions with antimonial drugs is reportedly effective [25], but it is impractical when the lesions are numerous or on sensitive locations such as the face. Other topical treatment options include cryotherapy and thermotherapy, but these require repeated applications with specialized equipment and are impractical.

Cases of "complicated" CL infection usually require systemic antileishmanial treatment (either oral or parenteral). Complicated CL includes infections due to species in the *Viannia* complex since "watchful waiting" or topical treatment will not protect against potential spread to the nasopharyngeal mucosa. Complicated infections also include situations with multiple or large-size ($\geq$5 cm) lesions, lesions on the face, fingers, or toes, subcutaneous nodules, immunosuppressed hosts, and some other circumstances. The options for systemic treatment are discussed later, but they all have significant drawbacks. Perhaps the biggest deficiencies are the limited options for oral therapy, since most patients with CL are capable of taking medicines by mouth.

Azole antifungal drugs such as ketoconazole and fluconazole are attractive options since they are administered orally; however, studies about their efficacy are conflicting, some being favorable [26, 27] and others unfavorable [28]. Azoles should probably not be used for CL due to *L. braziliensis* (and other species associated with mucosal leishmaniasis) [26, 29]. Ketoconazole and fluconazole act by inhibiting the sterol 14α-demethylase enzyme (CYP51) of the sterol biosynthesis pathway [30, 31]. Azoles are fungistatic at normal concentrations [32], and it seems likely that azoles may only have cytostatic activity on *Leishmania* too as inferred by their mediocre clinical efficacy. Ketoconazole is also problematic due to associated QT prolongation and risk of dangerous cardiac arrhythmias. Some case reports indicate activity of newer generation azoles (i.e. posaconazole) [33], although these have not been subjected to rigorous study.

The other oral option for treating CL is miltefosine. As discussed, this drug was developed for treating VL and has some safety/tolerability issues. Most of the data regarding its use in CL relates to New World CL where it appears to have

good efficacy against Viannia species in Columbia, Brazil, and Bolivia, but not in Guatemala [34–38]. Miltefosine is an alkylphosphocholine that exerts antileishmanial activity by multiple mechanisms of action that relate to interacting with membrane and organelle components of the parasite [39, 40]. It was approved by the FDA in 2014 for treating VL due to *L. donovani* and CL due to *L. braziliensis, L. guyanensis,* and *L. panamensis*.

The other treatment options for CL are the same parenteral therapies used for treating VL. Liposomal amphotericin B is generally preferred over amphotericin B deoxycholate due to better tolerability. The former compared favorably to sodium stibogluconate in patients with *L. braziliensis* (healing in 97% and 71% of patients, respectively) [41]. Treatment typically involves placing a peripherally inserted central catheter (PICC) for multiple injections given over a two- to three-week period with close clinical monitoring of blood chemistries and kidney function. The treatment is expensive not only from the high cost of the medicine but also due to infusion charges and monitoring expenses. Amphotericin B is a polyene antifungal drug that binds ergosterol precursors (also present in *Leishmania*) in preference to host cholesterol.

Parenteral pentavalent antimonial drugs have been used for decades to treat CL (both Old and New World disease). They include sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). Generally, their efficacy is good and serves as the bar against which other drugs are compared. However, parenterally administered antimonials, usually given for three weeks, are very poorly tolerated. Patients frequently report myalgias, rash, nausea, and headache, and run the risk of experiencing pancreatitis, hepatitis, or cardiotoxicity that can be life-threatening. Close clinical monitoring is imperative to avoid potentially disastrous side effects. The pentavalent antimony drugs act through a variety of nonspecific mechanisms, affecting leishmanial cell functions [42] as well as effecting cytokine responses in infected host macrophages [43].

6.3 Mucosal Leishmaniasis (ML)

This is a relatively uncommon manifestation of leishmaniasis, but one that commands attention because of the horrific disfigurement it can produce in the face and mouth of victims. Known as espundia, it can even result in fatal complications. ML is primarily associated with the leishmanial species of the Viannia subgenus, especially *L. braziliensis, L. guyanensis,* and *L. panamensis*. It can occur concurrently or following cutaneous lesions (as much as 10 years later). The risk of mucosal disease following primary CL is less than 10%. ML has also been described in Old World species (*L. infantum, L aethiopica, and L. tropica)*, but generally in immunocompromised hosts [44].

6.3.1 Current Treatment Regimens for ML

Treatment for ML is provided with systemically administered drugs that are usually given for longer durations than used for CL. The familiar selection of

antileishmanial drugs (antimonials, amphotericin B, and miltefosine) are known to be effective (~70% cure rates); but these drugs have significant failure rates, with the need for retreatment [45, 46].

6.4 Medicinal Chemistry

Drug discovery efforts for leishmaniasis suffered from the fact that it does not attract big pharmaceutical companies as it involves large cost of development followed by low return on investment. Further, the parasitic diseases suffer from a "translation innovation gap," where drugs rarely progress from *in vitro* drug screening to the preclinical phase [47]. One of the powerful approaches to fill this gap is through collaborative efforts of academia, industry, and nonprofit organizations [48]. There are well-established TPP for leishmaniasis by the Drugs for Neglected Diseases initiative (DND*i*) [49–51]. The TPPs of an optimized new chemical entity for the treatment of VL and CL are listed in Table 6.1.

The drug discovery approaches in the field of leishmaniasis have been reviewed by several authors [52–54]. There are several approaches which were effectively utilized for the leishmaniasis drug discovery efforts: (i) phenotypic screening approaches comprised of high-throughput screening (HTS) campaigns followed by hit-to-lead optimization, repurposing strategies, and fragment-based lead discovery; (ii) target-based approaches; and (iii) *in silico* computational approaches.

6.4.1 Phenotypic Screening Approach Versus Target-Based Approach

Both the approaches are widely utilized in drug discovery of neglected diseases (NDs) [55]. Phenotypic screening, especially in the drug discovery of NDs, has the advantage over target-based screening since the molecule is exposed to the fully functional biological system and the hits obtaining are actual actives with cell penetration. The target-based screening approach lacks the actual complexity of biological systems and oftentimes the hits obtained through this approach show no or poor efficacy in whole-organism or whole-cell assays. The disadvantage of target-based drug discovery over the phenotypic approach is that it does not account for polypharmacology or multi-target activity. With the careful selection of biological targets, the target-based drug discovery for neglected diseases can be successfully used; however, the limitation of this approach is that there are only few validated targets known for leishmaniasis and other NDs [55–57].

6.4.2 Phenotypic Screening Approaches

HTS with whole-cell phenotypic assays are often used in primary screenings where parasites cultured in macrophages provide complexity similar to that of the intracellular parasite in the human host [58]. The GlaxoSmithKline HTS program was the first parallel program against the three kinetoplastids most relevant to human disease, where 1.8 million compound libraries were screened

Table 6.1 Target product profile for leishmaniasis as determined by DND*i*.

	Visceral leishmaniasis (VL)		Cutaneous leishmaniasis (CL)	
Attribute	**Ideal**	**Acceptable**	**Ideal**	**Acceptable**
Species	All species	*L. donovani*	All species of *Leishmania*	*L. tropica* or *L. braziliensis*
Resistance	Active against resistant strains		—	—
Distribution	All areas	Either India or Africa	—	—
Safety and tolerability	No adverse events requiring monitoring	One monitoring visit in mid/endpoint	No adverse events requiring monitoring	No major safety concerns and monitoring by Primary Health Care (PHC)
Target population	Immunocompetent and immunosuppressed	Immunocompetent	Should be effective in immune-compromised patients	None
Clinical efficacy	>95%	>90%	>95%	60% for *L. tropica* 70% for *L. braziliensis*
Improved scar formation	—	—	Minimal scar	No worse than natural healing
Contraindications	None	Pregnancy/lactation	None	Can be assessed at PHC level
Drug–drug interactions	None	None for malaria, TB, and HIV therapies	—	—
Route of administration	Oral/im depot	Oral/im depot	Topical/oral	Non-parenteral or few doses if parenteral
Treatment regimen	Once per day for 10 days p.o. or three shots over 10 days	Bid for <10 days p.o. or <3 shots over 10 days	14 d topical or <7 days p.o.	28 days for topical or bid for 28 days p.o.
Product stability	Three years in relevant climates	Stable under conditions that can be reasonably achieved in the target region (>2 yr)	No cold chain, at least 3 yr at 37 °C	Two years at 4–8 °C
Cost	<$10 per course	<$80 per course	<$5 per treatment	<$50 per treatment

against *L. donovani, Trypanosoma cruzi, and Trypanosoma brucei* [59]. From the primary screening, 67 400 primary hits (>30% inhibition at 5 μM) were identified, with an overall hit rate of 4%. The primary hits were then narrowed down to 32 200 compounds, which were then tested in an intracellular assay of *L. donovani*–infected THP1-derived macrophages. The subsequent confirmation resulted in 5500 active compounds selective for *L. donovani* over macrophage cells. The confirmed active hits were then refined on the basis of physicochemical properties, and 351 noncytotoxic anti *L-donovani* compounds with MW <500 Da, calculated Property Forecast Index (cPFI) <8, and <5 aromatic rings were identified. Representative examples of *L. donovani* inhibitor hits identified through GSK HTS campaign are highlighted in Figure 6.1 [59].

One of the best medicinal chemistry approaches for the drug discovery of NTDs is rational medicinal chemistry optimization of the hit compounds identified via HTS screening. An excellent example was the discovery of GNF6702 by Novartis in 2013 [60]. The approach followed by Novartis involved screening of 3 million low-MW compounds against three kinetoplastids parasites, *viz. L. donovani*, *T. cruzi*, and *T. brucei* followed by refinement of active compounds over a selectivity window (EC_{50} < 10 μM and >fivefold selectivity over mammalian cells). This led to the identification of an azabenzoxazole compound, GNF5343, as a hit against *L. donovani* and *T. brucei*. The HTS screening was followed by extensive medicinal chemistry optimization of GNF5343 involving design and synthesis of ~300 compounds. Some of the key highlighted modifications are highlighted in Figure 6.2. The replacement of azabenzoxazole with imidazo- and triazolopyrimidine resulted in a boost in potency (20-fold, GNF2636). Further, the risk of toxicity issues related to the furan group was removed by replacing it with dimethyloxazole, and replacement of the chlorophenyl group with a

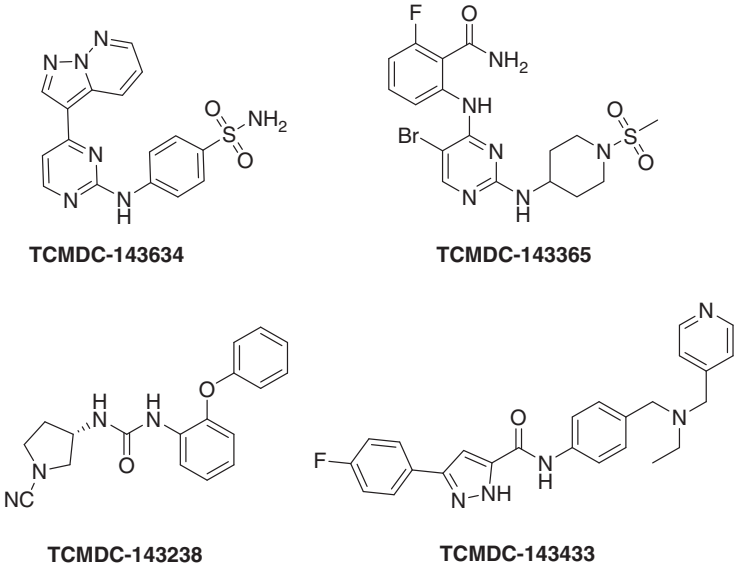


Figure 6.1 Representative hit compounds identified through the GSK-HTS campaign.

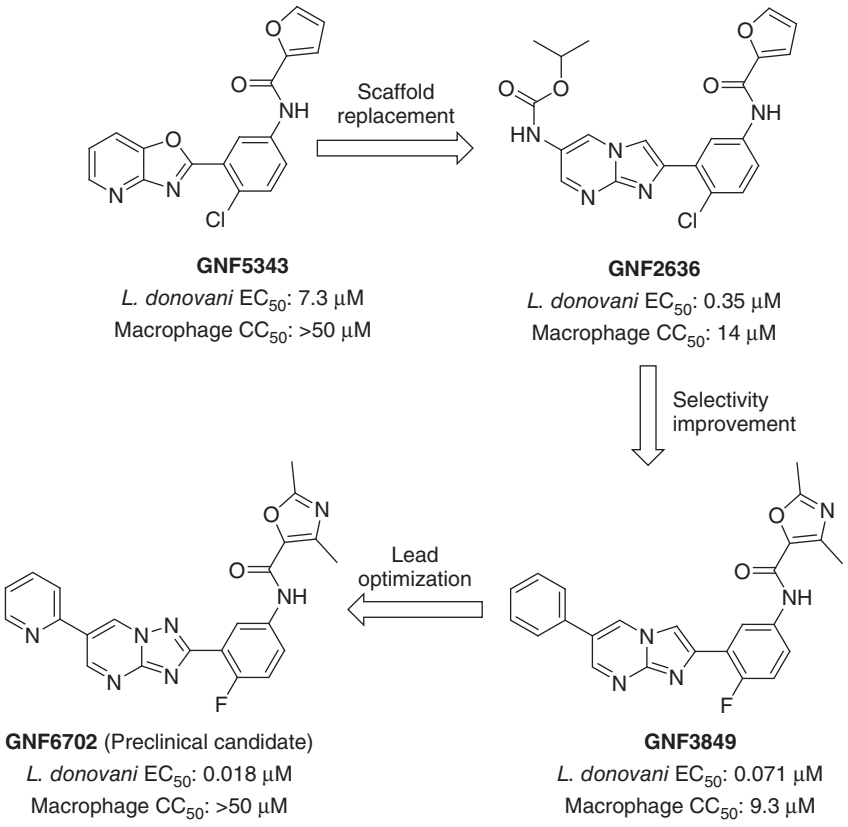


Figure 6.2 Medicinal chemistry optimization of hit compound GNF5343.

fluorophenyl resulted in GNF3849 with improved selectivity. The optimization of GNF3849 led to GNF6702 with low plasma clearance, high selectivity, and a 400-fold increase in potency compared to the original hit GNF5343. GNF6702 at 10 mg/kg twice daily orally was more effective in reducing liver parasite levels than miltefosine in mice infected with *L. donovani*. In CL efficacy model, 3 mg/kg twice-daily regimens of GNF6702 were superior to the 30 mg/kg once-daily miltefosine regimen [60].

The work done by DND*i* in developing amino-pyrazole ureas is another example of the HTS campaign followed by the hit-to-lead optimization studies. From the 95 000 small molecules library of Pfizer, a novel aminopyrazole hit was identified. The optimization of aminopyrazole (Figure 6.3) resulted in a lead compound with excellent *in vivo* efficacy via the oral route in a hamster model of VL. The treatment of an *L. infantum*–infected hamster with the lead compound at 50 mg/kg b.i.d. for five days resulted in 92.7% and 95% reduction in parasitemia levels in the liver and spleen, respectively [61]. Further, optimization efforts of the lead compound in collaboration with Takeda Pharmaceuticals resulted in a preclinical candidate, DNDI-5561 (structure not yet disclosed) [62].

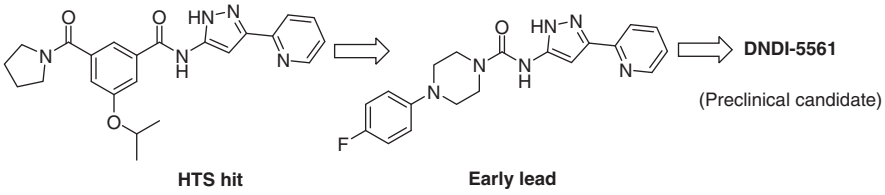


Figure 6.3 Hit-to-candidate optimization of amino-pyrazole ureas.

The drug discovery efforts and hit-to-lead optimization for NTDs are mostly performed in an academic environment with very few financial resources compared to big pharmaceutical companies. A popular strategy for academic laboratories is "repurposing," which can be drug repurposing, target repurposing, or lead repurposing [63]. The drug repurposing strategy for the treatment of leishmaniasis has been highlighted in other reviews [64, 65]. The classical example of drug repurposing is miltefosine. Miltefosine was originally developed as an anticancer candidate and later was developed as antileishmanial drug by WHO in public private partnership with a pharmaceutical company, Asta Medica [66]. The development of amphotericin B and paromomycin are the other examples of the drug repurposing strategy; these drugs were effectively utilized for the treatment of leishmaniasis [64]. The repurposing strategy was widely explored by the Pollastri research group for the identification of lead compounds for the treatment of NTDs [67–71]. The Pollastri group has repurposed human kinase inhibitors for neglected disease drug discovery, and medicinal chemistry optimization efforts have identified potent inhibitors of *L. major* (CL) **NEU-1923** [72] and **NEU-952** [73] by repurposing lapatinib and GW837016X, respectively (Figure 6.4).

Another promising effort made in the area of discovery of antileishmanial compounds using the repurposing strategy was done by the Drug Discovery Unit (DDU), University of Dundee, in collaboration with GSK, Tres Cantos, and DND*i*. The novel series of pyrazolopyrimidines were identified as an *L. donovani* inhibitor. The pyrazolopyrimidine series was developed by medicinal chemistry optimization of a *T. brucei* GSK3 kinase (*Tb*GSK3) inhibitor diaminothiazole compound, which was originally discovered from the DDU kinase set of compounds [74]. The optimization of the diaminothiazole *T. brucei* inhibitor by

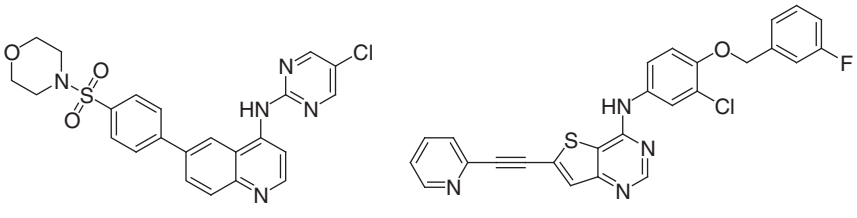


Figure 6.4 *L. major* inhibitors **NEU-1923** and **NEU-952**.

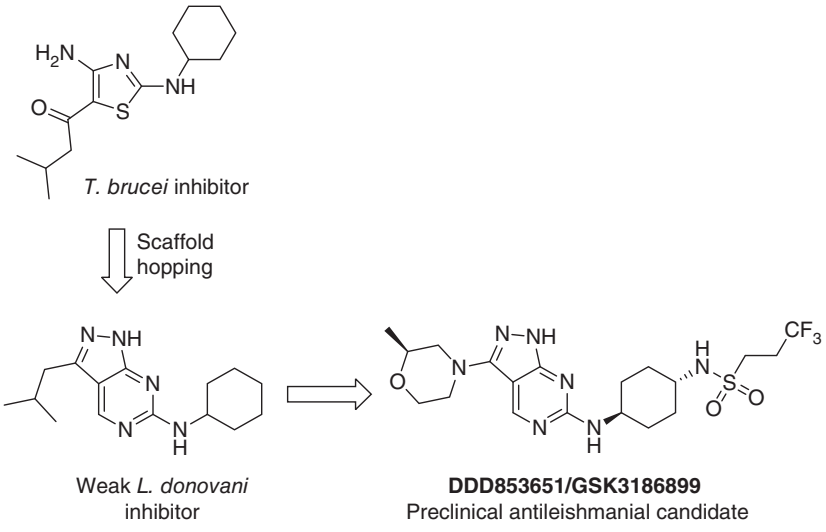


Figure 6.5 Evolution of pyrazolopyrimidines and DDD853651/GSK3186899 as antileishmanial preclinical candidates.

utilizing the "scaffold hopping" approach led to the discovery of the pyrazolopyrimidine compound as an *L. donovani* inhibitor. A further series of optimization efforts resulted in a preclinical candidate, DDD853651/GSK3186899 (Figure 6.5). In the mouse model of VL infection, DDD853651 demonstrated activity comparable to miltefosine and the parasitemia levels were reduced by 99% at a dose of 25 mg/kg p.o. twice a day for 10 days [75, 76].

The development of nitroimidazoles by DND*i* is another example of repurposing, where the 72 nitroimidazoles similar to antitubercular drug pretomanid (PA-824) were evaluated for antileishmanial activity and led to the identification of nitroimidazo-oxazole compound DNDI-VL-2098 as a preclinical candidate for the oral treatment of VL. It was effective in both mouse and hamster models of infection with ED_{90} values of 3.7 and <25 mg/kg, respectively [77, 78]. The development of DNDI-VL-2098 was terminated due to toxicity issues. The backup compound DNDI-0690 was later selected as a preclinical development. DNDI-0690 was developed by scaffold hopping from the antitubercular drugs delamanid and PA-824. DNDI-0690 was superior to DNDI-VL-2090 in terms of thermodynamic solubility, lower log *D* value. Furthermore, it had similar high-permeability properties and was better in terms of safety parameters. DNDI-0690 was not mutagenic (Ames test), had no CYP3A4 inhibition ($IC_{50} > 100\,\mu M$), and demonstrated low hERG inhibition ($IC_{50} > 30\,\mu M$), which makes it a better candidate for development than DNDI-VL-2098 [79]. Recently, DND*i* developed DNDI-8219 in their lead optimization program against VL, to mitigate development risks. The library of ~900 compounds with pretomanid-related scaffolds was screened against VL, followed by a systematic medicinal chemistry approach to develop a new lead compound. DNDI-8219 is a very promising VL backup candidate (Figure 6.6) with better solubility and PK

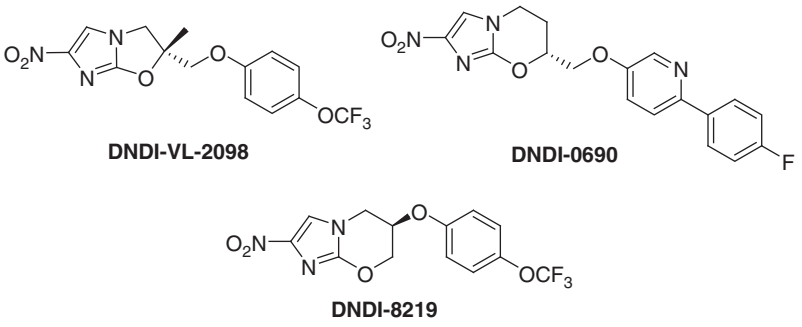


Figure 6.6 Nitroimidazole-based antileishmanial candidates.

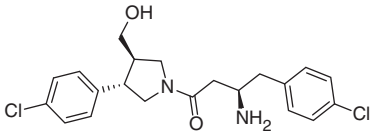
properties, and >97% parasite clearance in a hamster infection model at a dose of 25 mg/kg twice daily as well as minimal hERG inhibition ($IC_{50} > 30\,\mu M$) [49].

The boron-containing compounds, benzoxaboroles, have been explored against different kinetoplastid parasites by Anacor Pharmaceuticals [80–83]. The highlight was SCYX-7158, currently in phase IIb/III clinical trials for HAT diseases [80, 84]. Furthermore, in collaboration with DND*i*, they developed DNDI-6148 (*L. donovani* $IC_{50} = 1.62\,\mu M$; *in vivo* minimum effective dose in mice = 25 mg/kg twice daily for 10 days), a benzoxaborole compound (structure not yet disclosed) which completed preclinical studies in 2017. The decision has been taken to advance this compound to phase I clinical studies in healthy volunteers in parallel to the ongoing toxicological investigations [85].

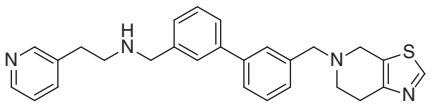
6.4.3 Target-Based Approaches

The phenotypic approach is favored over target-based approaches in the drug discovery of NDs, but careful selection of targets can make target-based drug discovery successful [55]. A good example of the target-based approach in leishmaniasis drug discovery is the inhibition of *Leishmania spp*. *N*-myristoyltransferase (NMT) [86–89]. NMT is a validated target for the development of new therapeutics against VL [90]. Groups at Imperial College London and University of York have discovered several potent antileishmanial compounds targeting the *L. donovani* NMT (*Ld*NMT). The Pfizer Global Diverse Representative Set consisting of 150 000 compounds was screened. All hit compounds ($IC_{50} < 5\,\mu M$) were counter-screened for selectivity against human NMT, and four compound series that displayed selectivity for *Leishmania* NMT over human NMT were identified [86–88]. The DDU at the University of Dundee also identified orally active trypanocidal NMT inhibitors that inhibited both *T. brucei* and *L. donovani* NMTs [91–93]. The selected examples of *L. donovani* NMT inhibitors are summarized in Figure 6.7.

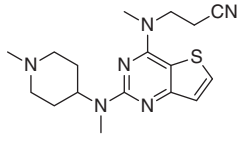
Protein kinases are potential drug targets against trypanosomatid parasites [94]. The Cdc2-related kinase (CRK), CRK3 of *Leishmania*, is essential for the proliferation of the parasite and can therefore be utilized as a potential drug target. CRK3 forms a complex with CYC6 and remains active in both the life cycle stages of the parasite, *viz*. the insect stage promastigote and the mammalian



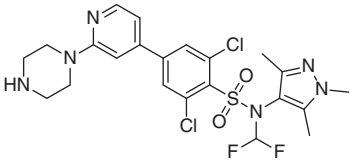
PF-03402623
L. donovani NMT, IC_{50} = 0.08 μM
L. donovani, EC_{50} = 12 μM



PF-00075634
L. donovani NMT, IC_{50} = 0.91 μM
L. donovani, EC_{50} = 16 μM



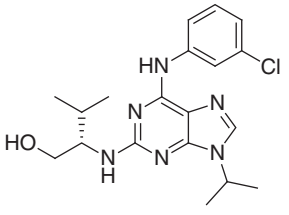
IMP-0000083
L. donovani NMT, IC_{50} = 0.28 μM
L. donovani, EC_{50} = 24 μM



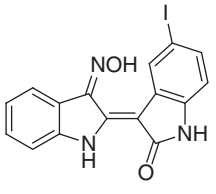
DDD100887
L. donovani NMT, IC_{50} = 0.02 μM
L. donovani, EC_{50} = 0.66 μM

Figure 6.7 Selected examples of *L. donovani* NMT inhibitors.

stage amastigote [55, 95]. The Mottram group at the University of Glasgow screened the chemical library of 634 compounds against *L. mexicana* CRK3, followed by validation of active hits against *L. donovani* amastigotes. The screen identified 12 compounds with 95–98% potent inhibition and 26 compounds with 90–95% inhibition against CRK3 kinase. The 27 most potent compounds were then screened against *L. donovani*–infected mouse peritoneal macrophages and the results were broadly similar [95]. The representative examples (NG58 and 98/146) are summarized in Figure 6.8. Similarly, DDU-Dundee screened 3383 compounds from the kinase library against *Leishmania* CRK3–CYC6. The primary screen identified 73 compounds with >40% inhibition at 30 μM, followed by confirmation of 46 compounds with IC_{50} values of ≤30 μM. This led to the identification of eight different chemical series and seven singletons. Out of the eight series, four were followed up with hit-to-lead optimization studies to obtain high-potency and high-selectivity compounds over the mammalian cyclin-dependent kinase 2–cyclin A complex (CDK2–CYCA). The optimization program led to the development of selective CRK3 inhibitors, but, unfortunately,

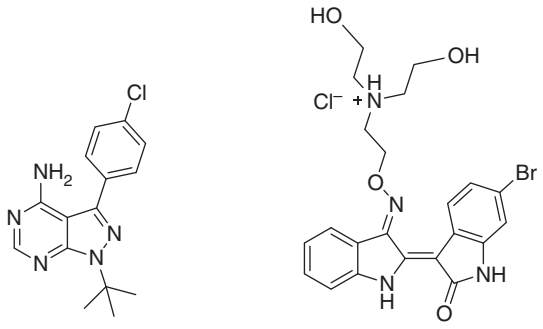


NG58
CRK3 IC_{50} = 2.1 μM
L. donovani EC_{50} = 3.93 μM



98/146
CRK3 IC_{50} = 1.2 μM
L. donovani EC_{50} = 5.77 μM

Figure 6.8 Selected examples of *Leishmania* CRK3 inhibitors.



PP2
LmCK1.2 IC_{50} = 1.6 μM
L. donovani EC_{50} = 1 μM

Compound 42
LmCK1.2 IC_{50} = 0.93 μM
L. donovani EC_{50} = 0.06 μM

Figure 6.9 LmCK1.2 inhibitors PP2 and compound **42**.

these compounds did not show promising activity against the tested parasite *L. major* [96], which demonstrates how potent inhibitor hits identified through a target-based approach do not always result in efficacy against the whole cell or the organism.

Leishmania casein kinase 1.2 (LmCK1.2) is an exoprotein kinase which has been recently shown to be essential for the proliferation and survival of the parasite [97, 98]. In an attempt to discover new compounds against leishmaniasis using the target-based approach, Duriue et al. screened 5018 compounds from the kinase library against LmCK1.2 and obtained 88 compounds with more than 50% inhibition at 10 μM. The active compounds were then screened against *L. donovani* cultures to validate the hits. Out of the 88 compounds tested, 75 were found to be active (>40% inhibition at 10 μM) against *L. donovani*. Two hit compounds PP2 and compound **42** (Figure 6.9) were selected further for their ongoing *Leishmania* program [97].

6.4.4 *In Silico* Computational Approaches

The *in silico* computational approach is an attractive method for developing new drugs against NDs, where the initial screening to identify primary hit can be speeded up and overall cost can be reduced using computational models and docking algorithms [99, 100]. The *in silico* approach can also be utilized for structure-based drug design (SBDD), which uses the structural knowledge of the target protein to design hits. Currently, 285 protein structures of *Leishmania* are available in the Protein Data Bank (PDB). The other popular *in silico* tool is ligand-based drug design (LBDD), where information of known active and inactive compounds are used to build computational models such as quantitative structure–activity relationships (QSAR), pharmacophore-based methods, and similarity searching methods [101].

The group at Imperial College has utilized the SBDD approach (Figure 6.10) in designing selective inhibitors against *Leishmania* NMT [89]. The binding modes were identified using the co-crystal structure of a known NMT inhibitor with the protein. The crystal structure was then used to enhance the protein affinity by

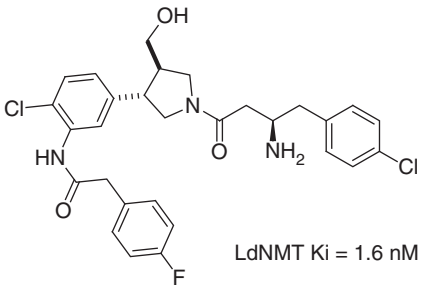


Figure 6.10 *L. donovani* *N*-myristoyltransferase (LdNMT) inhibitor discovered using the SBDD approach.

hybridization of two different binding modes, which led to the development of potent LdNMT inhibitors with improved solubility [89].

The NTD Drug Discovery Booster project was initiated by DND*i* in 2015 in collaboration with eight pharmaceutical companies: Abbvie, Astellas Pharma Inc., AstraZeneca, Celgene, Eisai, Merck, Shionogi & Co. Ltd., and Takeda Pharmaceuticals. This initiative was supported by the funding from Global Health Innovative Technology (GHIT) Fund. The aim of this project was to speed up the process, and to find new treatments for leishmaniasis and Chagas disease at low cost. This project involves computational approaches where collaborators mine their compound libraries and conduct a similarity structure search to identify molecules that could be used by DND*i* to design the next-generation oral treatment for Chagas and leishmaniasis. DND*i* shares the newly identified hit compound (the "seed") with weak or moderate activity against the parasite. The pharmaceutical companies then perform a similarity search in their compound libraries in order to identify similar compounds, followed by screening of the similar compounds by DND*i* against parasites, and then the company creates a large dataset of compounds around the initial seed. The results are shared between all partners, and they perform repeated structure mining for related compounds. These compounds are then sorted into different classes and provided to DND*i* for further optimization. To date, 17 seeds have been released by DND*i*, out of which 12 are currently being pursued and 5 have been stopped [102].

6.5 Conclusion

Leishmaniasis is a major health problem faced by many developing countries, and an estimated 700 000 to 1 million new cases occur annually. The emergence of resistance- and toxicity-related issues for available treatment makes it urgent to develop novel oral treatments with better safety and efficacy profiles. The initiatives of academia laboratories, nonprofit organizations, and public private partnerships have led to significant progress in the search for novel treatments for the diseases. The advancement of candidates like GNF6702, DNDI-5561, DNDI-8219, DNDI-0690, and benzoxaboroles are some of the noteworthy examples in the drug discovery efforts for leishmaniasis. However, there is still a

long road to travel, perhaps best achieved by increasing industrial–academic collaborations, along with better knowledge of validated targets and opportunities for repurposing of clinical candidates and approved drugs.

References

1 Mauricio, I.L., Stothard, J.R., and Miles, M.A. (2000). The strange case of Leishmania chagasi. *Parasitol. Today* 16: 188–189.

2 Alvar, J., Velez, I.D., Bern, C. et al. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7: e35671.

3 Ceccarelli, M., Rullo, E.V., Condorelli, F. et al. (2018). Unusual signs and symptoms in HIV-positive patients coinfected with *Leishmania spp*: the importance of neglected tropical disease in differential diagnosis. *Open Access Maced. J. Med. Sci.* 6: 843–847.

4 Dey, A. and Singh, S. (2006). Transfusion transmitted leishmaniasis: a case report and review of literature. *Indian J. Med. Microbiol.* 24: 165–170.

5 Bern, C. (2018). Visceral leishmaniasis: clinical manifestations and diagnosis. In: *UpToDate* (ed. P.F. Weller). Wolters Kluwer Health.

6 Lindoso, J.A., Cunha, M.A., Queiroz, I.T., and Moreira, C.H. (2016). Leishmaniasis-HIV coinfection: current challenges. *HIV AIDS (Auckl.)* 8: 147–156.

7 Berman, J.D., Badaro, R., Thakur, C.P. et al. (1998). Efficacy and safety of liposomal amphotericin B (AmBisome) for visceral leishmaniasis in endemic developing countries. *Bull. World Health Organ.* 76: 25–32.

8 Seaman, J., Mercer, A.J., Sondorp, H.E., and Herwaldt, B.L. (1996). Epidemic visceral leishmaniasis in southern Sudan: treatment of severely debilitated patients under wartime conditions and with limited resources. *Ann. Intern. Med.* 124: 664–672.

9 Sundar, S., Jha, T.K., Thakur, C.P. et al. (2002). Low-dose liposomal amphotericin B in refractory Indian visceral leishmaniasis: a multicenter study. *Am. J. Trop. Med. Hyg.* 66: 143–146.

10 Thakur, C.P. (2001). A single high dose treatment of kala-azar with Ambisome (amphotericin B lipid complex): a pilot study. *Int. J. Antimicrob. Agents* 17: 67–70.

11 Thakur, C.P., Pandey, A.K., Sinha, G.P. et al. (1996). Comparison of three treatment regimens with liposomal amphotericin B (AmBisome) for visceral leishmaniasis in India: a randomized dose-finding study. *Trans. R. Soc. Trop. Med. Hyg.* 90: 319–322.

12 Sundar, S., Jha, T.K., Thakur, C.P. et al. (2002). Oral miltefosine for Indian visceral leishmaniasis. *N. Engl. J. Med.* 347: 1739–1746.

13 Sundar, S., Jha, T.K., Sindermann, H. et al. (2003). Oral miltefosine treatment in children with mild to moderate Indian visceral leishmaniasis. *Pediatr. Infect. Dis. J.* 22: 434–438.

14 Bhattacharya, S.K., Sinha, P.K., Sundar, S. et al. (2007). Phase 4 trial of miltefosine for the treatment of Indian visceral leishmaniasis. *J. Infect. Dis.* 196: 591–598.

15 Sundar, S., Singh, A., Rai, M. et al. (2012). Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin. Infect. Dis.* 55: 543–550.
16 Rijal, S., Ostyn, B., Uranw, S. et al. (2013). Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. *Clin. Infect. Dis.* 56: 1530–1538.
17 Jha, T.K., Olliaro, P., Thakur, C.P. et al. (1998). Randomised controlled trial of aminosidine (paromomycin) v sodium stibogluconate for treating visceral leishmaniasis in North Bihar, India. *BMJ* 316: 1200–1205.
18 Sundar, S., Jha, T.K., Thakur, C.P. et al. (2007). Injectable paromomycin for Visceral leishmaniasis in India. *N. Engl. J. Med.* 356: 2571–2581.
19 Thakur, C.P., Kanyok, T.P., Pandey, A.K. et al. (2000). A prospective randomized, comparative, open-label trial of the safety and efficacy of paromomycin (aminosidine) plus sodium stibogluconate versus sodium stibogluconate alone for the treatment of visceral leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.* 94: 429–431.
20 Chunge, C.N., Owate, J., Pamba, H.O., and Donno, L. (1990). Treatment of visceral leishmaniasis in Kenya by aminosidine alone or combined with sodium stibogluconate. *Trans. R. Soc. Trop. Med. Hyg.* 84: 221–225.
21 Melaku, Y., Collin, S.M., Keus, K. et al. (2007). Treatment of kala-azar in southern Sudan using a 17-day regimen of sodium stibogluconate combined with paromomycin: a retrospective comparison with 30-day sodium stibogluconate monotherapy. *Am. J. Trop. Med. Hyg.* 77: 89–94.
22 Aronson, N. (2018). Cutaneous leishmaniasis: clinical manifestations and diagnosis. In: *UpToDate* (ed. P.F. Weller). Wolters Kluwer Health.
23 Herwaldt, B.L., Arana, B.A., and Navin, T.R. (1992). The natural history of cutaneous leishmaniasis in Guatemala. *J. Infect. Dis.* 165: 518–527.
24 Morizot, G., Kendjo, E., Mouri, O. et al. (2013). Travelers with cutaneous leishmaniasis cured without systemic therapy. *Clin. Infect. Dis.* 57: 370–380.
25 Solomon, M., Baum, S., Barzilai, A. et al. (2009). Treatment of cutaneous leishmaniasis with intralesional sodium stibogluconate. *J. Eur. Acad. Dermatol. Venereol.* 23: 1189–1192.
26 Navin, T.R., Arana, B.A., Arana, F.E. et al. (1992). Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconatzole for treating cutaneous leishmaniasis in Guatemala. *J. Infect. Dis.* 165: 528–534.
27 Alrajhi, A.A., Ibrahim, E.A., De Vol, E.B. et al. (2002). Fluconazole for the treatment of cutaneous leishmaniasis caused by Leishmania major. *N. Engl. J. Med.* 346: 891–895.
28 Morizot, G., Delgiudice, P., Caumes, E. et al. (2007). Healing of Old World cutaneous leishmaniasis in travelers treated with fluconazole: drug effect or spontaneous evolution? *Am. J. Trop. Med. Hyg.* 76: 48–52.
29 Prates, F.V., Dourado, M.E., Silva, S.C. et al. (2017). Fluconazole in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis*: a randomized controlled trial. *Clin. Infect. Dis.* 64: 67–71.
30 Berman, J.D., Holz, G.G. Jr., and Beach, D.H. (1984). Effects of ketoconazole on growth and sterol biosynthesis of Leishmania mexicana promastigotes in culture. *Mol. Biochem. Parasitol.* 12: 1–13.

31 Hargrove, T.Y., Wawrzak, Z., Liu, J. et al. (2011). Substrate preferences and catalytic parameters determined by structural characteristics of sterol 14alpha-demethylase (CYP51) from Leishmania infantum. *J. Biol. Chem.* 286: 26838–26848.

32 Hawser, S. and Islam, K. (1999). Comparisons of the effects of fungicidal and fungistatic antifungal agents on the morphogenetic transformation of Candida albicans. *J. Antimicrob. Chemother.* 43: 411–413.

33 Paniz Mondolfi, A.E., Stavropoulos, C., Gelanew, T. et al. (2011). Successful treatment of Old World cutaneous leishmaniasis caused by *Leishmania infantum* with posaconazole. *Antimicrob. Agents Chemother.* 55: 1774–1776.

34 Soto, J., Arana, B.A., Toledo, J. et al. (2004). Miltefosine for new world cutaneous leishmaniasis. *Clin. Infect. Dis.* 38: 1266–1272.

35 Velez, I., Lopez, L., Sanchez, X. et al. (2010). Efficacy of miltefosine for the treatment of American cutaneous leishmaniasis. *Am. J. Trop. Med. Hyg.* 83: 351–356.

36 Chrusciak-Talhari, A., Dietze, R., Chrusciak Talhari, C. et al. (2011). Randomized controlled clinical trial to access efficacy and safety of miltefosine in the treatment of cutaneous leishmaniasis Caused by Leishmania (Viannia) guyanensis in Manaus, Brazil. *Am. J. Trop. Med. Hyg.* 84: 255–260.

37 Soto, J., Rea, J., Balderrama, M. et al. (2008). Efficacy of miltefosine for Bolivian cutaneous leishmaniasis. *Am. J. Trop. Med. Hyg.* 78: 210–211.

38 Machado, P.R., Ampuero, J., Guimaraes, L.H. et al. (2010). Miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil: a randomized and controlled trial. *PLoS Negl.Trop. Dis.* 4: e912.

39 Pinto-Martinez, A.K., Rodriguez-Duran, J., Serrano-Martin, X. et al. (2018). Mechanism of action of miltefosine on Leishmania donovani involves the impairment of acidocalcisome function and the activation of the sphingosine-dependent plasma membrane Ca(2+) channel. *Antimicrob. Agents Chemother.* 62: e01614–17

40 Zhang, N., Prasad, S., Huyghues Despointes, C.E. et al. (2018). Leishmania parasitophorous vacuole membranes display phosphoinositides that create conditions for continuous Akt activation and a target for miltefosine in Leishmania infections. *Cell. Microbiol.* 20: e12889.

41 Solomon, M., Baum, S., Barzilai, A. et al. (2007). Liposomal amphotericin B in comparison to sodium stibogluconate for cutaneous infection due to Leishmania braziliensis. *J. Am. Acad. Dermatol.* 56: 612–616.

42 Berman, J.D., Gallalee, J.V., and Best, J.M. (1987). Sodium stibogluconate (Pentostam) inhibition of glucose catabolism via the glycolytic pathway, and fatty acid beta-oxidation in Leishmania mexicana amastigotes. *Biochem. Pharmacol.* 36: 197–201.

43 Pathak, M.K. and Yi, T. (2001). Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines. *J. Immunol.* 167: 3391–3397.

44 Shirian, S., Oryan, A., Hatam, G.R., and Daneshbod, Y. (2013). Three Leishmania/L. species – L. infantum, L. major, L. tropica – as causative agents of mucosal leishmaniasis in Iran. *Pathog. Glob. Health* 107: 267–272.

45 Amato, V.S., Tuon, F.F., Siqueira, A.M. et al. (2007). Treatment of mucosal leishmaniasis in Latin America: systematic review. *Am. J. Trop. Med. Hyg.* 77: 266–274.

46 Soto, J., Rea, J., Valderrama, M. et al. (2009). Efficacy of extended (six weeks) treatment with miltefosine for mucosal leishmaniasis in Bolivia. *Am. J. Trop. Med. Hyg.* 81: 387–389.

47 Nwaka, S., Ramirez, B., Brun, R. et al. (2009). Advancing drug innovation for neglected diseases-criteria for lead progression. *PLoS Negl.Trop. Dis.* 3: e440.

48 Ferrins, L. and Pollastri, M.P. (2018). The importance of collaboration between industry, academics, and nonprofits in tropical disease drug discovery. *ACS Infect. Dis.* 4: 445–448.

49 Thompson, A.M., O'Connor, P.D., Marshall, A.J. et al. (2018). Development of (6R)-2-nitro-6-[4-(trifluoromethoxy)phenoxy]-6,7-dihydro-5*H*-imidazo [2,1-b][1,3]oxazine (DNDI-8219): a new lead for visceral leishmaniasis. *J. Med. Chem.* 61: 2329–2352.

50 DND*i*. Target product profile for leishmaniasis. https://www.dndi.org/diseases-projects/leishmaniasis/leish-target-product-profile/ (accessed 24 September 2018).

51 Katsuno, K., Burrows, J.N., Duncan, K. et al. (2015). Hit and lead criteria in drug discovery for infectious diseases of the developing world. *Nat. Rev. Drug Discovery* 14: 751–758.

52 Mowbray, C.E. (2018). Anti-leishmanial drug discovery: past, present and future perspectives (Chapter 2). In: *Drug Discovery for Leishmaniasis*, 24–36. The Royal Society of Chemistry https://doi.org/10.1039/9781788010177-00024.

53 Nagle, A.S., Khare, S., Kumar, A.B. et al. (2014). Recent developments in drug discovery for leishmaniasis and human African trypanosomiasis. *Chem. Rev.* 114: 11305–11347.

54 Zulfiqar, B., Shelper, T.B., and Avery, V.M. (2017). Leishmaniasis drug discovery: recent progress and challenges in assay development. *Drug Discovery Today* 22: 1516–1531.

55 Gilbert, I.H. (2013). Drug discovery for neglected diseases: molecular target-based and phenotypic approaches. *J. Med. Chem.* 56: 7719–7726.

56 Reguera, R.M., Calvo-Alvarez, E., Alvarez-Velilla, R., and Balana-Fouce, R. (2014). Target-based vs. phenotypic screenings in Leishmania drug discovery: a marriage of convenience or a dialogue of the deaf? *Int. J. Parasitol. Drugs Drug. Resist.* 4: 355–357.

57 Swinney, D.C. (2013). Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clin. Pharmacol. Ther.* 93: 299–301.

58 Ortiz, D., Guiguemde, W.A., Hammill, J.T. et al. (2017). Discovery of novel, orally bioavailable, antileishmanial compounds using phenotypic screening. *PLoS Negl.Trop. Dis.* 11: e0006157.

59 Peña, I., Pilar Manzano, M., Cantizani, J. et al. (2015). New compound sets identified from high throughput phenotypic screening against three kinetoplastid parasites: an open resource. *Sci. Rep.* 5: 8771.

60 Khare, S., Nagle, A.S., Biggart, A. et al. (2016). Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature* 537: 229.

61 Mowbray, C.E., Braillard, S., Speed, W. et al. (2015). Novel amino-pyrazole ureas with potent in vitro and in vivo antileishmanial activity. *J. Med. Chem.* 58: 9615–9624.

62 DND*i* (2018). DNDI-5561 portfolio. https://www.dndi.org/diseases-projects/portfolio/dndi-5561/ (accessed 25 September 2018).

63 Klug, D.M., Gelb, M.H., and Pollastri, M.P. (2016). Repurposing strategies for tropical disease drug discovery. *Bioorg. Med. Chem. Lett.* 26: 2569–2576.

64 Andrade-Neto, V.V., Cunha-Junior, E.F., Dos Santos Faioes, V. et al. (2018). Leishmaniasis treatment: update of possibilities for drug repurposing. *Front. Biosci. (Landmark Ed.)* 23: 967–996.

65 Charlton, R.L., Rossi-Bergmann, B., Denny, P.W., and Steel, P.G. (2018). Repurposing as a strategy for the discovery of new anti-leishmanials: the-state-of-the-art. *Parasitology* 145: 219–236.

66 Sunyoto, T., Potet, J., and Boelaert, M. (2018). Why miltefosine-a life-saving drug for leishmaniasis-is unavailable to people who need it the most. *BMJ Glob. Health* 3: e000709.

67 Devine, W., Woodring, J.L., Swaminathan, U. et al. (2015). Protozoan parasite growth inhibitors discovered by cross-screening yield potent scaffolds for lead discovery. *J. Med. Chem.* 58: 5522–5537.

68 Diaz, R., Luengo-Arratta, S.A., Seixas, J.D. et al. (2014). Identification and characterization of hundreds of potent and selective inhibitors of Trypanosoma brucei growth from a kinase-targeted library screening campaign. *PLoS Negl.Trop. Dis.* 8: e3253.

69 Ochiana, S.O., Bland, N.D., Settimo, L. et al. (2015). Repurposing human PDE4 inhibitors for neglected tropical diseases. Evaluation of analogs of the human PDE4 inhibitor GSK-256066 as inhibitors of PDEB1 of Trypanosoma brucei. *Chem. Biol. Drug Des.* 85: 549–564.

70 Patel, G., Karver, C.E., Behera, R. et al. (2013). Kinase scaffold repurposing for neglected disease drug discovery: discovery of an efficacious, lapatinib-derived lead compound for trypanosomiasis. *J. Med. Chem.* 56: 3820–3832.

71 Patel, G., Roncal, N.E., Lee, P.J. et al. (2014). Repurposing human Aurora kinase inhibitors as leads for anti-protozoan drug discovery. *Medchemcomm* 5: 655–658.

72 Devine, W., Thomas, S.M., Erath, J. et al. (2017). Antiparasitic lead discovery: toward optimization of a chemotype with activity against multiple protozoan parasites. *ACS Med. Chem. Lett.* 8: 350–354.

73 Woodring, J.L., Behera, R., Sharma, A. et al. (2018). Series of alkynyl-substituted thienopyrimidines as inhibitors of protozoan parasite proliferation. *ACS Med. Chem. Lett.* https://doi.org/10.1021/acsmedchemlett.8b00245.

74 Woodland, A., Grimaldi, R., Luksch, T. et al. (2013). From on-target to off-target activity: identification and optimisation of Trypanosoma brucei

GSK3 inhibitors and their characterisation as anti-Trypanosoma brucei drug discovery lead molecules. *ChemMedChem* 8: 1127–1137.

75 Wyllie, S., Thomas, M., Patterson, S. et al. (2018). Cyclin-dependent kinase 12 is a drug target for visceral leishmaniasis. *Nature* 560: 192–197.

76 Miles, T.J. and Thomas, M.G. (2016). Pyrazolo[3,4-d]pyrimidin derivative and its use for the treatment of leishmaniasis. US patent US20170369496A1.

77 Gupta, S., Yardley, V., Vishwakarma, P. et al. (2015). Nitroimidazo-oxazole compound DNDI-VL-2098: an orally effective preclinical drug candidate for the treatment of visceral leishmaniasis. *J. Antimicrob. Chemother.* 70: 518–527.

78 Thompson, A.M., O'Connor, P.D., Blaser, A. et al. (2016). Repositioning antitubercular 6-Nitro-2,3-dihydroimidazo[2,1-*b*][1,3]oxazoles for neglected tropical diseases: structure–activity studies on a preclinical candidate for visceral leishmaniasis. *J. Med. Chem.* 59: 2530–2550.

79 Thompson, A.M., O'Connor, P.D., Marshall, A.J. et al. (2017). 7-Substituted 2-nitro-5,6-dihydroimidazo[2,1-b][1,3]oxazines: novel antitubercular agents lead to a new preclinical candidate for visceral leishmaniasis. *J. Med. Chem.* 60: 4212–4233.

80 Jacobs, R.T., Nare, B., Wring, S.A. et al. (2011). SCYX-7158, an orally-active benzoxaborole for the treatment of stage 2 human African trypanosomiasis. *PLoS Negl.Trop. Dis.* 5: e1151.

81 Van Bocxlaer, K., Gaukel, E., Hauser, D. et al. (2018). Topical treatment for cutaneous leishmaniasis: dermato-pharmacokinetic lead optimization of benzoxaboroles. *Antimicrob. Agents Chemother.* 62: e02419–17.

82 Nare, B., Wring, S., Bacchi, C. et al. (2010). Discovery of novel orally bioavailable oxaborole 6-carboxamides that demonstrate cure in a murine model of late-stage central nervous system african trypanosomiasis. *Antimicrob. Agents Chemother.* 54: 4379–4388.

83 Zhang, N., Zoltner, M., Leung, K.F. et al. (2018). Host-parasite co-metabolic activation of antitrypanosomal aminomethyl-benzoxaboroles. *PLoS Pathog.* 14: e1006850.

84 DND*i* (2016). Prospective study on efficacy and safety of SCYX-7158 in patients infected by human African trypanosomiasis due to T.b. Gambiense. https://ClinicalTrials.gov/show/NCT03087955 (accessed 7 March 2019).

85 DND*i* (2018). DNDI-6148 portfolio. https://www.dndi.org/diseases-projects/portfolio/oxaborole-dndi-6148/ (accessed 27 September 2018).

86 Paape, D., Bell, A.S., Heal, W.P. et al. (2014). Using a non-image-based medium-throughput assay for screening compounds targeting N-myristoylation in intracellular Leishmania amastigotes. *PLoS Negl.Trop. Dis.* 8: e3363.

87 Bell, A.S., Mills, J.E., Williams, G.P. et al. (2012). Selective inhibitors of protozoan protein N-myristoyltransferases as starting points for tropical disease medicinal chemistry programs. *PLoS Negl.Trop. Dis.* 6: e1625.

88 Brannigan, J.A., Roberts, S.M., Bell, A.S. et al. (2014). Diverse modes of binding in structures of Leishmania major N-myristoyltransferase with selective inhibitors. *IUCrJ* 1: 250–260.

89 Hutton, J.A., Goncalves, V., Brannigan, J.A. et al. (2014). Structure-based design of potent and selective leishmania N-myristoyltransferase inhibitors. *J. Med. Chem.* 57: 8664–8670.
90 Brannigan, J.A., Smith, B.A., Yu, Z. et al. (2010). N-myristoyltransferase from Leishmania donovani: structural and functional characterisation of a potential drug target for visceral leishmaniasis. *J. Mol. Biol.* 396: 985–999.
91 Brand, S., Cleghorn, L.A.T., McElroy, S.P. et al. (2012). Discovery of a novel class of orally active trypanocidal N-myristoyltransferase inhibitors. *J. Med. Chem.* 55: 140–152.
92 Frearson, J.A., Brand, S., McElroy, S.P. et al. (2010). N-myristoyltransferase inhibitors as new leads to treat sleeping sickness. *Nature* 464: 728–732.
93 Brand, S., Wyatt, P., Thompson, S. et al. (2010). N-myristoyl transferase inhibitors. International Patent WO2010/026365.
94 Merritt, C., Silva, L.E., Tanner, A.L. et al. (2014). Kinases as druggable targets in trypanosomatid protozoan parasites. *Chem. Rev.* 114: 11280–11304.
95 Grant, K.M., Dunion, M.H., Yardley, V. et al. (2004). Inhibitors of Leishmania mexicana CRK3 cyclin-dependent kinase: chemical library screen and antileishmanial activity. *Antimicrob. Agents Chemother.* 48: 3033–3042.
96 Cleghorn, L.A., Woodland, A., Collie, I.T. et al. (2011). Identification of inhibitors of the Leishmania cdc2-related protein kinase CRK3. *ChemMedChem* 6: 2214–2224.
97 Durieu, E., Prina, E., Leclercq, O. et al. (2016). From drug screening to target deconvolution: a target-based drug discovery pipeline using leishmania casein kinase 1 isoform 2 to identify compounds with antileishmanial activity. *Antimicrob. Agents Chemother.* 60: 2822–2833.
98 Allocco, J.J., Donald, R., Zhong, T. et al. (2006). Inhibitors of casein kinase 1 block the growth of Leishmania major promastigotes in vitro. *Int. J. Parasitol.* 36: 1249–1259.
99 Ekins, S., Williams, A.J., Krasowski, M.D., and Freundlich, J.S. (2011). In silico repositioning of approved drugs for rare and neglected diseases. *Drug Discovery Today* 16: 298–310.
100 Crowther, G.J., Shanmugam, D., Carmona, S.J. et al. (2010). Identification of attractive drug targets in neglected-disease pathogens using an in silico approach. *PLoS Negl.Trop. Dis.* 4: e804.
101 Roca, C., Sebastián-Pérez, V., and Campillo, N.E. (2018). In silico tools for target identification and drug molecular docking in leishmania (Chapter 7). In: *Drug Discovery for Leishmaniasis*, 130–152. The Royal Society of Chemistry https://doi.org/10.1039/9781788010177-00130.
102 DND*i* (2015). NTD drug discovery booster. https://www.dndi.org/diseases-projects/open-innovation/drug-discovery-booster/ (accessed 28 September 2018).

Part III

Helminths

7

Onchocerciasis Drug Discovery

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7.1 Introduction

Onchocerciasis or river blindness is a vector-borne disease caused by *Onchocerca volvulus*, a filarial nematode transmitted by blackflies (*Simulium* spp.) [1].

In 1875, Irish naval surgeon John O'Neill recovered what he described as microfilariae from skin samples of local habitants on the Gold Coast (Ghana) who were suffering from "craw craw" [2]. Subsequently, in 1893 Rudolf Leuckart, a German zoologist, described the adult parasites [3] and Rodolfo Robles, a Guatemalan physician, associated these subcutaneous nodules with the eye lesions [4]. In 1926, Donald Blacklock, a Scottish physician, elucidated the full life cycle of *O. volvulus* (Figure 7.1) [5].

7.1.1 The Vector

The prevalent vector of river blindness is the black flies of *Simulium damnosum* complex. The *S. damnosum* complex is composed of several species with similar morphology, but different capacity in transmitting *O. volvulus* L3 larvae. In West Africa, pairs of black flies of the *S. damnosum* complex spread the disease in savannah regions (*S. damnosum–Simulium sirbanum* and *Simulium sanctipauli–Simulium leonese*) and in forest regions (*Simulium squamosum–Simulium yahense*). In East and Central Africa, *Simulium neavei* also transmit the disease in addition to the black flies of the *S. damnosum* complex [6]. *S. damnosum* flies breed in large rivers or in small streams with adequate water flow. Larvae develop in "whitewater" rapids created by exposed rocks, and the female flies feed on humans within a range of a few kilometers surrounding the breeding site. Wind facilitates their flight over several hundred kilometers, allowing proliferation to different breeding sites. In some instances, female flies can also feed on bovines, horses, and small ruminants [7, 8].

Clinical manifestation of river blindness is different in forest areas compared to savannah areas; in the latter, blinding lesions are more severe. This phenomenon

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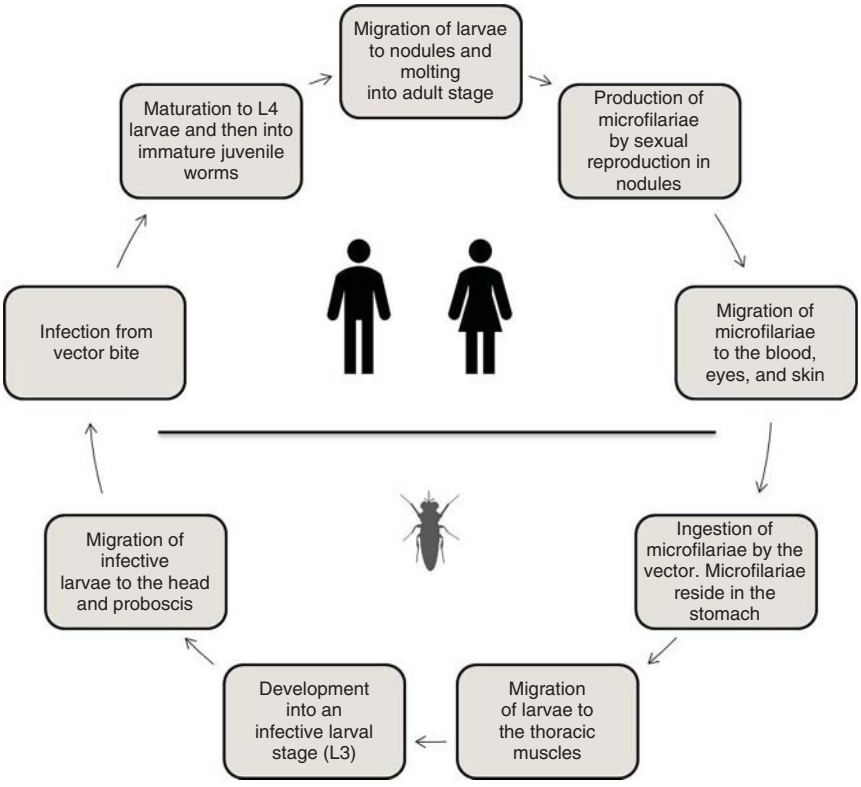


Figure 7.1 Life cycle and transmission of *O. volvulus*.

can be explained by the existence of different strains of *O. volvulus*. The use of PCR techniques, however, established that the vector transmits both strains equally in forest and savannah regions. Another explanation lies in the environmental differences between savannah and forest regions. Savannah regions have a dry atmosphere with exposure to dust, and the increased photophobic effect and ultraviolet radiation may cause exacerbation of clinical symptoms [9–11].

7.1.2 Life Cycle of *O. volvulus*

In onchocerciasis, adult worms (macrofilaria) live in subcutaneous nodules or in subcutaneous tissues, whereas the larval stage (microfilariae) is predominantly present in nodules, upper dermis, and eyes. The microfilariae migrate to these areas from adjacent skin or, in the case of severe infection, from blood, urine, or other body fluids. Transmission to the vector occurs when the microfilariae present in the skin are consumed upon feeding. Few microfilariae are able to migrate from the gut of the blackfly into their thoracic muscles and mature into the infective larvae, L3 stage, via molting twice. The series of molts require 6–12 days, and the subsequent transmission to a human host occurs in the course of the next blood meal. The L3 stage larvae migrate to the subcutaneous tissues where

they develop into adult stage over several months, requiring two additional molts. Mature adult female worms release microfilariae in the skin 10 to 15 months following infection. The estimated life span of an adult female worm is between 9 and 11 years. During this period, it is estimated that 500–1500 microfilariae are produced per day on average by each adult female worm. The severity of an infection in humans can be determined by measuring the number of microfilariae per milligram of skin [7, 8].

7.2 Epidemiology

It is estimated that 198 million people living in the regions of Africa, the Americas, and the Eastern Mediterranean are exposed to transmission of *O. volvulus*. In 2013, a modeling approach approximated that 17 million of those exposed to the parasite developed onchocerciasis. In 2015, onchocerciasis was estimated to contribute 1.1 million disability-adjusted life-years (DALYs).

The control of onchocerciasis relies on preventive chemotherapy (PC) programs analogous to the other neglected tropical diseases (PC-NTDs): lymphatic filariasis, schistosomiasis, soil-transmitted helminths, and trachoma. Onchocerciasis elimination depends on the ivermectin (**IVM**) donation program run by Merck & Co. Merck made this commitment in 1987 and agreed to make available as many treatments as needed for as long as the program was necessary [12].

Prior to 2016, the African Programme for Onchocerciasis Control (APOC) organized and empowered local communities to combat river blindness in their own villages. APOC coordinated community-directed treatment initiatives with **IVM** (CDTI) to relieve clinical symptoms, interrupt transmission, and increase coverage in order to ensure elimination goals. In 2015–2016, a total of 251 million **IVM** treatments were distributed, providing 67% overall coverage in Africa. In 2016, during the 69th session of the World Health Assembly and following the end of APOC, AFRO (the World Health Organization's Regional Office for Africa) announced the launch of the five-year Expanded Special Project for Elimination of Neglected Tropical Diseases (ESPEN). The aim of this project was to provide technical and fundraising support for control and elimination of the five PC-NTDs [13, 14].

The Onchocerciasis Elimination Program for the Americas (OEPA) approved the regional strategic plan of Action for Neglected Infectious Diseases and Post Elimination Actions (CD55/15). The target date for elimination in all regions has been set for 2022. In four countries, elimination has already been verified: Colombia, Ecuador, Mexico, and Guatemala. In the north-east region of the Bolivarian Republic of Venezuela, requirements have been met for posttreatment surveillance, as defined in the 2016 WHO guidelines. The Mass Drug Administration (MDA) program remains active in two foci of the Yanomami Indigenous Territory transmission zone, which is shared with the State of Brazil and the Bolivarian Republic of Venezuela. To achieve elimination goals and expand MDA programs, a mapping of the infection is required. A result of this mapping has been the identification of potential transmission areas such as Yemen that are outside the well-known transmission zone [13, 14].

7.3 Clinical Manifestation

7.3.1 Skin Lesions

The stages of onchocerciasis are classified through a grading system of cutaneous alteration. The recognized categories are acute popular onchodermatitis, chronic popular onchodermatitis, lichenified onchodermatitis, skin atrophy, and skin depigmentation. In many cases, a combination of these categories coexists in the same individual [8, 15]. For example, in Africa, skin lesion and depigmentation are common on the legs but may appear on the entire body. These lesions are caused by death of microfilariae in the skin. Many patients afflicted by onchocerciasis live in endemic regions; therefore, reinfection is constant. As a result, the disease gradually progresses over many years. The lesions determine the severity of the disease. Itching and rash are the most common and earliest clinical manifestation of onchocerciasis. The latter is caused by small abscesses which appear as papules and can cause an intense itching, often resulting in a secondary infection due to scratching. Over time, this condition can cause lichenification or thickening of the skin (lizard skin). Subsequent stages of the disease cause the skin to lose elasticity due to premature aging. Finally, degradation of the dermal collagen and thickening of the epidermis cause loss of pigment called leopard skin [8, 16]. Sowda is a clinical manifestation of river blindness most common in Yemen, but not exclusive to this region. Sowda is a term derived from Arabic, meaning black or dark. It is a local form of onchodermatitis caused by an intense immune response, which is characterized by strong itching. As a result, the skin becomes swollen and dark with scaly papules [8].

7.3.2 Nodules

Formation of granulomas is the immune system's response to confining the adult worms in tissues. These granulomas are called onchocercomata or nodules, which are formed most often in subcutaneous tissues. It is estimated that approximately 25% of nodules form in deep tissues, with sizes ranging from a few millimeters to several centimeters [8, 17]. It is believed that localization of nodules on the body is dependent on the site vector inoculation [18]. In Africa, palpable nodules are commonly found in the pelvic girdle, abdomen, chest wall, head, or limbs; while in Central America, they are typically found on the head. Nodules are believed to be harmless for patients; however, they are perceived as unsightly and are removed surgically whenever possible [7].

7.3.3 Eye Lesions

The eyes are an important target organ for onchocerciasis. Lesions can occur in both the anterior and posterior chamber of the eyeball [7, 8, 19]. Anterior segment lesions, punctate keratitis, or snowflake opacities are the result of acute inflammation caused by microfilariae in the eye. These lesions, which are analogous to papules in the skin, are reversible and often occur in young patients. During the course of the infection, excessive scarring of the cornea occurs, starting

at the limbus, which can result in blindness. Furthermore, the death of microfilariae in the ciliary body causes an inflammation of the iris, iridocyclitis, and formation of synechiae [19]. Posterior segment lesion blindness can be caused due to chronic inflammation of the choroid, called chorioretinitis. The choroid is the vascular layer of the eye, containing connective tissues, lying between the retina and the sclera. Atrophy of the optic nerve causes visual impairment such as decrease of visual acuity or constriction of the visual field. Treatment with diethylcarbamazine (**DEC**) (Figure 7.2), a drug causing rapid death of microfilariae, has been linked with accelerating damage to the optic nerve [19].

7.3.4 Nodding Syndrome

Characterized by the nodding of the head, nodding syndrome affects the neurological system, causing stunted growth and dysfunction of cognitive aptitude. There is no direct correlation between nodding syndrome and individuals infected with onchocerciasis. Nonetheless, this condition has been reported in children between 5 and 15 years in hyperendemic areas for *O. volvulus*, the United Republic of Tanzania, South Sudan, and Uganda [20].

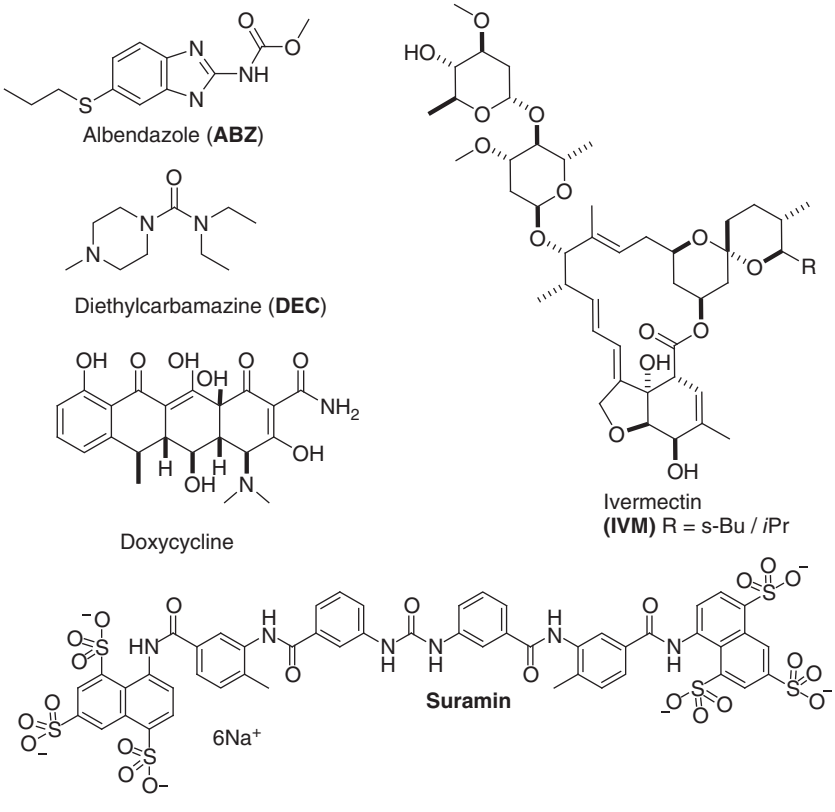


Figure 7.2 Current known therapies for onchocerciasis.

7.4 Diagnostics

7.4.1 Clinical Diagnosis

In regions endemic for onchocerciasis where transmission of *O. volvulus* has not been interrupted by PC programs, the observation of skin lesions, eye lesions, and/or subcutaneous nodules can lead to diagnosis. Onchodermatitis (itching), which is a typical clinical manifestation of onchocerciasis, can be caused by other infections such as *Mansonella streptocerca* or scabies. Infection with *Treponema pallidum* causes yaws, in particular tertiary yaws, superficial mycoses, leprosy, and chronic eczema and can confuse the diagnosis of an *O. volvulus* infection [7, 8].

7.4.2 Ultrasonography

Ultrasonography is a technique allowing the detection of nodules in patient's tissues. This technique can be used to detect deep and non-palpable nodules [21].

7.4.3 Mazzotti Test

In 1948, patients affected by onchocerciasis developed reactions following administration of **DEC**, the Mazzotti reaction. Clinical manifestations of fever, urticaria, swollen and tender lymph nodes, tachycardia, hypotension, arthralgia, edema, and abdominal pain were observed within seven days of administration of **DEC**. During the test, a controlled reaction is induced by either topical or oral administration of a subtherapeutic dose of **DEC** (50 mg for an adult). The severity of the Mazzotti reaction correlates with the intensity of infection [22, 23].

7.4.4 Parasitological Diagnosis

Parasitological diagnosis is based on identification of microfilariae from skin snips. Clinical manifestation of onchocerciasis in patients is often accompanied by positive skin snips; however, this is not always the case. Skin snips are performed either with a needle and a razor blade or with a Walser corneoscleral punch and should not contain any blood. Approximately, 1 mg of sampled skin is immersed in a 0.9% saline solution and within 24 hours microfilariae move out of the sample and are counted using a microscope. *O. volvulus* is differentiated from other microfilariae species which may contaminate the skin snips, such as *Mansonella streptocerca* in Africa, *Mansonella ozzardi* in South America, and other blood microfilariae such as *Wuchereria bancrofti*, *Loa loa*, and *Mansonella perstans* based on its characteristic length (270–320 µm) and shape (head and pointed tail) [24, 25]. The sensitivity of this method depends on the number of performed skin snips, the intensity of the infection, and the optimal location for skin snips. In Africa, the iliac crest or below is the preferred location to sample patients. In Mexico, the deltoid or the scapula regions are more appropriate.

7.4.5 Immunological Tests and PCR

Several immunodiagnostic tests have been developed for onchocerciasis using antigens of different *Onchocerca* host species. Tests based on the detection of antibodies, however, have low sensitivity to *O. volvulus* infection, low specificity to non-helminth infections and cross-reactivity to other helminth infections. Specificity of tests has been improved by the detection of specific IgG4 antibodies and the identification of specific antigens. An ELISA test, using a mixture of *O. volvulus*-specific antigen, Ov16, has demonstrated high specificity and sensitivity. Recently, a rapid diagnostic test package with a positive control for Ov16 was developed with stability under field conditions for over 15 weeks.

Another ELISA assay based on different linear epitopes of the *O. volvulus* motif peptide has been evaluated. The sensitivity, specificity, and cross-reactivity were determined using several panels of clinical isolates. Using a combination of two *O. volvulus* motif peptides, excellent results for sensitivity, specificity, and cross-reactivity were obtained. It was also demonstrated that these peptides were reactive to IgG but not to IgG4; thus, there is no correlation with the Ov16 IgG4, making them promising candidates to complement the existing test [26–28]. An antigen-specific and sensitive, neurotransmitter-derived secretion metabolite from *O. volvulus, N*-acetyltyramine-*O*-glucuronide (NATOG), has been identified in the urine of infected patients, opening up the possibility to develop urine diagnostic tests [29, 30]. *O. volvulus* microfilariae-recovered skin biopsies and third-stage infective larvae, L3, recovered from black flies, can be distinguished from other parasite strains using PCR [26, 31].

7.5 Current Therapies and Approaches

Therapeutic approaches for targeting filarial diseases include utilizing direct-acting agents to target parasitic nematodes, an antibacterial approach to target the endiosymbiotic bacteria, *Wolbachia*, or surgical removal of nodules.

7.5.1 Direct-Acting Approach

Current therapies for filarial diseases rely on three direct-acting agents for MDA of **DEC**, **IVM**, and albendazole (**ABZ**) (Figure 7.2) as either single agents or combinations. The current strategy for controlling human filarial infections is focused on killing microfilariae, larval stage, by repeated administration of **IVM** or **DEC**, usually in combination with **ABZ**. Concerns of serious *Loa loa*-related post-**IVM** serious adverse events and the possible emergence of **IVM**-resistant *O. volvulus* have prompted the need for compounds that exhibit adult-stage selectivity (macrofilaricidal) or long-lasting sterilizing effects. However, due to emergence of drug resistance, there is also a need for new microfilaricides, chemical entities that target the larval stage (microfilariae) [32]. It has been suggested that **IVM** treatments will probably not lead to onchocerciasis elimination in Africa [12]. Future therapies for targeting onchocerciasis include a macrofilaricidal regimen that increases the rate of elimination, is safe to use in co-endemic regions

of *O. volvulus* and *L. loa*, and provides a rapid method to resolve recrudesced infections [33]. An ideal chemotherapy for treatment of onchocerciasis should target the adult stage of the parasite by eliminating adult worms, macrofilaria, or by inducing a long-term sterility by preventing appearance of microfilariae. The preferred target product profile (TPP) should provide a 70–95% macrofilaricidal effect, wherein there is an improvement in clinical signs and symptoms by 12 months. An ideal treatment regimen would be once a day (QD) oral (PO) dosing up to three days; however, twice a day (BID) and up to 14 days of treatment or one single intramuscular (IM) or subcutaneous (SC) injection, biweekly, would be acceptable.

7.5.1.1 Diethylcarbamazine

DEC, a potent microfilaricide with an unknown mechanism of action and with varying degrees of macrofilaricidal activity, has been used extensively to treat lymphatic filariasis since 1947. Loiasis, a rare filariasis that is caused by the nematode worm, *Loa loa*, has become increasingly important as a result of the serious adverse neurological events that were observed when patients infected with *L. loa* underwent treatment for onchoceriasis. The neurological events in a limited patient population treated with **IVM** in areas of Cameroon and the Democratic Republic of Congo (DRC) where loiasis and onchoceriasis are co-endemic included cases of coma, some of which result in encephalopathy, Parkinsonism, or death [34]. Although **DEC** is effective against loaisis, it is not recommended in areas of high levels of *Loa loa* microfilaremia due to severe adverse side effects, including encephalitis, retinal hemorrhage, and Manzotti reaction, all caused by rapid killing of microfilariae [1, 32].

7.5.1.2 Ivermectin

IVM, another potent microfilaricide and a member of the avermectin class, is the standard of care for the treatment of onchocerciasis due to its safety profile [1]. **IVM** functions by binding selectively to glutamate-gated chlorine channels in invertebrate nerve and muscle cells. In a study held in the 1980s [35], it was reported that more than 80% of the microfilariae were cleared from the skin within 48 hours of administration, and low levels of microfilariae were maintained for months before a gradual repopulation occurred. The induction period before repopulation can be explained by temporary sterility of adult females of approximately four months. After one year, it has been observed that the microfilariae population reaches 20% of pretreatment levels [36, 37]. Due to these observations in a study, it was implied that treatment should be repeated annually or biannually [38]. Recent concerns have been raised regarding the suboptimal response to **IVM** treatment. Rapid repopulation of microfilariae in communities after up to 18 years of treatment suggests that **IVM** is unable to suppress reproduction and has reduced efficacy due to emerging drug resistance [39]. Additional investigations were performed, using the analogous host–parasite system *Onchocerca ochengi* in cattle, to determine whether the protective immunity might develop in Africa where transmission has not been eliminated and **IVM**-treated populations continue to be exposed to infective larva (L3). Results showed no evidence of protection, the

three-month **IVM** treatment group was significantly ($P < 0.05$) hypersusceptible and the microfilarial densities and the rate of increase in the microfilarial loads were significantly higher ($P < 0.05$) in the **IVM**-treated groups versus the control animals. These findings raise concerns for **IVM**-based control of human onchocerciasis, suggesting that humans exposed to ongoing transmission in endemic areas while receiving **IVM** are unlikely to develop immunity and will have increased susceptibility to infection upon drug withdrawal [40, 41].

7.5.1.3 Albendazole

ABZ is a broad-spectrum anthelminthic agent of the benzimidazole class commonly used to treat enteric helminthiasis and, more recently, to eliminate lymphatic filariasis. **ABZ** is used primarily in combination with **DEC** or **IVM**. The benzimidazoles target free β-tubulin, an essential protein component of microtubules, leading to the inhibition of tubulin polymerization and loss of cytoplasmic microtubules in worms [42].

7.5.1.4 Suramin

Suramin is a polysulfonated polyaromatic urea (Figure 7.2) developed by Bayer in 1916. Formerly a cancer therapy, suramin has been used extensively for macrofilaricide treatment of African river blindness and African sleeping sickness. The diverse range of biologically important targets that suramin has been reported to inhibit is attributed to its nonspecific mode of binding. As a result, its clinical applications are significantly limited because this leads to side effects and high toxicity. Recently, the use of suramin for treatment of onchocerciasis was halted due to safety concerns [43, 44].

7.5.2 Antibacterial Approach

Targeting *Wolbachia* endobacteria in filarial species has emerged as a new promising target for the treatment of human filariasis [45, 46]. *O. volvulus* harbors rickettsial endobacteria, of the genus *Wolbchia*, as symbionts, while *L. loa* does not harbor these endosymbionts, and, therefore, would not lead to adverse events following treatment [47–50]. It has been reported that use of tetracycline-based antibiotics results in the slow death of adult worms [51]. It has also been demonstrated in animal experiments with *O. ochengi* that the elimination of this endobacteria causes an inhibition of embryogenesis and a macrofilaricidal effect [52]. Trials with human onchocerciasis patients using doxycycline (**DOX**), an antibiotic that is used in the treatment bacterial infections, demonstrated a long-term sterilizing activity [53].

7.5.2.1 Tetracycline Derivatives

Administration of **DOX** (100 mg/d for six weeks) causes elimination of endobacteria, decreased insemination of female worms, and an interruption of embryogenesis [54]. This was observed for up to 18 months, the longest period of female sterility achieved by an antifilarial drug without severe side effects [46, 55]. Although the new strategy showed promising results, **DOX** is limited in its use because it is contraindicated in children under the age of nine and

women who are pregnant or breastfeeding [56]. Minocycline (**MIN**), another broad-spectrum tetracycline antibiotic, also showed depletion of *Wolbachia* in *Wolbachia* containing the C6/36 insect cell line. Recent clinical data reported have suggested that **MIN** (200 mg/d for three weeks) may be more efficacious than **DOX** (200 mg/d for three weeks), since **MIN** showed an improved trend for the absence of *Wolbachia* and the inhibition of normal embryogenesis. These differences, however, were not statistically significant, as the pilot trial was not sufficiently designed in power to detect such differences [57].

7.5.3 Nodulectomy

Nodulectomy is the surgical removal of nodules. The symptoms of onchocerciasis cannot be controlled through nodulectomy due to the difficulty in completely removing all of the nodules. Some nodules are impalpable because they are located in deep tissues and a systematic removal of nodules by surgery would not be feasible in large-scale endemic areas. Although nodulectomy is generally not effective for treatment of disease symptoms, it is often performed for cosmetic reasons or because of nodule location [8].

7.6 Discovery Models

Testing of compounds for macrofilaricidal activity, against adult parasites, has progressed significantly in recent years through refinement of culture conditions and implementation of high-throughput automated imaging. However, a major challenge for drug discovery is finding suitable preclinical animal models since the parasite can only develop fully in humans and primates. The only known permissive hosts for the human parasite, *O. volvulus*, are chimpanzees and mangabey monkeys. This requires the use of surrogate parasites for both *in vitro* and *in vivo* evaluation.

7.6.1 Primary *In Vitro* Assays

Compounds are assessed *in vitro* on filarial worm motility, migration, feeding, and development as well as on worm viability using MTT (3-(4,5-dimethylthiazola-2-yl)-2, *S*-diphenyl tetrazolium bromide) [58]. Based on previous reports, *Brugia* spp., such as *B. malayi*, a human pathogen, and *B. pahangi* along with *Litomosoides sigmodontis*, are the most suitable sources of viable microfilariae and adult worms for high-throughput screening. Surrogate parasites for *O. volvulus* include *onchocerca* spp., such as *O. gutturosa* and *O. ochengi*, with *O. ochengi* being the closest relative to the human parasite in that it forms nodules that resemble those of *O. volvulus* [59].

To determine depletion of *Wolbachia*, whole-organism cell-based assays with a *Wolbachia*-containing *Aedes albopictus* cell line (C6/36 Wp) are used as the primary *in vitro* assay [60]. *In vitro* counter screening, using either adult *O. gutturosa* or *B. malayi*, determine direct-acting effects.

7.6.2 *In Vivo* Efficacy Models

For *in vivo* studies, established animal models of filarial infection utilize *L. sigmodontis* in mice or Mongolian jirds [61, 62] and *B. malayi* in jird [63]. Patent infections with circulating microfilariae can be established in the Mongolian jird (*Meriones unguiculatus*) [64]. The *in vivo* models with *B. malayi* or *L. sigmodontis* in jirds allow for evaluation of macrofilaricidal activity, effects on female fertility, and microfilarial production [59].

For models of *onchocerca*, *O. ochengi*, the cattle, its natural host, forms nodules that closely resemble those of *O. volvulus* and can be enumerated noninvasively or removed for analysis during studies [59]. Recently, there have been reports of small animal models utilizing *O. ochengi* infections [65, 66].

In all *in vivo* models, the reduction of *Wolbachia* load following treatment is measured by qPCR. For anti-*Wolbachia* depletion, *in vivo* screening using *L. sigmodontis* in mice yields a visible and quantifiable phenotype of larvae with retarded growth. Further evaluation in jirds assesses the reduction of the *Wolbachia* load as a predictor of macrofilaricidal activity [45].

7.7 Medicinal Chemistry Approaches

Current efforts toward finding new therapies for onchocerciasis have been primarily focused on reevaluating known molecules.

7.7.1 Benzimidazoles

7.7.1.1 Flubendazole (FLBZ)

FLBZ (Figure 7.3), a methylcarbamate benzimidazole (**BZD**), was initially developed as a paste by Janssen Pharmaceutica N.V. for protection against gastrointestinal nematodes in dogs and cats. **FLBZ** demonstrates lethal effects on many filarial species in animal host models and is reported to be the best macrofilaricidal molecule within the benzimidazole group [67]. Approved for human use in Europe, **FLBZ** is a good candidate for therapy against filariasis. Unfortunately, like other benzimidazoles, **FLBZ** has limited water solubility, and the commercially available oral human administration as tablets or suspensions provides low systemic bioavailability [68]. In 2012, Janssen and several partners, including Drugs for Neglected Diseases *initiative* (DND*i*) and AbbVie, reported investigating an improved formulation of **FLBZ** as a macrofilaricide with enhanced bioavailability in blood and tissues as a potential treatment of parasitic diseases that cause lymphatic filariasis and onchocerciasis [69]. Efforts to improve bioavailability have been reported. The systemic exposure of **FLBZ**, formulated as either aqueous hydroxypropyl-β-cyclodextrin (CD) or aqueous carboxymethyl cellulose (CMC) suspension or a Tween 80-based formulation (TWEEN) in rats and jirds (*Meriones unguiculatus*), was evaluated. **FLBZ**, 5 mg/kg, was administered by both the oral or subcutaneous routes, and it was shown that it was hydrolyzed (**H-FLBZ**) and reduced (**R-FLBZ**) (Figure 7.3). Both metabolites were recovered in the plasma samples collected from rats

and jirds treated with the different **FLBZ** formulations. In rats, **FLBZ** systemic exposure (AUC0-LOQ) was significantly higher after the **FLBZ**-CD treatments, both orally (4.8 ± 0.9 mg h/ml) and subcutaneously (7.3 ± 0.6 mg h/ml) when compared to oral administration of **FLBZ**-CMC suspension (0.93 ± 0.2 mg h/ml). Similar differences were observed in jirds. It was concluded that the enhanced systemic exposure is observed with the CD-based formulations and has significant therapeutic implications for a drug with poor or erratic bioavailability [67].

Preclinical data showed that **FLBZ** dosed orally at 0.2, 0.6, and 1.5 mg/kg, decreased the number of *B. malayi* microfilariae in *Meriones unguiculatus* by 43%, 61%, and 68%, respectively, compared to untreated animals. **FLBZ** was also reported to show significant reductions in motile worm burdens in a SCID mouse model implanted with *O. ochengi* male worms when treated with either five daily SC injections of standard **FLBZ** formulation (10 mg/kg) or a single SC injection (10 mg/kg) [70, 71].

FLBZ interferes with microtubules and has reproductive toxicity [72]. It is reported that embryotoxicity had been observed at concentrations above 0.25 µg/ml *in vivo* [73–75]. The assessment of the results of the preclinical toxicology studies will determine whether an oral formulation can be developed clinically for MDA. Current information on whether, and when, an oral formulation of **FLBZ** has, or is expected to enter clinical development, is not publicly available at this time.

7.7.1.2 UMF-078

The University of Michigan researchers have reported 5-substituted benzimidazole carbamate ester compounds as soluble analogs of **FLBZ** [76]. It was reported that **UMF-078**, methyl (±)-[5-[amino(4-fluorophenyl) methyl]-1*H*-benzimidazol-2-yl]-carbamate (Figure 7.3), cured *B. pahangi*-infected dogs when administered by IM injection over doses ranging from 12.5–50 mg/kg/d for two to three days. In addition, the dihydrochloride hydrate

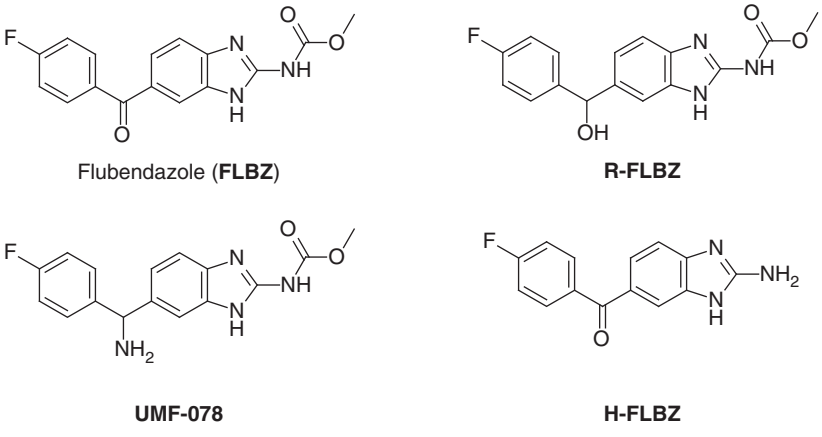


Figure 7.3 Benzimidazole analogs reported by Janssen and the University of Michigan.

salt, **UMF-289**, cured *B. pahangi*-infected dogs when administered orally at a dose of 200 mg/kg/d (155 mg/kg/d base equivalent) for one, three, or five days in 10% suspension in HEC-Tween 80. In contrast to IM injection of **FLBZ**, **UMF-078** does not cause inflammation at the injection site. Administered orally, **UMF-078** is rapidly absorbed, and gives higher plasma levels relative to IM dosing. **UMF-289** has an enhanced absorption rate with a significantly shorter T_{max} than the freebase, but it does not increase the extent of absorption [77]. When delivered at 150 mg/kg IM, **UMF-078** is a potent macrofilaricide against *O. ochengi*. Due to data indicating that benzimidazoles can induce genotoxicity and cytotoxicity, the development of **UMF-078** has been halted [78].

7.7.1.3 Boron-Derived Benzimidazoles

Anacor (a subsidiary of Pfizer) reported several small-molecule boron-based molecules for the potential treatment of river blindness. Fifteen compounds (Figure 7.4) have been exemplified in this class, and were shown to kill adult worms of *B. malayi* in one to two days at 10 mM *in vitro*. Compounds **J13** and **J15** were exemplified, with **J15** being one of the most potent compounds reported, killing 100% of the worms in 24 hours when administered at 0.01 mM [79]. No further data on the oxaboroles has been disclosed to date.

7.7.2 Macrocyclic Lactones

7.7.2.1 Milbemycins

Milbemycins were discovered prior to the avermectins, but their anthelmintic properties were realized only after that of the avermectins [80]. One member of this class is moxidectin (Figure 7.5), a macrocyclic lactone glutamate-gated chloride channel (GuCl) binder, approved for veterinary medicine. The milbemycins are structurally related to the avermectins; however, they differ by the absence of the R-L-oleandrosyl-R-L-oleandrosyl disaccharide at position 13 and aliphatic substitutions at position 25. The spectrum of biological activity of moxidectin is derived from its unique methoxine and dimethylbutenyl side chains. Moxidectin has shown macrofilaricidal activity in animal models of onchocerciasis and lymphatic filariasis. Preclinical studies have shown that a single treatment produces slow death of adult worms in birds and dogs, and sterilization of worms in cattle. Compared to **IVM**, moxidectin has a considerably longer half-life in plasma of 20 days compared to 2 days, allowing for either less frequent treatment or improved efficacy with similar frequency of treatment compared to **IVM**. It is also effective in animal helminth infections that are resistant to **IVM** [81]. Medicines Development for Global Health (MDGH), through the Global Health Investment Fund

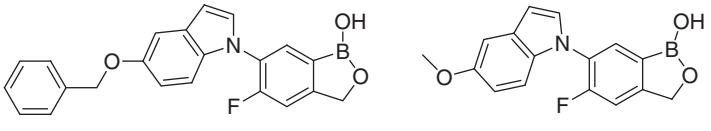


Figure 7.4 Benzoxaborole derivative analogs reported by Anacor.

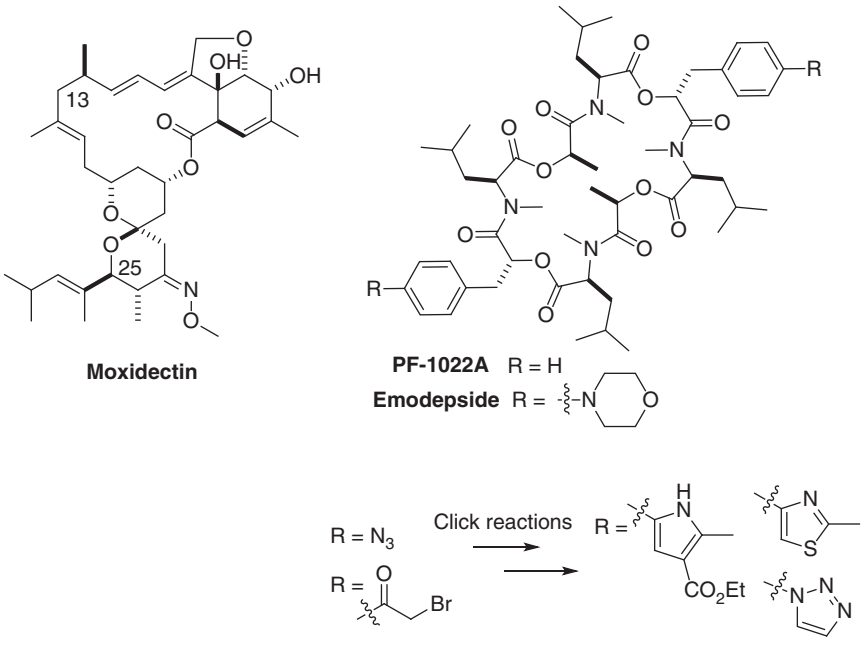


Figure 7.5 Milbemycin and cyclooctadepsipeptide analogs.

(GHIF) program, reported development of moxidectin as a potential oral treatment of African river blindness [72].

In March 2012, a phase III study comparing the efficacy, safety, and tolerability of moxidectin and **IVM** in subjects infected with *O. volvulus* was completed. The data suggested that moxidectin was efficacious and had a safety profile consistent with MDA. In November 2015, further clinical data were reported. Using skin microfilariae density (microfilariae per milligram of skin) 12 months posttreatment as the primary efficacy outcome, the moxidectin group (adjusted geometric mean 0·6 [95% CI 0·3–1·0]) was lower than that in the **IVM** group (4·5 [3·5–5·9]; difference 3·9 [3·2–4·9], $p < 0·0001$; treatment difference 86%) concluding that moxidectin was shown to be superior to **IVM** [38].

7.7.2.2 Cyclooctadepsipeptides

Emodepside (**BAY 44-4400**) is a semisynthetic analog of **PF-1022A**, a metabolite of *Mycelia sterilia* (Rosselinia sp.). Both emodepside and **PF-1022A** (Figure 7.5) are 24-membered cyclooctadepsipeptides with known anthelmintic activity [82]. Structure–activity relationships (SARs) suggested that incorporation of basic groups at the para position of the phenylalanine moieties in **PF-1022A** leads to improved *in vivo* anthelmintic activity. Incorporation of the morpholine group into **PF-1022A** led to emodepside [80].

Emodepside is registered in Europe and the United States as a combination product for the treatment of parasitic diseases in companion animals. Recently, there has been interest in the use of emodepside for the treatment of human helminthiases due to its potency against adult filarial worms. Emodepside

paralyzes adult filarial worms, via a mode of action distinct from previous anthelmintics, and therefore is of interest as a new treatment for onchocerciasis [72]. It is a novel modulator of the BK/SLO-1 Ca^{2+}-activated K^+ channels in nematodes, insects, and humans [83]. In 2014, DND*i* and Bayer HealthCare completed a legal agreement for collaboration on development of emodepside. A first-in-human (FIH) double-blind, placebo-controlled study of single ascending doses in healthy Caucasian men has been completed with favorable preliminary results. A repeat dose study was completed in March 2017, and evaluated pharmacokinetic, safety, and tolerability of the liquid service formulation given over 10 days [84].

Second-generation anthelmintic cyclooctadepsipeptides were recently reported (Figure 7.5). Focusing on modifications to para position of phenylalanine residues in **PF-1022A** was a means to improve pharmacological properties. Click reactions such as the Paal–Knorr and Hantzsch syntheses or the Huisgen azide-alkyne cycloaddition were utilized in order to replace the morpholine of emodepside with heteroaromatic-substituted phenyl ring systems such as phenylpyrrole, phenylthiazole, and phenyltriazole. New heteroaromatic-substituted **PF-1022A** analogs, which appear to be structurally similar to emodepside, were disclosed; however, no data to support potential improved anthelmintic and/or pharmacological properties have been provided [82].

7.7.2.3 Tylosins

AbbVie reported identification of Tylosin A (**TylA**) through screening of its representative anti-infectives collection. Tylosin A (Figure 7.6) is a veterinary antibiotic with potent anti-*Wolbachia* activity, EC_{50} = 24–36 nM. Tylosin A (50 mg/kg/d) shows anti-*Wolbachia* efficacy, 99.9%, when dosed intraperitoneally (IP) for 14 days, but it is not sufficiently effective when dosed PO, 50.4%. **TylA** is an effective macrofilaricidal agent; however, the pharmacokinetic properties of **TylA** make it unsuitable for oral administration. Medicinal chemistry efforts were focused on improving drug exposure upon oral delivery while maintaining anti-*Wolbachia* activity. Over 150 4″-0-substituted **TylA** analogs were reported with an increase in potency of over 1000-fold over **TylA** [85].

In the *Brugia malayi* larval infection mouse model, **A-1535469** reduced the *Wolbachia* load by 99.9% at two weeks postinfection (wpi), when dosed QD at 50 mg/kg/d for 14 days. The *Wolbachia* load was also reduced by 99.8% at a lower a dose of 25 mg/kg/d. A 99.8% reduction of *Wolbachia* was still observed at the same dose when treatment duration was reduced to seven days. These results are superior when compared to the standard of care, doxycycline (50 mg/kg/d for 14 days, 98.3% reduction). **ABBV-4083** reduced *Wolbachia* by 99.5% at 2wpi, when dosed PO at 50 mg/kg/d for seven days. A reduction of 97.9% was observed at the lower dose of 25 mg/kg/d. Both compounds are superior to doxycycline, which yielded a 77.4% reduction dosing at 50 mg/kg/d for seven days.

In a preclinical lymphatic filariasis model, the adult *Brugia malayi* jird model, PO administration of **ABBV-4083** at both 10 and 50 mg/kg daily for 14 days resulted in a >90% reduction in *Wolbachia*. A dose-dependent reduction of 70.1%

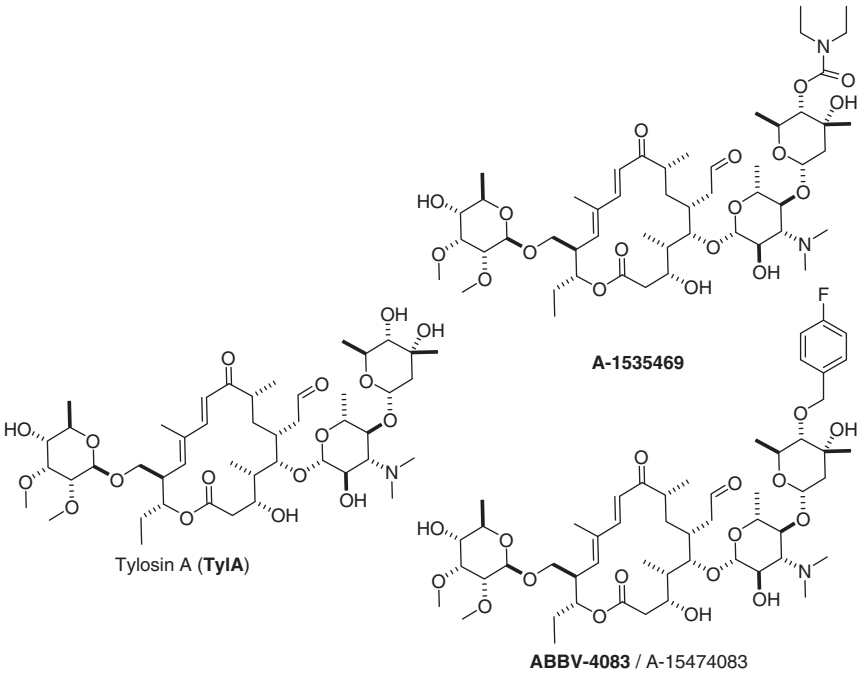


Figure 7.6 Tylosin analogs reported by AbbVie.

and 99.6% was observed in motile microfilariae recovered from the peritoneum with 10 and 50 mg/kg daily PO treatment, respectively [85].

In the preclinical onchocerciasis model, the *O. ochengi* model, adult male mice were treated with **ABBV-4083** (75 mg/kg QD, seven days) **and A-1535469** (250 mg/kg QD, 14 days) in separate studies, reducing *Wolbachia* loads (97.2% and 99.7%, respectively) in a shortened dose time frame relative to the tetracyclines. Both compounds demonstrated the potential of a shorter course of treatment over minocycline, and doxycycline [85].

In an *ex vivo* assay of microfilaricidal assessment against changes in *L. Ioa* microfilariae motility, **ABBV-4083** and **A-1535469** showed no effect in cessation of microfilariae motility when compared with **IVM**, which rendered microfilariae completely immotile after a period of 7 days and induced >90% reduction in motility in +2 days. It can be concluded that **ABBV-4083** and **A-1535469** are ineffective against *L. Ioa* microfilariae, allowing for safe treatment of patients coinfected with loaisis and onchocerciasis or lymphatic filariasis [85, 86].

7.7.3 Natural Products

7.7.3.1 Corallopyronin A

Investigation of natural products as potential new anti-*Wolbachia* agents revealed corallopyronin A (**Cor A**, Figure 7.7). Corallopyronin A is a non-competitive inhibitor of bacterial DNA-dependent RNA polymerase (RNAP) which is active against gram-positive bacteria. In the C6/36 cell line infected

with *A. albopictus Wolbachia*, corallopyronin A (1 μg/ml) depleted *Wolbachia* to levels equivalent to that of doxycycline (4 μg/ml). In BALB/c mice infected with *L. sigmodontis*, treatment with **Cor A** for 28 days at 35 mg/kg/d resulted in a >99.9% reduction in the *Wolbachia* load (4.7-log reduction) compared to the control and a larger reduction than doxycycline (3.9-log reduction). The phenotype of significantly shorter worm length is indicative of effective therapy and the blocking of larval development and was seen in mice treated with **Cor A**. **Cor A**, at this dosing regimen and a shorter time, was equivalent to treatment with doxycycline, at a higher dose. It was also reported that **Cor A** at 35 mg/kg/d for 28 days was well tolerated by the mice, with no visually apparent toxic effects [87–89].

SAR studies suggested that for myxopyronin A, changes in the left-side portion of the molecule, which is identical to that of corallopyronin A, result in diminished antibacterial effects. It has been reported that the left-side portion of the molecule interacts with the target RNAP in multiple ways and is sensitive to structural changes. The improved antibiotic activity of corallopyronin A in comparison to myxopyronin A has been attributed to the longer lipophilic left-side chain of corallopyronin A, which occupies an extra binding pocket in the RNAP. Metabolites and semisynthetic analogs of corallopyronin A were prepared and evaluated, and revealed that modifications at the hydroxy group at carbon C-24 on the left-side chain of **Cor A** altered binding at the target site. Acylation and oxidation of the C-24 hydroxyl group resulted in less active or inactive molecules. However, precorallopyronin A (**PreCor A)**, the des-OH analog of **Cor A**, demonstrated *in vitro* activity against *Wolbachia* that was comparable to that of **Cor A**, suggesting that the OH group at C-24 minimally enhances the antibiotic activity of **Cor A**. Replacement of the central pyrone ring with the open-form analog, methyl-*N*-(6-[sphingosyl-*N*-carbonyl]hexyl)carbamate (Figure 7.7), resulted in no antibacterial activity, showing that the pyrone ring is a critical feature for

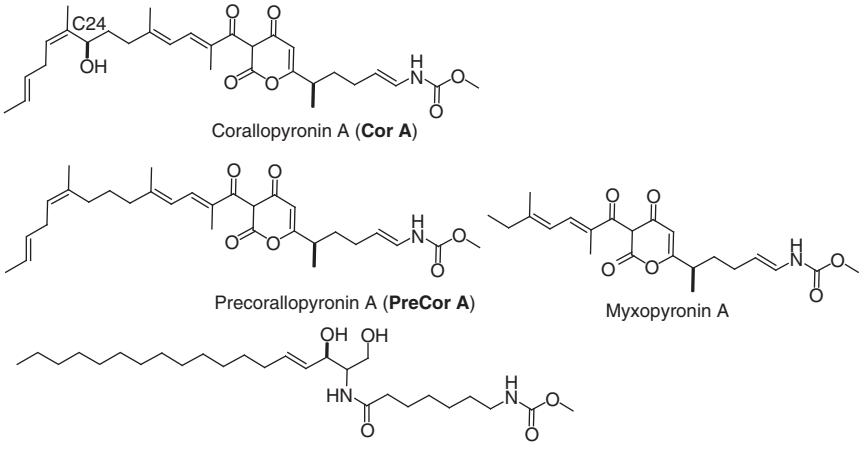
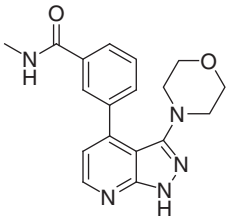


Figure 7.7 Structures of corallopyronins.



AWZ-8051

Figure 7.8 7-Amino pyrazolopyridine analog.

arranging **Cor A** in the binding pocket. *In silico* analyses of **Cor A** and **PreCor A** in the RNAP binding pocket were performed to explain the antimicrobial effects. Any increase in bulk or rigidity off the left-side chain results in loss of activity due to the tight binding pocket of the RNAP target [90]. **Cor A** appears to be an attractive target for further development as an anti-*Wolbachia* antibiotic for the control and elimination of filarial nematode infections.

7.7.4 Small Molecules

7.7.4.1 Pyrazolopyridine

Limited data has been reported on a series of 7-amino-pyrazolopyridine analogs as potential treatment for lymphatic filariasis and onchocerciasis [91]. Pyrazolopyridine analog AWZ-8051 (Figure 7.8) shows activity against *Wolbachia*, $EC_{50} = 19$ nM. It was reported that AWZ-8051 has a preferred pharmacokinetic profile (2 mg/kg IV, 50 mg/kg PO) in SCIDD mice, Cl = 2.6 l/h/kg, $V_{dss} = 1.8$ l/kg, $t_{1/2} = 1.3$ h, $C_{max} = 15\,833$ ng/ml, $T_{max} = 0.81$ h, and AUC (0 to t) = 44 980 ng h/ml. No further data regarding *in vivo* efficacy has been reported.

7.8 Conclusion

The current strategies for elimination of onchocerciasis have limitations since the standard treatments are ineffective against the macrofilaria, or adult worms, and require repeated and prolonged treatments. This is also further complicated by the threat of emerging resistance. The need for a novel macrofilaricide is imperative to achieve eradication of this disease. However, given the threat of drug resistance, the need for novel microfilaricides is equally important and should not be discounted. Future therapies for targeting onchocerciasis should include a macrofilaricidal regimen that increases the rate of elimination and is safe to use in co-endemic regions of *O. volvulus* and *L. loa*.

References

1 Kati yar, D. and Singh, L.K. (2011). Filariasis: current status, treatment and recent advances in drug development. *Curr. Med. Chem.* 18 (14): 2174–2185.
2 O'Neill, S.J. (1875). On the presence of a filaria in "craw-craw". *Lancet* 105 (2686): 265–266.

3 Heyneman, D., Karapelou, J.W., Grave, E.V. et al. (2012). *Parasitic Diseases*. New York: Springer.
4 Richards, F. Jr., Rizzo, N., Diaz Espinoza, C.E. et al. (2015). One hundred years after its discovery in Guatemala by Rodolfo Robles, *Onchocerca volvulus* transmission has been eliminated from the central endemic zone. *Am. J. Trop. Med. Hyg.* 93 (6): 1295–1304.
5 Cox, F.E.G. (2017). The Golden Age of parasitology-1875-1925: the Scottish contributions. *Parasitology* 144 (12): 1567–1581.
6 WHO (1995). Onchocerciasis and its control. Report of a WHO Expert Committee on Onchocerciasis control. *World Health Organ. Tech. Rep. Ser.* 852: 1–104.
7 Selendy, J.M.H. (2011). *Water and Sanitation-Related Diseases and the Environment: Challenges, Interventions, and Preventive Measures*. Wiley.
8 Cook, G.C. and Zumla, A. (2009). *Manson's Tropical Diseases*. Saunders.
9 Higazi, T.B., Filiano, A., Katholi, C.R. et al. (2005). Wolbachia endosymbiont levels in severe and mild strains of *Onchocerca volvulus*. *Mol. Biochem. Parasitol.* 141 (1): 109–112.
10 Toe, L., Tang, J., Back, C. et al. (1997). Vector-parasite transmission complexes for onchocerciasis in West Africa. *Lancet* 349 (9046): 163–166.
11 Zimmerman, P.A., Dadzie, K.Y., De Sole, G. et al. (1992). Onchocerca volvulus DNA probe classification correlates with epidemiologic patterns of blindness. *J. Infect. Dis.* 165 (5): 964–968.
12 Boussinesq, M. (2008). Onchocerciasis control: biological research is still needed. *Parasite* 15 (3): 510–514.
13 WHO (2017). 681 Progress report on the elimination of human onchocerciasis, 2016-2017. *Wkly Epidemiol. Rec.* 92 (45): 681–694.
14 WHO (2016). Guidelines for stopping mass drug administration and verifying elimination of human onchocerciasis: criteria and procedures. In *Guidelines for Stopping Mass Drug Administration and Verifying Elimination of Human Onchocerciasis: Criteria and Procedures*, Geneva.
15 Murdoch, M.E., Asuzu, M.C., Hagan, M. et al. (2002). Onchocerciasis: the clinical and epidemiological burden of skin disease in Africa. *Ann. Trop. Med. Parasitol.* 96 (3): 283–296.
16 Murdoch, M.E., Hay, R.J., Mackenzie, C.D. et al. (1993). A clinical classification and grading system of the cutaneous changes in onchocerciasis. *Br. J. Dermatol.* 129 (3): 260–269.
17 Leichsenring, M., Troger, J., Nelle, M. et al. (1990). Ultrasonographical investigations of onchocerciasis in Liberia. *Am. J. Trop. Med. Hyg.* 43 (4): 380–385.
18 Browne, S.G. (1961). The localization of onchocercomata. *Trans. R. Soc. Trop. Med. Hyg.* 55 (3): 258–262.
19 Abiose, A. (1998). Onchocercal eye disease and the impact of Mectizan treatment. *Ann. Trop. Med. Parasitol.* 92 (Suppl. 1): S11–S22.
20 Colebunders, R., Hendy, A., and van Oijen, M. (2016). Nodding syndrome in onchocerciasis endemic areas. *Trends Parasitol.* 32 (8): 581–583.
21 Mand, S., Marfo-Debrekyei, Y., Debrah, A. et al. (2005). Frequent detection of worm movements in onchocercal nodules by ultrasonography. *Filaria J.* 4 (1): 1.

22 Ozoh, G., Boussinesq, M., Bissek, A.C. et al. (2007). Evaluation of the diethylcarbamazine patch to evaluate onchocerciasis endemicity in Central Africa. *Trop. Med. Int. Health* 12 (1): 123–129.

23 Boatin, B.A. and Richards, F.O. Jr. (2006). Control of onchocerciasis. *Adv. Parasitol.* 61: 349–394.

24 Neafie, R.C. (1972). Morphology of *Onchocerca volvulus*. *Am. J. Clin. Pathol.* 57 (5): 574–586.

25 Duke, B.O. (1962). A standard method of assessing microfilarial densities on onchocerciasis surveys. *Bull. World Health Organ.* 27 (4-5): 629–632.

26 Lagatie, O., Verheyen, A., Nijs, E. et al. (2018). Evaluation of the diagnostic performance of *Onchocerca volvulus* linear epitopes in a peptide enzyme-linked immunosorbent assay. *Am. J. Trop. Med. Hyg.* 98 (3): 779–785.

27 Golden, A., Stevens, E.J., Yokobe, L. et al. (2016). A recombinant positive control for serology diagnostic tests supporting elimination of *Onchocerca volvulus*. *PLoS Negl. Trop. Dis.* 10 (1): e0004292.

28 Golden, A., Faulx, D., Kalnoky, M. et al. (2016). Analysis of age-dependent trends in Ov16 IgG4 seroprevalence to onchocerciasis. *Parasit. Vectors* 9 (1): 338.

29 Globisch, D., Specht, S., Pfarr, K.M. et al. (2015). Litomosoides sigmodontis: a jird urine metabolome study. *Bioorg. Med. Chem. Lett.* 25 (24): 5804–5807.

30 Globisch, D., Moreno, A.Y., Hixon, M.S. et al. (2013). *Onchocerca volvulus* – neurotransmitter tyramine is a biomarker for river blindness. *Proc. Natl. Acad. Sci. U. S. A.* 110 (11): 4218, S4218/1–4223, S4218/8.

31 Lagatie, O., Batsa Debrah, L., Debrah, A., and Stuyver, L.J. (2017). Plasma-derived parasitic microRNAs have insufficient concentrations to be used as diagnostic biomarker for detection of *Onchocerca volvulus* infection or treatment monitoring using LNA-based RT-qPCR. *Parasitol. Res.* 116 (3): 1013–1022.

32 Townson, S., Ramirez, B., Fakorede, F. et al. (2007). Challenges in drug discovery for novel antifilarials. *Expert Opin. Drug Discovery* 2 (s1): S63–S73.

33 Geary, T.G. (2016). A step toward eradication of human filariases in areas where Loa is endemic. *MBio* 7 (2): e00456–e00416.

34 Holmes, D. (2013). Loa loa: neglected neurology and nematodes. *Lancet Neurol.* 12 (7): 631–632.

35 Whitworth, J.A., Morgan, D., Maude, G.H., and Taylor, D.W. (1988). Community-based treatment with ivermectin. *Lancet* 2 (8602): 97–98.

36 Walker, M., Walker, M., Basanez, M.-G. et al. (2017). Macrofilaricidal efficacy of repeated doses of ivermectin for the treatment of river blindness. *Clin. Infect. Dis.* 65 (12): 2026–2034.

37 Gardon, J., Boussinesq, M., Kamgno, J. et al. (2002). Effects of standard and high doses of ivermectin on adult worms of *Onchocerca volvulus* : a randomised controlled trial. *Lancet* 360 (9328): 203–210.

38 Opoku, N.O., Bakajika, D.K., Kanza, E.M. et al. (2018). Single dose moxidectin versus ivermectin for *Onchocerca volvulus* infection in Ghana, Liberia, and the Democratic Republic of the Congo: a randomised, controlled, double-blind phase 3 trial. *Lancet* 392 (10154): 1207–1216.

39 Lustigman, S. and McCarter, J.P. (2007). Ivermectin resistance in *Onchocerca volvulus* : toward a genetic basis. *PLoS Negl.Trop. Dis.* 1 (1): e76.

40 Glickman, L.T., Glickman, N.W., Moore, G.E. et al. (2006). Comparative effectiveness of sustained-release moxidectin (proheart 6) and ivermectin (heartgard plus) for the prevention of heartworm infection in dogs in the United States. *Int. J. Appl. Res. Vet. Med.* 4 (4): 339–354.

41 Njongmeta, L.M., Nfon, C.K., Gilbert, J. et al. (2004). Cattle protected from onchocerciasis by ivermectin are highly susceptible to infection after drug withdrawal. *Int. J. Parasitol.* 34 (9): 1069–1074.

42 Taman, A. and Azab, M. (2014). Present-day anthelmintics and perspectives on future new targets. *Parasitol. Res.* 113 (7): 2425–2433.

43 McGeary, R.P., Bennett, A.J., Tran, Q.B. et al. (2008). Suramin: clinical uses and structure-activity relationships. *Mini Rev. Med. Chem.* 8 (13): 1384–1394.

44 Zaniboni, A. (1990). Suramin: the discovery of an old anticancer drug. *Med. Oncol. Tumor Pharmacother.* 7 (4): 287–290.

45 Taylor, M.J., Hoerauf, A., Townson, S. et al. (2014). Anti-Wolbachia drug discovery and development: safe macrofilaricides for onchocerciasis and lymphatic filariasis. *Parasitology* 141 (1): 119–127.

46 Hoerauf, A., Adjei, O., and Buttner, D.W. (2002). Antibiotics for the treatment of onchocerciasis and other filarial infections. *Curr. Opin. Invest. Drugs* 3 (4): 533–537.

47 Taylor, M.J., Voronin, D., Johnston, K.L., and Ford, L. (2013). Wolbachia filarial interactions. *Cell Microbiol.* 15 (4): 520–526.

48 Taylor, M.J. and Hoerauf, A. (2001). A new approach to the treatment of filariasis. *Curr. Opin. Infect. Dis.* 14 (6): 727–731.

49 Townson, S., Hutton, D., Siemienska, J. et al. (2000). Antibiotics and Wolbachia in filarial nematodes: antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against *Onchocerca gutturosa*, *Onchocerca lienalis* and *Brugia pahangi*. *Ann. Trop. Med. Parasitol.* 94 (8): 801–816.

50 Hoerauf, A., Volkmann, L., Hamelmann, C. et al. (2000). Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. *Lancet* 355 (9211): 1242–1243.

51 Turner, J.D., Tendongfor, N., Esum, M. et al. (2010). Macrofilaricidal activity after doxycycline only treatment of *Onchocerca volvulus* in an area of Loa loa co-endemicity: a randomized controlled trial. *PLoS Negl. Trop. Dis.* 4 (4): e660.

52 Johnston, K.L., Ford, L., Umareddy, I. et al. (2014). Repurposing of approved drugs from the human pharmacopoeia to target Wolbachia endosymbionts of onchocerciasis and lymphatic filariasis. *Int. J. Parasitol. Drugs. Drug Resist.* 4 (3): 278–286.

53 Walker, M., Specht, S., Churcher, T.S. et al. (2015). Therapeutic efficacy and macrofilaricidal activity of doxycycline for the treatment of river blindness. *Clin. Infect. Dis.* 60 (8): 1199–1207.

54 Hoerauf, A., Specht, S., Buttner, M. et al. (2008). Wolbachia endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study. *Med. Microbiol. Immunol.* 197 (3): 295–311.

55 Hoerauf, A., Mand, S., Volkmann, L. et al. (2003). Doxycycline in the treatment of human onchocerciasis: kinetics of Wolbachia endobacteria reduction and of inhibition of embryogenesis in female Onchocerca worms. *Microbes Infect.* 5 (4): 261–273.

56 Taylor, M.J., Ford, L., Hoerauf, A. et al. (2012). Drugs and targets to perturb the symbiosis of Wolbachia and filarial nematodes. *Drug Discovery Infect. Dis.* 3 (Parasitic Helminths): 251–265.

57 Klarmann-Schulz, U., Specht, S., Debrah, A.Y. et al. (2017). Comparison of doxycycline, minocycline, doxycycline plus albendazole and albendazole alone in their efficacy against onchocerciasis in a randomized, open-label, pilot trial. *PLoS Negl. Trop. Dis.* 11 (1): e0005156/1–e0005156/22.

58 Marcellino, C., Gut, J., Lim, K.C. et al. (2012). WormAssay: a novel computer application for whole-plate motion-based screening of macroscopic parasites. *PLoS Negl. Trop. Dis.* 6 (1): e1494.

59 Allen, J.E., Adjei, O., Bain, O. et al. (2008). Of mice, cattle, and humans: the immunology and treatment of river blindness. *PLoS Negl. Trop. Dis.* 2 (4): e217.

60 Johnston, K.L., Wu, B., Guimaraes, A. et al. (2010). Lipoprotein biosynthesis as a target for anti-Wolbachia treatment of filarial nematodes. *Parasit. Vectors* 3.

61 Hubner, M.P., Torrero, M.N., McCall, J.W., and Mitre, E. (2009). Litomosoides sigmodontis: a simple method to infect mice with L3 larvae obtained from the pleural space of recently infected jirds (Meriones unguiculatus). *Exp. Parasitol.* 123 (1): 95–98.

62 Hoerauf, A., Nissen-Pahle, K., Schmetz, C. et al. (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode Litomosoides sigmodontis and results in filarial infertility. *J. Clin. Invest.* 103 (1): 11–18.

63 Ash, L.R. and Riley, J.M. (1970). Development of Brugia pahangi in the jird, Meriones unguiculatus, with notes on infections in other rodents. *J. Parasitol.* 56 (5): 962–968.

64 Lok, J.B. and Abraham, D. (1992). Animal models for the study of immunity in human filariasis. *Parasitol. Today* 8 (5): 168–171.

65 Mbah, G.E., Ayiseh, R.B., and Cho-Ngwa, F. (2016). Development and validation of an Onchocerca ochengi microfilarial hamster model for onchocerciasis drug screens. *BMC Infect. Dis.* 16 (1): 404.

66 Halliday, A., Guimaraes, A.F., Tyrer, H.E. et al. (2014). A murine macrofilaricide pre-clinical screening model for onchocerciasis and lymphatic filariasis. *Parasit. Vectors* 7: 472.

67 Ceballos, L., Mackenzie, C., Geary, T. et al. (2014). Exploring the potential of flubendazole in filariasis control: evaluation of the systemic exposure for different pharmaceutical preparations. *PLoS Negl. Trop. Dis.* 8 (5): e2838/1–e2838/10.

68 Moreno, L., Alvarez, L., Mottier, L. et al. (2004). Integrated pharmacological assessment of flubendazole potential for use in sheep: disposition kinetics, liver metabolism and parasite diffusion ability. *J. Vet. Pharmacol. Ther.* 27 (5): 299–308.

69 J&J, D., and AbbVie (2012). Johnson & Johnson joins public and private partners in the largest coordinated action to date to eliminate or control neglected tropical diseases. https://www.jnj.com/media-center/press-releases/johnson-johnson-joins-public-and-private-partners-in-the-largest-coordinated-action-to-date-to-eliminate-or-control-neglected-tropical-diseases (accessed 7 March 2019).

70 Sjoberg, H., Pionnier, N., Steven, A. et al. (2016). Lack of significant microfilaricidal efficacy of parenteral or oral bioavailable flubendazole formulations in a *Brugia malayi* microfilaraemic mouse model. In *65th American Society of Tropical Medicine & Hygiene, Atlana, GA*.

71 Hübner, M.P., Quirynen, L., Lachau-Durand, S. et al. (2016). Efficacy of single and repeated oral and subcutaneous doses of flubendazole in litomosoides sigmodontis infected jirds. In *65th American Society of Tropical Medicine & Hygine, Atlanta, GA*.

72 Kuesel, A.C. (2016). Research for new drugs for elimination of onchocerciasis in Africa. *Int. J. Parasitol. Drugs Drug. Resist.* 6 (3): 272–286.

73 Tweats, D.J., Johnson, G.E., Scandale, I. et al. (2016). Genotoxicity of flubendazole and its metabolites in vitro and the impact of a new formulation on in vivo aneugenicity. *Mutagenesis* 31 (3): 309–321.

74 Longo, M., Zanoncelli, S., Messina, M. et al. (2014). In vivo preliminary investigations of the effects of the benzimidazole anthelmintic drug flubendazole on rat embryos and fetuses. *Reprod. Toxicol.* 49: 33–42.

75 Longo, M., Zanoncelli, S., Colombo, P.A. et al. (2013). Effects of the benzimidazole anthelmintic drug flubendazole on rat embryos in vitro. *Reprod. Toxicol.* 36: 78–87.

76 Townsend, L.B., Wise, D.S., Ram, S. (1993). Soluble alkyl [5-[amino(phenyl)methyl]-1H-benzimidazol-2-yl]carbamate anthelmintics. WO9323037A1.

77 Theplertboon, R., Fleckenstein, L., Dzimianski, M.T., and McCall, J.W. (2000). Pharmacokinetics of UMF-078, a candidate antifilarial drug, in infected dogs. *Am. J. Trop. Med. Hyg.* 62 (1): 86–91.

78 Bronsvoort, B.M.D., Makepeace, B.L., Renz, A. et al. (2008). UMF-078: a modified flubendazole with potent macrofilaricidal activity against onchocerca ochengi in African cattle. *Parasit. Vectors* 1.

79 Zhang, Y.-K., Zhou, H., Ding, C. et al. (2011). Boron-containing small molecules as antihelminth agents. WO2011063293A1.

80 Meinke, P.T. (2001). Perspectives in animal health: old targets and new opportunities. *J. Med. Chem.* 44 (5): 641–659.

81 Awadzi, K., Opoku, N.O., Attah, S.K. et al. (2014). A randomized, single-ascending-dose, ivermectin-controlled, double-blind study of moxidectin in *Onchocerca volvulus* infection. *PLoS Negl. Trop. Dis.* 8 (6): e2953.

82 Sivanathan, S., Korber, F., and Scherkenbeck, J. (2016). Semisynthetic routes to PF1022H--A precursor for new derivatives of the anthelmintic cyclooctadepsipeptide PF1022A. *Bioorg. Med. Chem.* 24 (4): 873–876.

83 Crisford, A., Ndukwe, K., O'Connor, V. et al. (2015). The cyclooctadepsipeptide anthelmintic emodepside differentially modulates nematode, insect and

human calcium-activated potassium (SLO) channel alpha subunits. *PLoS Negl. Trop. Dis.* 9 (10): e0004062.

84 DNDi (2016). First in man clinical trial of emodepside (BAY 44-4400), NCT02661178. clinicaltrials.gov (accessed 8 March 2019).

85 Von Geldern, T.W., Kempf, D.J., Marsh, K.C. et al. (2016). Treatment of filarial diseases. WO2016112317A1.

86 Von Geldern, T.W., Kempf, D.J., Marsh, K. et al. (2017). *Tylosin Analogs As Anti-Filarial Agents: Medicinal Chemistry Optimization and Candidate Selection*, COMP-226. American Chemical Society.

87 Schiefer, A., Vollmer, J., Lammer, C. et al. (2013). The ClpP peptidase of Wolbachia endobacteria is a novel target for drug development against filarial infections. *J. Antimicrob. Chemother.* 68 (8): 1790–1800.

88 Schiefer, A., Schmitz, A., Schaberle, T.F. et al. (2012). Corallopyronin A specifically targets and depletes essential obligate Wolbachia endobacteria from filarial nematodes in vivo. *J. Infect. Dis.* 206 (2): 249–257.

89 Pfarr, K.M., Hoerauf, A., Koenig, G.M. et al. (2012). Pharmaceutical compositions comprising corallopyronins for treatment of filariasis. EP2520293A1.

90 Schaberle, T.F., Schiefer, A., Schmitz, A. et al. (2014). Corallopyronin A – a promising antibiotic for treatment of filariasis. *Int. J. Med. Microbiol.* 304 (1): 72–78.

91 Charoensutthivarakul, S., Hong, W. David, G. et al. (2016). Discovery and development of pyrazolopyridines and indazoles targeting wolbachia symbiont of lymphatic filariasis and onchocerciasis. In *XXIV International Symposium on Medicinal Chemistry* (30 August 2016).

8

Drug Discovery and Development for Schistosomiasis

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8.1 Schistosomiasis: The Disease and the One Drug We Have for Treatment, Praziquantel

Schistosomiasis is a neglected tropical disease (NTD) associated with poverty in the developing world. This disease is caused by trematode flatworms of the genus *Schistosoma*. There are more than 200 million people infected [1], with as many as 700 million people at risk of infection [2, 3] and an estimated 280 000 deaths annually [2–4]. Vulnerable populations include farmers, car washers, fisherfolk, migrants, and women and children who use the water for work and/or recreation [1, 2, 5]. Infection arises via direct contact with freshwater (ponds, lakes, irrigation canals, and streams) that harbor the intermediate snail hosts.

Schistosome infections can last a lifetime [6–9], contribute to anemia and malnutrition [9, 10], and are often associated with other parasitic infections, e.g. roundworms and malaria. Chronic schistosomiasis is debilitating and painful, leading to lost school days and a decreased stamina to perform manual labor, which, in subsistence communities, is particularly consequential [9, 11, 12]. Often, infection symptoms are mistaken for simply being the norm [9].

The methods for control of schistosomiasis include education on the risks, provision of piped water and sanitation infrastructure, snail removal, and drug treatment [13]. The easiest and least expensive to engage and sustain, "preventative chemotherapy," via mass drug administration (MDA) programs has been

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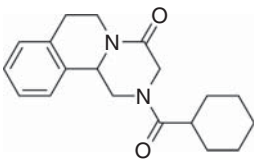


Figure 8.1 Praziquantel.

the mainstay control approach for over 40 years, as recommended by the WHO [13, 14]. Preventative chemotherapy delivers drug to those communities where infection prevalence is highest in order to decrease morbidity and the transmission potential [13]. Note that the emphasis of preventative chemotherapy is not cure, but control of infection at the population level. The method evolved as the drug deployed, the pyrazinoisoquinoline derivative praziquantel (PZQ; Figure 8.1) is safe, including during pregnancy, reasonably (but not completely) effective against all clinically relevant schistosome species, and affordable. PZQ causes tetanic paralysis in the parasite and damage to the surface, potentially aiding immune clearance [15, 16]; however, the exact mechanism of action is unclear. Very recent evidence has shown that the clinically active enantiomer, (*R*)-PZQ, acts as a GPCR ligand, engaging the human 5-HT2B receptor [17], consistent with earlier reports showing that PZQ antagonizes the increased motor activity of schistosome caused by 5HT [18]. PZQ may also act on voltage-gated calcium channels [19] or destabilize the surface membrane [20]. Successful control programs using PZQ have been implemented in China, Brazil, and Egypt [13, 21]. These programs serve as an example and help bring international interest to the control of schistosomiasis in sub-Saharan Africa where the disease is most prevalent [13]. As of 2015, 66.5 million people have received treatment.

PZQ has a number of pharmaceutical and pharmacological problems [22, 23] that warrant discussion, including in the context of alternative drugs as exemplified in this chapter. These include being rarely curative at the single dose offered, poor metabolic stability, and an unpalatable taste especially relevant for children who are most often the primary target population [24–26]. Also, being synthesized as a racemate, half of the rather high 40 mg/kg recommended dose is clinically useless. Finally, for reasons unknown, PZQ is much less effective against immature schistosomes, meaning that individuals harboring parasites of different ages go on to develop morbidity in spite of treatment. In this context, discussions have included the combination of PZQ with other drugs that better target the developing stages while potentially decreasing the risk of the development of resistance [27, 28].

With the reliance on just one drug, the biggest fear is the emergence and establishment of drug resistance. This concern is amplified in that there are no well-supported transnational drug discovery and development partnerships for schistosomiasis akin to the Medicines for Malaria venture (MMV) or the Drugs for Neglected Diseases *initiative* (DND*i*, which includes protozoan and filarial parasites in its drug portfolio). Laboratory studies in rodents [29–31] and in the intermediate snail host [32] demonstrate that increased tolerance to PZQ can be selected for. Incidences of lower than anticipated cure rates with PZQ have been reported clinically in Egypt, sub-Saharan Africa and Brazil [33, 34].

As a result of increased international support over the past decade for MDA programs coordinated by, for example, the Schistosomiasis Control Initiative (SCI), the availability of PZQ is expanding [5]. This potentiates further the threat of drug resistance [23, 35, 36]. Thus, there is a need to identify new drugs and drug targets, and this chapter illustrates some of the exciting research being performed to fulfill this need.

8.2 Drug Discovery for Schistosomiasis: Strategies, Tools, Targets, and a Note on the Target Product Profile

In the absence of transnational agencies that support *new* chemical development, efforts to discover and validate new drugs and drug targets have come to rely on the academic sector. Hit identification typically relies on *in vitro* phenotypic screens (see subsequent text) with a variety of quantitative or partially quantitative readouts [37, 38]. Phenotypic screens have been applied to a number of small-molecule chemical libraries, including FDA drug libraries [39] and natural products [40], as well as collections arising from industry [38] or product development partnerships such as the MMV, which has released the Malaria Box [41] and Pathogen Box [42], each of which contains 400 Lipinski-compliant chemistries with validated antiparasitic activities. Subsequent chemical "lead" optimization has also employed phenotypic- and, where possible, target-based modalities. Finally, for proof-of-principle studies, there is a well-established mouse model for *Schistosoma mansoni* and *Schistosoma japonicum* infections. *Schistosoma haematobium* infections in mice are less robust and hamsters are used as an alternative animal model.

Backing up these tools, there is now a well-annotated *S. mansoni* genome [43, 44], and improving *S. japonicum* [45] and *S. haematobium* [46] genomes. These data have facilitated both the identification of potential drug targets and their subsequent validation via chemistry [47] or reverse genetics, which is essentially limited to transient RNA interference (RNAi) [48–50], although the development and deployment of (CRISPR)/Cas9 will likely aid future efforts to understand gene function [51–53]. As elaborated later, among the targets identified *and validated* are molecules specific to the schistosome parasite, e.g. thioredoxin glutathione reductase [54] and hematin formation [55], or orthologs of human proteins, e.g. cysteine proteases [56], hydroxymethylglutaryl coenzyme A reductase [47, 57], and histone deacetylases [58]. For well-conserved genes, *Caenorhabditis elegans* has also served as a tool to validate gene function and, potentially, optimize chemical matter [38]. Finally, comparative analyses of gene function have also employed the free-living flatworm *Girardia tigrina* [59] and *Schmidtea mediterranea* [60].

Along with the improvement in tools, there has been a better appreciation of the need to conceptualize the desired properties of any drug candidate using PZQ as the gold standard comparator (target product profile [TPP]). Taking a cue from [22, 61], many of the more obvious TPP provisions to establish early in a development program would be (i) efficacy in a single oral dose, including against the

PZQ-refractory juvenile worms; (ii) efficacy against all species of schistosomes or at least against *S. mansoni* and *S. haematobium* which are sometimes co-endemic; (iii) efficacy at a lower dose relative to PZQ; and (iv) that the molecule of interest belong to a different chemical class and/or involves a different molecular target such that cross-resistance is less likely to occur (even though it is not entirely clear what the target of PZQ is [61]). Other desirable properties that may only become clear as a development program progresses involve possessing a wide margin of safety, including in young children and pregnant and nursing mothers (PZQ is offered under minimal medical supervision), and a low risk regarding potential drug–drug interactions. Finally, stability (shelf life) and cost of goods will be important factors in delivering drugs to resource-constrained communities.

For the remainder of this chapter, we discuss in more detail the current approaches and the key chemistries that have been or are under investigation to identify and characterize new drugs and drug targets. We focus wherever possible on original papers published in the period 2015 to 2017. We also briefly summarize the most promising data from schistosomiasis drug repurposing investigations, structure-based drug design, phenotypic approaches, organometallics, and natural products. In addition, a perspective on kinases as drug targets is included and, finally, two case studies regarding biarylalkyl carboxylic acids (BACAs) and arylmethylamino steroids (AASs) are highlighted. Excluded from consideration are meeting abstracts; natural product extracts or mixtures; biochemical matter including fatty acids, vitamins, cytokines, and other proteins; formulations of existing drugs; compounds that may affect schistosome pathology but do not inhibit parasite growth; and enzyme inhibitor, *in silico*, or theoretical studies without antischistosomal activity data. The reader is referred to a number of excellent medicinal chemistry–type antischistosomal reviews published from 2007 to 2017 [22, 62–70].

8.3 Drug Repurposing

Drug repurposing for schistosomiasis has borne some fruit, including the identification of new starting points for medicinal chemistry [67, 71, 72]. The earliest and most widely investigated drug class are the semisynthetic artemisinins, including dihydroartemisinin, artemether, and artesunate, which are effective against the juvenile parasite stages but less so against adults [73, 74] (Figure 8.2). Animal model and clinical trial data suggest that the artemisinins are synergistic with PZQ and may be useful in the chemoprophylaxis and prevention of schistosomiasis [55, 75].

As reviewed by Keiser and Utzinger [55] and Thétiot-Laurent et al. [62], other classes of antimalarial peroxide compounds also have substantial antischistosomal activity (Figure 8.2). For example, single 400 mg/kg oral doses of the ozonide carboxylic acid, OZ418 (**1**) administered to *S. mansoni*-, *S. japonicum*-, or *S. haematobium*-infected mice, achieved adult worm burden reductions (WBRs) of 80%, 69%, and 86%, respectively [73, 76]. Thus, **1** is effective against all three important schistosome species in the mouse model. Like the artemisinins, **1** is

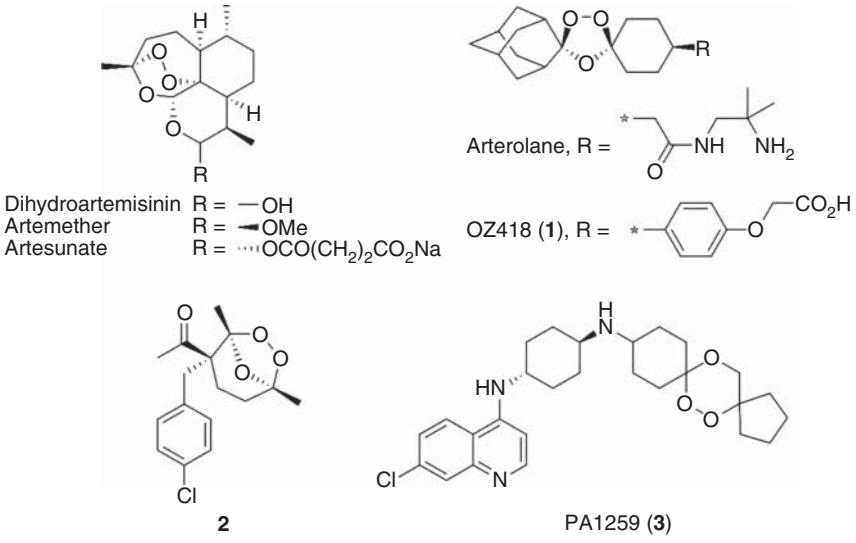


Figure 8.2 The semisynthetic artemisinins, dihydroartemisinin, artemether, and artesunate, are effective against juvenile schistosomes, but less so against the adult form of the parasite [73, 74]. Other classes of antimalarial peroxide compounds also have substantial antischistosomal activity [55, 62].

even more effective against the juvenile form of the parasite; single 200 mg/kg oral doses of artemether and **1** administered to *S. mansoni*-infected mice reduced juvenile (21-day-old) worm burdens by 81% and 100%, respectively [55]. However, with IC_{50} values of 44 and 39 μM against *ex vivo S. mansoni* and *S. japonicum*, **1** has relatively low *in vitro* activity [76, 77]. Even so, a pharmacokinetic study of **1** showed that a single 400 mg/kg dose achieved plasma levels above its *S. mansoni* IC_{50} value for >72 hours [77]. Arterolane (ozonide OZ277), developed as a three-dose combination product with piperaquine (Synriam®) to treat uncomplicated malaria, also has antischistosomal potential [78]. Administration of a single 100 mg/kg oral dose of arterolane or a single fixed-dose combination of 40 mg/kg arterolane and 200 mg/kg piperaquine to mice infected with adult *S. mansoni* reduced worm burden by 81% in both experiments [78]. In a subsequent clinical trial, Barda et al. [79] demonstrated that Synriam had low efficacy in individuals infected with *S. haematobium* and *S. mansoni* with respective cure rates of just 11% and 7%.

Another structurally distinct ozonide (**2**) [80] had an IC_{50} value of 4.2 μM against *ex vivo S. mansoni*, but administration of a single 400 mg/kg oral dose of this ozonide to *S. mansoni*-infected mice led to a statistically insignificant adult WBR of 44%. The 4-aminoquinoline trioxane, PA1259 (**3**), was comparable to PZQ in its *ex vivo* antischistosomal effects [81]. Administration of four 50 mg/kg oral doses of **3** to *S. mansoni*-infected mice reduced juvenile and adult worm burdens by 53% and 41%, respectively [81]. The same authors also demonstrated that **3** alkylates heme in *S. mansoni*-infected mice, suggesting a shared antimalarial and antischistosomal mechanism of action.

The antimalarial, mefloquine, demonstrated promising antischistosomal efficacy in animal models [55] (Figure 8.3). For example, WBR values ranging from 88% to 94% were observed when single 200 mg/kg oral doses of this quinoline methanol were administered to mice infected with juvenile or adult stages of all three major schistosome species. However, mefloquine did not increase the efficacy of PZQ in *S. haematobium*-infected patients [82].

After a chemical and genetic validation of *S. mansoni* 3-hydroxy-3-methylglutaryl coenzyme A reductase (*Sm*HMGR) as a promising drug target, Rojo-Arreola et al. [47] demonstrated that the two statin drugs, simvastatin and lovastatin, had ED_{50} values of 90 and 190 nM against cultured *S. mansoni* schistosomula; the two drugs were 2 orders of magnitude less potent against adult *S. mansoni*. Worm death was prevented by excess mevalonate, the enzymatic product of *Sm*HMGR. Other more hydrophilic statin drugs were less effective, including pravastatin and the nitrogen heterocycle-containing fluvastatin, atorvastatin, and rosuvastatin. Using an *in silico* chemogenomics approach, Neves et al. [83] predicted that the selective serotonin reuptake inhibitors (SSRIs) antidepressant drug paroxetine would inhibit serotonin transporters in *S. mansoni*. A subsequent *in vitro* study showed that paroxetine decreased *S. mansoni* schistosomula and adult worm viability with EC_{50} values ranging from 2.5 to 10 μM [84].

A phenotypic screen of *S. mansoni* with a 2160 compound collection, including 821 known drugs, identified the antibiotics, anisomycin and lasalocid, and the molluscicide and anti-tapeworm drug, niclosamide, as the promising leads [39] (Figure 8.3). In this study, compounds at 1 μM were first tested against schistosomula and then against adult parasites *in vitro* before progressing to *in vivo* efficacy experiments. Administration of four 100 mg/kg daily oral doses of anisomycin to mice infected with adult *S. mansoni* did not reduce worm burden but did decrease hepatic egg burden by 36%; administration of four 100 mg/kg twice-daily doses killed all of the mice. In this same experiment, lasalocid was both more effective and better tolerated; four 100 mg/kg oral doses of lasalocid reduced worm burden by 44% and hepatic egg burden by 39%. In contrast, niclosamide at total doses up to 800 mg/kg administered either orally or intraperitoneally was completely ineffective. Consequently, the authors tested closantel, oxyclozanide, rafoxanide, and nitazoxanide, four salicylanilide analogs of niclosamide [39]. Of these, rafoxanide was by far the most effective; administration of four 50 mg/kg oral doses of this salicylanilide reduced worm burden by 50–56% and decreased hepatic egg count by 49%.

In another antischistosomal phenotypic screen of 1600 drugs, the authors identified the antileprotic, clofazimine, and the veterinary anti-parasitic, doramectin, as the most active leads [37] (Figure 8.3). In this study, compounds were successively tested against schistosomula (10 μM) and adult *S. mansoni* (33 μM) *in vitro* before progressing to *in vivo* efficacy testing in mice infected with adult *S. mansoni*. Doramectin and clofazimine had IC_{50} values for viability of 21 and 17 μM against adult *S. mansoni in vitro*. Single oral doses of doramectin (10 mg/kg) and clofazimine (400 mg/kg) reduced worm burden by 60% and 83%, respectively; a lower 200 mg/kg dose of clofazimine was completely ineffective. The same authors [37] noted that ivermectin, a close structural analog of

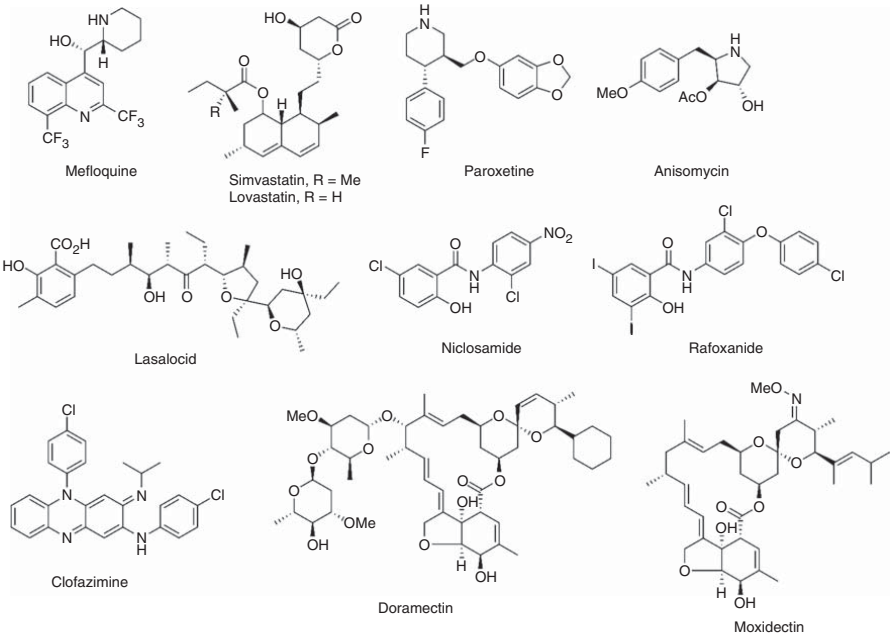


Figure 8.3 Compounds tested in phenotypic screens of the schistosome parasite. Compounds pictured from the first screen of 2160 compounds, including 821 known drugs, identified the antibiotics, anisomycin and lasalocid, and the molluscicide and anti-tapeworm drug, niclosamide as the most promising leads [39]. In the second phenotypic screen of 1600 drugs, the antileprotic, clofazimine, and the veterinary antiparasitic, doramectin, were identified as the most promising leads [37].

doramectin, had minimal antischistosomal efficacy in animal models and in clinical trials [85]. A recent clinical trial with the veterinary anthelmintic, moxidectin, showed that this chemical cousin of doramectin had low efficacy in patients infected with *S. haematobium*, but had some promise in reducing egg production in patients infected with *S. mansoni* [79].

Eissa et al. [86] showed that cultured adult *S. mansoni* exposed to the anticancer drug chlorambucil at 5 μg/ml for 72 hours led to complete worm death. In the same study, administration of five 2.5 mg/kg oral doses of chlorambucil to *S. mansoni*-infected mice reduced juvenile and adult worm burdens by 76% and 72%, respectively. Beckmann and Grevelding [87] showed that cultured adult *S. mansoni* exposed to 10 μM of the anticancer protein kinase inhibitor, imatinib, for 96 hours led to >60% mortality; the treated worms had a notably disintegrated gastrodermis (Figure 8.4). In a subsequent phenotypic screen of an anticancer drug library, Cowan and Keiser [88] discovered that trametinib and vandetanib, two other anticancer protein kinase inhibitors, had promising antischistosomal activities (Figure 8.4). The compounds were successively tested against schistosomula and adult *S. mansoni in vitro* before progressing to *in vivo* efficacy experiments. Trametinib and vandetanib had respective IC_{50} values for viability of 4.1 and 9.5 μM against *ex vivo* adult *S. mansoni*. Single 400 mg/kg oral doses of trametinib and vandetanib administered to mice infected with adult *S. mansoni* reduced worm burden by 64% and 48%, respectively. Repositioning anticancer protein kinase inhibitors for schistosomiasis chemotherapy has been thoroughly reviewed by Dissous and Grevelding [89] and Gelmedin et al. [90].

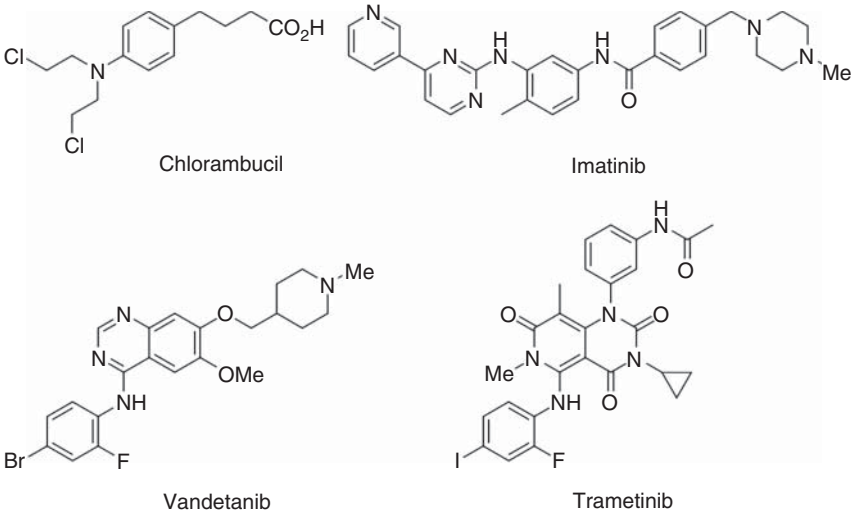


Figure 8.4 Anticancer drugs that are also antischistosomal. Adult *S. mansoni* exposed to 10 μM of the anticancer protein kinase inhibitor, imatinib, for 96 hours leads to >60% mortality and a notably disintegrated gastrodermis [87]. Trametinib and vandetanib, two other anticancer protein kinase inhibitors, also have promising antischistosomal activities [88].

8.4 Structure-Based Drug Design

A milestone in structure-based drug design for schistosomiasis was the identification of several inhibitors of *S. mansoni* thioredoxin glutathione reductase (*Sm*TGR) [91] (Figure 8.5). The most promising of these was the nitric oxide donor, oxadiazole 2-oxide (**4**), which irreversibly inhibited both the glutathione reductase (IC_{50} = 0.32 μM) and thioredoxin reductase (IC_{50} = 1.7 μM) activities of *Sm*TGR, completely inhibited adult *S. mansoni* at 10 μM within 24 hours, and after five intraperitoneal 10 mg/kg doses to *S. mansoni*-infected mice, reduced juvenile and adult worm burdens by 89% and 94% [91]. 1,4-Naphthoquinone ethers were recently identified as inhibitors of *Sm*TGR [95]. As exemplars of this compound series, **5** and **6** had respective IC_{50} values of 0.48 and 0.046 μM against *Sm*TGR and were an order of magnitude less potent against human glutathione reductase and selenium-dependent thioredoxin reductase. At 50 μM, these 1,4-naphthoquinone ethers killed *ex vivo* adult *S. mansoni* within 24 hours, but none of these had any activity in the *S. mansoni* mouse model. Using a QSAR-based virtual screening approach against *Sm*TGR, Neves et al. [92] identified the new antischistosomal chemotypes, **7** and **8**. Although no experimental *Sm*TGR inhibition data was disclosed for these compounds, **7** and **8** inhibited *ex vivo* adult *S. mansoni* with respective IC_{50} values of 6.1 and 13 μM. As a measure of cytotoxicity, **7** and **8** inhibited the growth of the human epithelial WSS-1 cell line with respective IC_{50} values of 16 and 28 μM.

Based on an X-ray structure of an *S. mansoni* histone deacetylase 8 (*Sm*HDAC8)–ligand complex [96], hydroxamic acid **9** was identified as a new lead. Compound **9** had an IC_{50} of 0.075 μM against *Sm*HDAC8 compared to IC_{50} values ranging from 0.026 to 6.3 μM against three human HDAC isoforms.

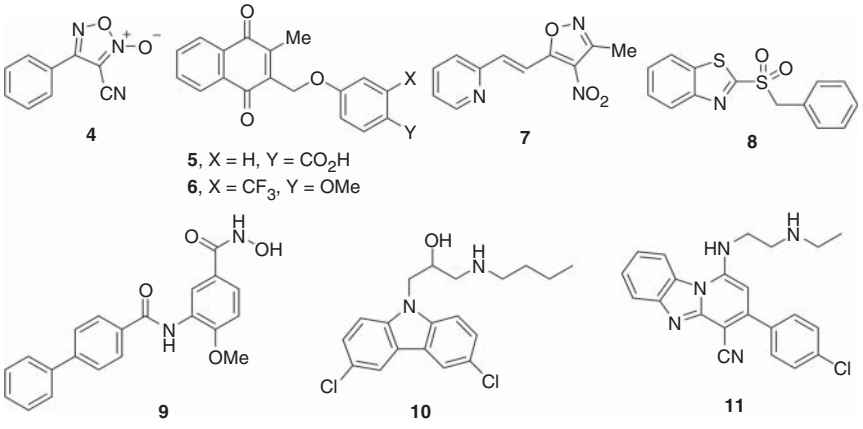


Figure 8.5 Identification of *S. mansoni* thioredoxin glutathione reductase (*Sm*TGR) [91] inhibitors represents a milestone in schistosomiasis structure-based drug design (SBDD). These *Sm*TGR inhibitors include the irreversible inhibitor, oxadiazole 2-oxide (**4**), and exemplar compounds in a naphthoquinone series **(5**, **6)** [92]. Other antischistosomal compounds include new chemotypes (**7**, **8**), hydroxamic acid (**9**), and new inhibitors (**10** and **11**) of β-hematin (hemozoin) formation [93, 94].

Compound **9** was lethal to *S. mansoni* schistosomula and human HEK293 cells with respective IC_{50} values of 16 and 200 μM, and thus appeared to possess reasonable antischistosomal selectivity. The corresponding carboxylic acid and esters of **9** had no activity against either *Sm*HDAC8 or schistosomula.

In a search for new inhibitors of β-hematin (hemozoin) formation, Wang et al. [93] identified **10** as a new lead. This carbazole amino alcohol inhibited β-hematin formation, *ex vivo* adult *S. mansoni*, and the W138 lung fibroblast cell line with respective IC_{50} values for mortality of 92, 14, and 7.9 μM. The data from the compound series was suggestive of a correlation between inhibition of β-hematin formation and parasite survival. In another investigation of β-hematin inhibitors, Okombo et al. [94] identified **11** as a new lead. This pyrido[1,2-*a*]benzimidazole inhibited β-hematin formation, *ex vivo* adult *S. mansoni*, and the CHO mammalian cell line with respective IC_{50} values of 52, 2.4, and 9.6 μM. There was no correlation between inhibition of β-hematin formation and parasite survival for this compound series. In *S. mansoni*-infected mice, a single 400 mg/kg oral dose of **11** reduced worm burden by 59% despite its relatively low exposure due to solubility-limited absorption and high hepatic clearance [94].

8.5 Phenotypic Approaches

We now briefly review the search for new antischistosomal lead compounds using phenotypic approaches (Figure 8.6). Taking decoquinate as a lead, Wang et al. [93] discovered that 10 μM of the amide derivative **12** was 100% lethal to *ex vivo* adult *S. japonicum*; in contrast, decoquinate at concentrations up to 100 μM had no effect. As a measure of cytotoxicity, the IC_{50} of **12** against HeLa cells was approximately 70 μM.

As part of an investigation of indole chemistry, Jiang et al. [97] discovered that 10 μM **13** was 100% lethal to *ex vivo* adult *S. japonicum*. With an objective of targeting *S. mansoni* aldose reductase, Mäder et al. [98] conducted a small structure–activity relationship (SAR) study starting with biaryl carboxylic acid inhibitors of human aldose reductase (see Section 8.9 for more details). Qualitative data from this study indicated that **14** and **15** had measurable activity against *ex vivo* adult *S. mansoni* and neither compound at 100 μM had significant cytotoxicity against the HepG2 and LS174T cell lines. de Brito et al. [99] showed that 100 μM of the iminobenzoxazine **16** led to, respectively, a 50% and 100% mortality of female and male *S. mansoni* adult worms in culture. As a measure of cytotoxicity, 100 μM **16** inhibited the growth of four mammalian cell lines by 12–28%. In a study of cinnamic acid esters, **17** had the most potent antischistosomal activity [102]. *S. mansoni* schistosomula exposed to 5 μM **17** for 24 hours were degenerated and contained vacuoles; at 72 hours, all the parasites were dead. However, 10 μM **17** had no effect on *ex vivo* adult *S. mansoni*. As a measure of cytotoxicity, the IC_{50} of **17** against J774 cells was 32 μM. There was no correlation between antischistosomal activity and cytotoxicity for this compound series. An enone and unsubstituted phenyl ring in the cinnamic acid substructure were required for optimal activity. The authors suggested that **17** interferes with autophagy.

Based on preliminary data indicating the schistosomicidal potential of various imidazolidines, de Silva et al. showed that administration of five 250 mg/kg doses of compound PT-09 (**18**) to *S. mansoni*-infected mice reduced adult worm burden by 54% (Figure 8.6). Data from this and lower doses of **18** indicated that WBR was greater for female versus male worms. Pereira et al. [100] reported the antischistosomal profile of a dinitro semisynthetic derivative (**19**) of the lignan cubebin isolated from *Piper cubeba*. Compound **19** had LC_{50} values of 20, 180, and 100 μM against *ex vivo* schistosomula and juvenile and adult *S. mansoni*, respectively. As a measure of cytotoxicity, the IC_{50} of **19** against V79 cells was 890 μM. Administration of five 50 mg/kg intraperitoneal doses of **19** to

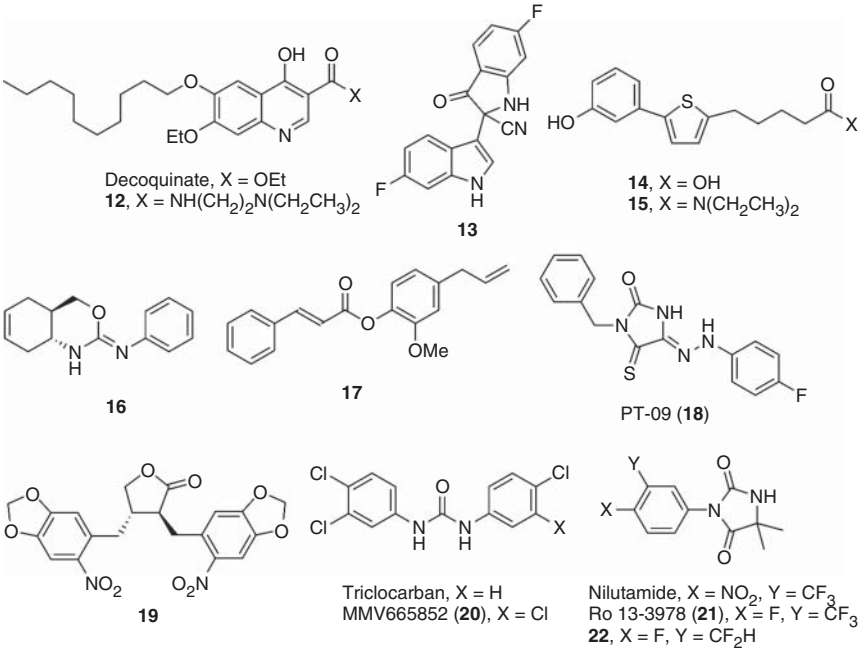


Figure 8.6 Antischistosomal compounds identified using phenotypic approaches. Decoquinate (**12**) at concentrations above 100 μM was determined to be 100% lethal against adult *S. japonicum* [93]. Compound **13** (10 μM) is 100% lethal against adult *S. japonicum* [97]. Qualitative data from a SAR study testing biaryl carboxylic acid inhibitors of human aldose reductase showed that **14** and **15** had measurable activity against *ex vivo* adult *S. mansoni* [98]. The imino-benzoxazine **16** (100 μM) led to 50% and 100% mortalities of female and male *S. mansoni* adult worms, respectively, in culture [99]. *S. mansoni* schistosomula exposed to 5 μM **17** for 24 hours became degenerate and contained vacuoles; and were dead at 72 hours. Administration of five 250 mg/kg doses of compound PT-09 (**18**) to *S. mansoni*-infected mice reduced adult worm burden by 54%. A dinitro semisynthetic derivative (**19**) of the lignan cubebin isolated from *Piper cubeba* also has antischistosomal activity [100]. MMV665582 (**20**), identified in a phenotypic screen of the 200-member MMV Malaria Box compound library [82], identified a symmetrical *N,N′*-diaryl urea as a promising lead. Ro 13-3978 (**21**), identified by scientists at Hoffmann La-Roche in the 1980s, is a promising antischistosomal lead compound with high efficacy against the three major schistosome species, *S. mansoni*, *S. haematobium*, and *S. japonicum,* in a range of animal models [101].

S. mansoni-infected mice reduced juvenile and adult worm burdens by 52% and 49%, respectively. A subsequent proteomic investigation revealed that **19** seems to act by targeting carbohydrate metabolism in the parasite [103].

In a phenotypic screen of the 200-member MMV Malaria Box compound library, Ingram-Sieber et al. [82] identified the symmetrical *N,N′*-diaryl urea, MMV665582 (**20**), as the most promising lead (Figure 8.6). The structure of the relatively lipophilic **20** (Log *P* 5.2) is very similar to that of triclocarban [104], an antibacterial agent used in detergents, cosmetics, and other products. With an IC_{50} of 0.8 μM against *ex vivo* adult *S. mansoni*, **20** is only fourfold less potent than PZQ and it has a 64-fold *in vitro* selectivity index. A single 400 mg/kg oral dose of **20** administered to *S. mansoni*-infected mice reduced worm burden by 53%. *N,N′*-diaryl urea **20** has a half-life of 4.7 hours and C_{max} of 4.4 μM at a 46.3 mg/kg oral dose [82]. Two subsequent SAR studies [80, 105] of this compound series established that substitution at positions 3 and 4 of the phenyl rings with H, F, Cl, CN, and CF_3 groups was optimal, substitution at positions 3 and 4 of the phenyl rings with OCH_3, NH_2 and other electron-donating groups diminished activity, replacement of one of the phenyl rings with alkyl substituents diminished or abolished activity, cyclization of the urea to imidazoline-2-ones abolished activity, and replacement of the urea with carbamates, thioureas, sulfonamides, or oxalamides diminished or abolished activity.

In the 1980s, the aryl hydantoin, Ro 13-3978 (**21**), was identified by scientists at Hoffmann La-Roche as a promising antischistosomal lead compound with high efficacy against the three major schistosome species, *S. mansoni*, *S. haematobium*, and *S. japonicum,* in a range of animal models ([101]; Figure 8.6). Confirming these data, Keiser et al. [106] showed that **21** had single-dose ED_{50} values of 15 and 140 mg/kg against adult and juvenile *S. mansoni* in a mouse model. Surprisingly, high concentrations of **21** had little effect on *ex vivo* adult *S. mansoni* [106]. Data generated so far [93, 106] indicate that active metabolites do not account for the disparity between the *in vitro* and *in vivo* antischistosomal activities.

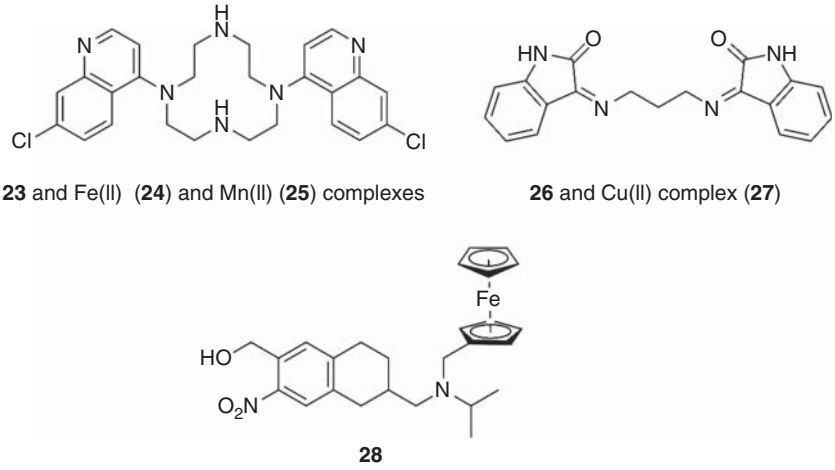
Given the structural similarity of **21** and the anti-androgenic drug nilutamide, the latter and three structurally diverse drugs, flutamide, bicalutamide, and cyproterone acetate were assessed for their *in vivo* antischistosomal activity [107]. Of these, only nilutamide had any activity, although it was weak. As schistosome species do not appear to have androgen receptors [108], Wang et al. [109] hypothesized that for **21** and similar aryl hydantoins, the structural requirements for antischistosomal efficacy and androgen receptor binding interactions are dissimilar. In this respect, **21** did not interact with the androgen receptor in a ligand competition assay [109], but it did weakly inhibit dihydrotestosterone-induced cell proliferation in the LAPC4 androgen-dependent cell line [110].

The SAR of this aryl hydantoin compound series from Hoffmann La-Roche [101] established that a combination of halogens and/or trifluoromethyl groups at positions 3 and 4 of the phenyl ring were optimal, methoxy and dimethylamino at these same positions reduced activity, and 4-imino and various N^1-substituted derivatives were active. A subsequent SAR study guided by incorporation of substructures and functional groups known to reduce ligand–androgen receptor interactions revealed that the hydantoin

core of **21** is required for high antischistosomal activity [110]. The same study identified several compounds with high antischistosomal efficacy that were less antiandrogenic than **21**, as exemplified by **22**. In *S. mansoni*-infected mice, single 100 mg/kg oral doses of **21** and **22** produced almost identical WBRs of 95% and 94%, respectively. However, **21** (IC_{50} = 4.4 μM), but not **22** ($IC_{50} \geq 10$ μM), inhibited dihydrotestosterone-induced cell proliferation in the LAPC4 androgen-dependent cell line [110]. Plasma exposures in mice to single 100 mg/kg oral doses of **21** and **22** were similar with respective C_{max} values of 124 and 112 μM, and AUC_{0-last} values of 2740 and 3019 μMxh [110].

8.6 Organometallics

Several recent studies have explored the antischistosomal potential of organometallics (Figure 8.7). Khan et al. [111] found that bisquinoline tetrazamacrocycle **23** and its Fe(II) (**24**) and Mn(II) (**25**) metal complexes had respective IC_{50} values of 1.6, 1.3, and 4.1 against *ex vivo* adult *S. mansoni* and respective cytotoxicity IC_{50} values of 8.2, 2.7, and 5.9 μM against the L6 rat skeletal myoblast cell line. Despite this cytotoxicity, single 400 mg/kg oral doses of **24** and **25** administered to mice infected with adult *S. mansoni* reduced worm burden by 75% and 88%, respectively; **23** was inactive in this experiment. de Moraes et al. [112] found that the Cu(II) (**27**) but not the Zn(II) or vanadyl(II) complexes of bisoxindolimine **26** had moderate, but selective, antischistosomal activity. Complex **27** had an IC_{50} of 39 μM against *ex vivo* adult *S. mansoni* and an IC_{50} > 500 μM against the Vero African green monkey kidney fibroblast cell line.



23 and Fe(II) (**24**) and Mn(II) (**25**) complexes

26 and Cu(II) complex (**27**)

28

Figure 8.7 Organometallics as antischistosomals. Bisquinoline tetrazamacrocycle [111] (**23**) and its Fe(II) (**24**) and Mn(II) (**25**) metal complexes have activity against adult *S. mansoni* and Cu(II) (**27**) complexes of bisoxindolimine (**26**) have selective antischistosomal activity [112]. Finally, a ferrocene derivative of oxamniquine (**28**) has demonstrated promising activity against *S. mansoni* [113].

Hess et al. [113] found that a ferrocene derivative of oxamniquine (**28**) had promising activity against *S. mansoni*, and, unlike oxamniquine, also against *S. haematobium*. Compound **28** had an IC_{50} of 11 μM against *ex vivo* adult *S. mansoni* and an IC_{50} of 78 μM against the L6 mammalian cell line. Oxamniquine was inactive against cultured adult *S. mansoni*, and had an IC_{50} value > 90 μM against the L6 mammalian cell line. Administration of single 100 mg/kg oral doses of **28** and oxamniquine to *S. mansoni*-infected mice reduced worm burdens by 81% and 99%, respectively. Compound **28** at 100 μM killed all worms in an *ex vivo* culture of adult *S. haematobium*; as predicted, oxamniquine was without effect in this same experiment. The ferrocene substructure was not required for high activity as the corresponding ruthenocenyl and benzyl analogs of **28** had very similar *in vitro* and *in vivo* antischistosomal activities to that of **28**. The antischistosomal properties of these and other organometallics have been reviewed by Hess et al. [66].

8.7 Natural Products

Finally, we consider recent studies of antischistosomal natural products (Figure 8.8). In a screen of 38 monoterpenes, sesquiterpenes, and phenylpropanoids, Mafud et al. [119] discovered that the monoterpene alcohol, dihydrocitronellol (**29**), had the best antischistosomal potency with an IC_{50} of 52 μM against *ex vivo* adult *S. mansoni*. Silva et al. [114] showed that a single 400 mg/kg dose of the sesquiterpene alcohol, nerolidol (**30**), to *S. mansoni*-infected mice reduced adult worm burden by 70%, but it had no effect in mice infected with juvenile *S. mansoni*. Eraky et al. [115] showed that the diterpene alcohol, phytol (**31**), produced a concentration- and time-dependent reduction in the motility of *ex vivo* adult *S. haematobium*. At a concentration of 150 μg/ml, **31** was uniformly fatal to the worms. In a study of eight Brazilian plants for their antischistosomal effects, Viegas et al. [116] identified *Miconia willdenowii* as the most promising. After bioassay-guided fractionation, the author identified primin (**32**) as the active constituent. This benzoquinone natural product had respective IC_{50} values of 34 and 260 μM against *ex vivo S. mansoni* and peripheral human blood mononuclear cells.

Based on the diverse biological activity of garcinelliptone FC (**33**), a constituent of the Brazilian plant, *Platonia insignis*, Silva et al. [117] profiled the antischistosomal activity of this prenylated benzophonone natural product. Compound **33** was uniformly fatal to *ex vivo S. mansoni* at 12.5 μM, but it had no effect on the growth of the Vero mammalian cell line at 50 μM. Eraky et al. [115] demonstrated that administration of single 100 mg/kg oral doses of the triterpene furanolactone, limonin (**34**), to mice infected with adult and juvenile *S. mansoni* reduced worm burdens by 60% and 83%, respectively. The authors also noted less severe hepatic pathology in the treated animals. Kang et al. [118] demonstrated that administration of five consecutive twice-daily 400 mg/kg intravenous doses of the triterpene glycoside hederacoside C (**35**) to mice infected with adult and juvenile *S. japonicum* reduced worm burdens by 48% and 44%, respectively. The authors

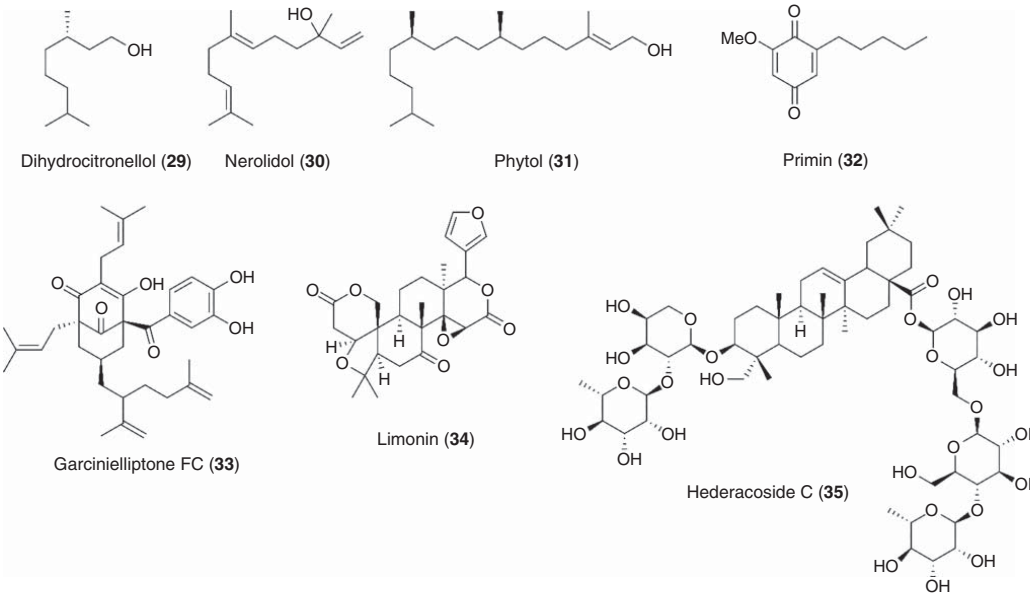


Figure 8.8 Natural products as antischistosomals. The monoterpene alcohol dihydrocitronellol (**29**) has antischistosomal activity against adult *S. mansoni* [114]. The sesquiterpene alcohol, nerolidol (**30**), reduced *S. mansoni*-infected mice adult worm burden by 70%. The diterpene alcohol, phytol (**31**), produced a concentration- and time-dependent reduction in the motility of adult *S. haematobium* [115]. In a study of eight Brazilian plants for their antischistosomal effects, *Miconia willdenowii*, with primin as its active constituent (**32**), was determined to be the most promising [116]. Garcinelliptone FC (**33**), a constituent of the Brazilian plant, *Platonia insignis* [117] and a benzophonone natural product, has antischistosomal activity. Administration of five consecutive twice-daily 400 mg/kg intravenous doses of the triterpene glycoside hederacoside C (**35**) to mice infected with adult and juvenile *S. japonicum* reduced worm burdens by 48% and 44%, respectively [118].

also noted less hepatic inflammation, reduced expression of IgG, and decreased TNF-alpha, IL-4, and IL-17 levels in the treated animals. The antischistosomal properties of these and other natural products have been reviewed by de Moraes [65] and Neves et al. [67].

8.8 Perspective on Schistosome Kinases as Potential Drug Targets

Kinases have emerged as one of the most successful drug targets of the twenty-first century, with 46 small inhibitors approved for use by the FDA, primarily for oncology indications [120]. This success has been built on decades of consistent and diligent research into kinase signaling and kinase medicinal chemistry by academic laboratories, biotech companies, and large pharmaceutical companies. The scientific community has recognized that this massive investment in human kinases can and should be leveraged to find treatments for underserved diseases, be they rare cancers that affect but a few people, or NTDs such as malaria and schistosomiasis that affect millions [90].

The structure and function of kinases are well enough conserved across species that compounds synthesized as inhibitors of human kinases often show cross-reactivity against, for example, parasite kinases. The Tres Cantos Antimalarial Set (TCAMS) is a set of compounds from GlaxoSmithKline that were found to be active against *Plasmodium falciparum* in a phenotypic screen [121, 122]. A large number of these compounds are human kinase inhibitors. Although the molecular targets for these compounds' activity against the malaria parasite are, for the most part, unknown, a simple and compelling hypothesis is that they target essential plasmodial kinases. An even clearer example of cross-species activity comes from efforts to target *Trypanosoma brucei*, the parasite that causes human African trypanosomiasis. In an elegant chemical proteomics experiment, Urbaniak et al. were able to use a set of promiscuous human kinase inhibitors immobilized on beads to pull out more than 50 *T. brucei* kinases from cell extracts [123]. The experiments clearly demonstrate that a broad range of human kinase inhibitors can inhibit the kinases of the parasite *T. brucei*. Accordingly, schistosomal kinases should be no different.

The *S. mansoni* genome encodes 357 kinases with representatives homologous to human kinases from each of the kinome subfamilies [124, 125]. Of these, 351 are transcribed in the adult stage, with 268 and 83 representing protein kinases and 83 nonprotein kinases, respectively [125]. Certain human kinase signaling networks, such as the MAPK pathway, seem to be largely recapitulated in the parasite kinome and a number of MAPK pathway members have demonstrated contributions to worm development and reproduction [126]. Even with the success of human kinase drug discovery, many human kinases have been largely ignored and details of their biology are thus poorly understood [127]. This is also the case for the schistosome kinome, although particular functions have been suggested via RNAi, as already noted [126] and inferred by analogy. That stated, there are efforts to explore the schistosome kinome in order to identify potential drug targets. Gasser et al. recently defined the kinome of *S. haematobium*

and predicted essential targets that can be prioritized as potential drug targets [128]. They noted that most kinases of this *S. haematobium* are very similar to the kinases of *S. mansoni*, which means it may be possible to target both species with the same compounds.

In light of the clinical success of human kinase inhibitors and the high likelihood of kinase involvement in critical pathogen biology such as the development of *S. mansoni* reproductive organs, [126, 129] efforts have been made to screen kinase inhibitors in the hope of identifying starting points for schistosomiasis drug discovery programs. Several examples are highlighted here, with structures of the compounds discussed shown in Figure 8.9. Imatinib, the first small molecule ATP competitive kinase inhibitor approved by the FDA, has been shown to inhibit *Sm*Abl2, one of three Abl kinases in *S. mansoni*. Dosing of the compound affected the morphology of the parasite *in vitro*, leading to death. Unfortunately, these results were not recapitulated in an *in vivo* mouse infection model due to impedance of compound efficacy by infection-induced circulating alpha-1 acid glycoprotein [130].

The National Cancer Institute's approved oncology drug set was tested against both schistosomula and adult stages of *S. mansoni* [88]. Several kinase inhibitors had IC_{50} values below 5 μM against both stages. The kinase inhibitors bosutinib, ponatinib, afatinib, vandetanib, and crizotinib all exhibited sub-micromolar IC_{50} values against the larval stage of the parasite. Trametinib and vandetanib (Figure 8.4) demonstrated some *in vivo* activity, reducing worm burdens in mice by 64% and 48%, respectively. Dissous and colleagues sought to take advantage of the similarity of the active sites of the kinases, *Sm*VKR1, *Sm*VKR2, *Sm*IR1, and *Sm*IR2, and found one molecule that could inhibit all four, and thus serve as a multipronged starting point to target schistosomiasis [131]. By screening a set of tyrosine kinase inhibitors, they identified AG1024 as an inhibitor of all four enzymes, albeit at different potency levels. First, this work highlights an advantage of targeting kinases. The active site similarity can be exploited, allowing one to pursue targeted polypharmacology and perhaps exploit two or more parasite vulnerabilities simultaneously, ideally leading to more efficient killing and enhanced *in vivo* activity. Secondly, the study also highlights an area that needs to be addressed. AG1024 is a relatively old kinase inhibitor, and many would not consider it a good lead. A much larger set of kinase inhibitors should be screened against these targets, and when hits emerge, a medicinal chemistry program should be initiated to drive to clinical candidates.

Long et al. identified *Sm*PLK1 as a potential target for treatment of schistosomiasis [132]. *Sm*PLK1 is involved in mitotic processes in the parasite, and is important in parasite reproduction. A human PLK1 inhibitor, BI2536, inhibited *Sm*PLK1 in *Xenopus* oocytes. In addition, dosing of BI2536 led to significant morphology changes in the reproductive organs, consistent with the high expression of *Sm*PLK1 in these organs. This may prove important for schistosome drug discovery as female schistosomes that transmit disease and are primarily responsible for disease pathology produce large numbers of eggs each day. As a follow-up to these initial findings, an expanded set of human PLK1 inhibitors, in part pulled from the GlaxoSmithKline Published Kinase Inhibitor sets 1 and 2 (PKIS1, PKIS2), were screened in phenotypic assays of

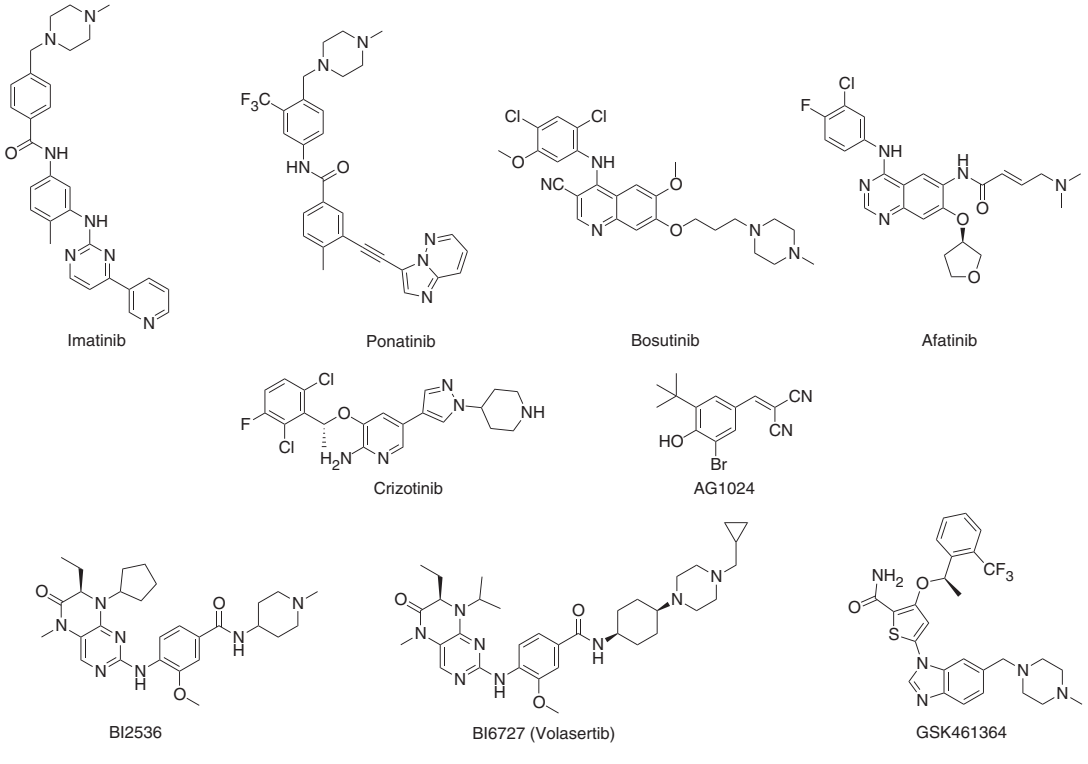


Figure 8.9 Human kinase inhibitors that have been explored as antischistosomals.

both schistosomula and adult parasites [133]. A range of phenotypic descriptors such as changes in movement and changes in shape, translucence, and surface (tegument) integrity were noted and recorded. Based on these microscopical observations, each compound was given a severity score in order to facilitate compound comparison. Importantly, some compounds active against the adult parasites were also active against the schistosomula.

There are several points of particular interest from this study that are worthy of discussion in the context of exploring kinase inhibitors as potential schistosomiasis treatments. Importantly, two different PLK1 inhibitor chemotypes (BI6727/volasertib [pteridinone series] and GSK461364 [benzimidazole thiophene series]) were active in the phenotypic assays. In general, recapitulation of a phenotypic response with a variety of chemotypes that inhibit the same target builds confidence that the phenotypic response is actually due to the target being queried. In addition, this is one of the only schistosome studies where multiple analogs from a particular chemotype have been explored. Screening multiple exemplars in phenotypic assays allows the medicinal chemist to learn the features of the molecules that enhance activity. This is important to guide the synthesis of new and improved molecules.

The clinical success of human kinase inhibition is built on two key features. First, kinases are involved in critical biological processes that lead to the initiation, progression, and/or maintenance of disease. Current literature suggests, although there is much still to learn, that signaling through kinases is vitally important in the life cycle of the schistosome parasite, and thus targeting the appropriate schistosome kinases should lead to death. Second, kinases are eminently druggable. The ATP binding pocket of kinases can be targeted efficiently with small molecules, and these molecules can be designed such that they possess the features that allow progression over the various hurdles that arise in a drug discovery program. These features include cellular potency, adequate selectivity, appropriate physical properties, and pharmacokinetic properties suitable for desired dosing regimens. Selectivity is indeed a major challenge to be overcome, but the medicinal chemistry literature is replete with examples of careful optimization leading to compounds with the appropriate, and sometimes exquisite, levels of selectivity.

In order to successfully bring a parasite kinase inhibitor through to registration and patients, a few recommendations are in order. First, simply screening marketed kinase inhibitors will not get the field to the drug molecules needed. These compounds represent a very small fraction of the human kinase inhibitor chemical diversity explored over the past 20 years or so. At best, these compounds can serve as starting points for discovery projects. Rather, one should screen much larger sets of kinase inhibitors, perhaps in collaboration with industrial partners who have these compounds. Open science appears to be gaining steam, and arguments can be made that open collaboration will make drug discovery faster and more efficient [134, 135]. In addition, full-blown medicinal chemistry projects need to be undertaken using both relevant phenotypic screens and target-based assays for those targets for which there is good evidence of parasite essentiality. Descriptions of many traditional medicinal chemistry campaigns can be found for malaria, for example, but very few for schistosomiasis. Because kinases are so

tractable and the vast and still growing human kinase experience is translatable to the parasite problem, medicinal chemistry programs based on identified targets and integrated with appropriate parasite screens will lead to the creation of molecules that possess the requisite features to become antischistosomal drugs.

8.9 Case Study 1: Biarylalkyl Carboxylic Acids (BACAs) as Antischistosomals

Originally developed as options to prevent long-term complications of diabetes [136], BACAs are also believed to impact metabolic processes involving drug-conjugating liver enzymes that combat cancer [137, 138]. The target enzymes include aldose reductases (ARs) [139] or UDP-glucuronosyltransferases (UGTs) [137, 138]. Because schistosomes live in a hostile environment, they have developed strategies to neutralize toxic products, either produced during intrinsic metabolic processes or due to host responses. Among the many insights into schistosome biology obtained from the genome projects, a number of genes with putative detoxification roles, such as putative AR and UGT orthologs (ARs: Smp_150700, Smp_053220; UGT: Smp_083130), were discovered in *S. mansoni* [43]. Gene expression studies indicated that one of the ARs, Smp_150700, has a function in larval and juvenile stages, whereas the other, Smp_053220, is found in adults (http://www.genedb.org). *S. mansoni* UGT, is also well expressed in adults. Recently performed RNA-seq analyses confirmed the adult-biased expression profiles of Smp_053220 and Smp_083130 [140, 141]. With the help of an established *in vitro* culture system [142, 143], BACA derivatives were tested and modified on the basis of their activity in a phenotypic screening.

From 32 BACA derivatives tested against adult *S. mansoni in vitro*, 18 induced abnormal egg production. Of these, 12 also affected vitality and/or impaired the integrity of the tegument and/or gut as demonstrated by confocal laser scanning microscopy (CLSM; Figure 8.10). Some of these derivatives also caused the separation of females and males, the degeneration of oocytes and/or their mislocalization within the ovary, an accumulation of eggs in the uterus, tumor-like outgrowths, spasm-like convulsions, and/or the disintegration of internal tissue structures. This remarkable variety of phenotypes suggests that one ubiquitously expressed target is being targeted or that different molecules are being engaged.

A parallel study of the AR ortholog from *S. japonicum* aimed at finding an inhibitor targeting this enzyme. A molecule containing two linked anthraquinone scaffolds, called bianthrone, inhibited the activity of the *S. japonicum* AR ortholog inducing >90% mortality of adult worms during a three-day treatment period *in vitro* [145]. Anthraquinones are structurally different from the BACA derivatives, and it is assumed that they bind to different target molecules. However, like the BACA derivative–induced phenotypes in *S. mansoni*, it was interesting to note that bianthrone induced similar phenotypes in *S. japonicum* such as a decrease in vitality and tegumental defects including the formation of surface blebs. Therefore, it is tempting to speculate that bianthrone and some BACA derivatives may share one or more targets or affect the same target processes.

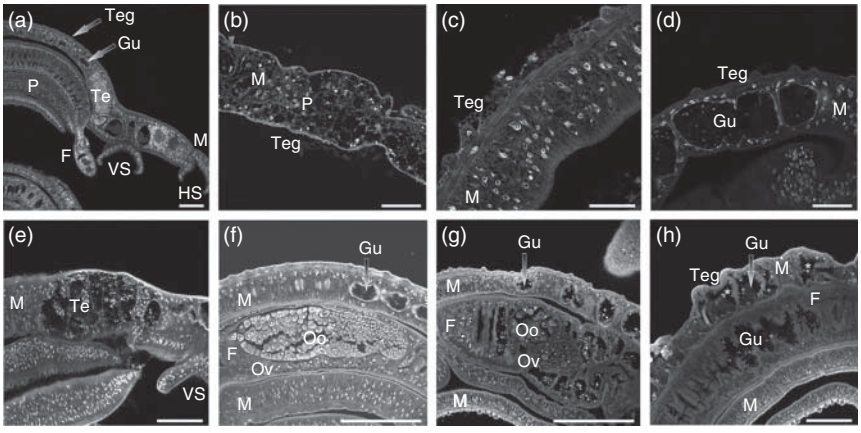


Figure 8.10 Phenotypic alterations induced by biarylalkyl carboxylic acids and arylmethylamino steroids. A selection of phenotypes obtained by incubating adult *S. mansoni* (a, f: controls; a, anterior part of a control couple, untreated; f, close-up of the ovary of a paired adult female, untreated) with derivatives of biarylalkyl carboxylic acids (b–e) or arylmethylamino steroids (g, h; see Section 8.11) and analyzed by confocal laser scanning microscopy [98, 144]. A loss of integrity of inner structures (b), including the testes and the ovary (e, g), was observed. Tegument disruption (c) and/or deformation (h), reduced numbers of spermatogonia (e) or oogonia (g), gut dilatation (d, h), and the formation of aggregates, probably representing precipitates of degraded gastrodermis tissue (h, see asterisks), were also observed. Scale bars: 100 μM.

As to the question whether BACA derivatives engage one or more targets, both scenarios are inherently attractive. In the first case, a function important for schistosome biology and survival may have been shut down. In the alternative scenario, engaging different targets by a single compound, or a compound mix, will diminish the probability of the development of resistance. A prerequisite for the latter would be that more than one gene has to be mutated at the same time under drug selection pressure. In summary, the obtained data suggest that BACA derivatives are fatal to adult *S. mansoni* and, thus, are interesting candidates for further development (see Section 8.10).

With the identification of two schistosomal AR orthologs and the antioxidant requirements of the parasite living in the hostile environment of the vascular system, it is reasonable to speculate that *Schistosoma* AR might also participate in antioxidant pathways and protect the parasite from host reactive oxygen species. Therefore, a selection of inhibitors of human aldose-reductase (hAR; Figure 8.11) available from previous studies was tested for their potential *in vitro* activity against adult *S. mansoni* [136].

All of the compounds tested have a biaryl-4-oxo butanoic acid or biaryl butanoic acid moiety and, with the exception of compound **36**, which lacks an electron-withdrawing substituent in position 3 or 4 of the terminal ring (compounds **37**–**41**). In the absence of recombinantly expressed *Schistosoma* AR, the compound series was tested *in vitro* against adult worms. As assay parameters, pairing stability, gut peristalsis, vitality, motility, and egg production were initially investigated at a concentration of 100 μM [143]. Unlike the control

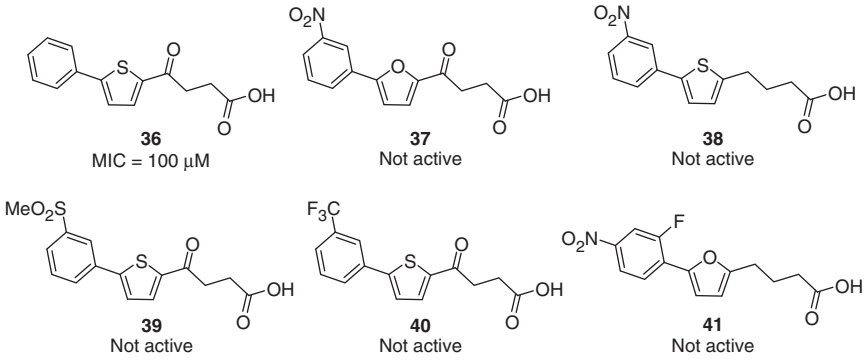


Figure 8.11 Structures of selected human aldose-reductase (hAR) inhibitors that were initially tested against adult *S. mansoni*.

drug, PZQ, which causes tegumental damage and paralysis of treated worms (up to concentrations of 5 μM *in vitro*), the only active compound (**36**, Figure 8.11) produced a different phenotype and significantly reduced egg production (20 versus 2318 eggs counted for the untreated control after 72 hours). Furthermore, the eggs were greatly deformed, which indicates an additional effect on egg synthesis. Likewise, the pairing stability of the worm couples was decreased (only 50% of couples remained paired compared to the control [100%]), whereas gut peristalsis and motility were normal. All other compounds (**37**–**41**) tested were inactive, suggesting that the electron-withdrawing substituents on the phenyl ring led to a loss of activity, which is in contrast to the SARs observed against hAR. Based on these results, BACA derivatives were synthesized (Figure 8.12).

Primarily, the substitution pattern of the phenyl residue was investigated and the following SARs were determined (Figure 8.12, Table 8.1). As shown in the initial compound series tested, electron-withdrawing substituents led to a loss of activity. Electron-donating, lipophilic, aromatic, and hydrophilic residues were introduced and variations of the substitution pattern were performed in all possible positions of the terminal aromatic moiety. Only compounds with substitutions at the meta-position (compounds **42**–**45**) were active; compounds with substitutions at other ring positions were inactive. Introduction of methyl- (**43**) or alkoxy-substituents (**44**, **45**) resulted in activities comparable to that of the parent compound **36**, including a significant decrease in egg production, with the remaining eggs being as deformed as post exposure to **36**. Likewise, the pairing stability of the worm couples was decreased, and gut peristalsis and worm motility were slightly decreased. Moreover, bioisosteric substitution of the phenyl ring with thiophene revealed the same results as the parent compound **36**. Introduction of more spacious, lipophilic residues such as naphthyl led to inactivity. Interestingly, derivatives carrying a hydrophilic 3-hydroxyphenyl moiety (**42**) caused an activity increase and resulted in additional phenotypes. In addition to a decline in egg production after 72 hours at 100 μM, pairing stability decreased within the same period. Furthermore, worms showed slowed gut peristalsis, whereas general motility seemed to be normal. Some males showed slight tegumental

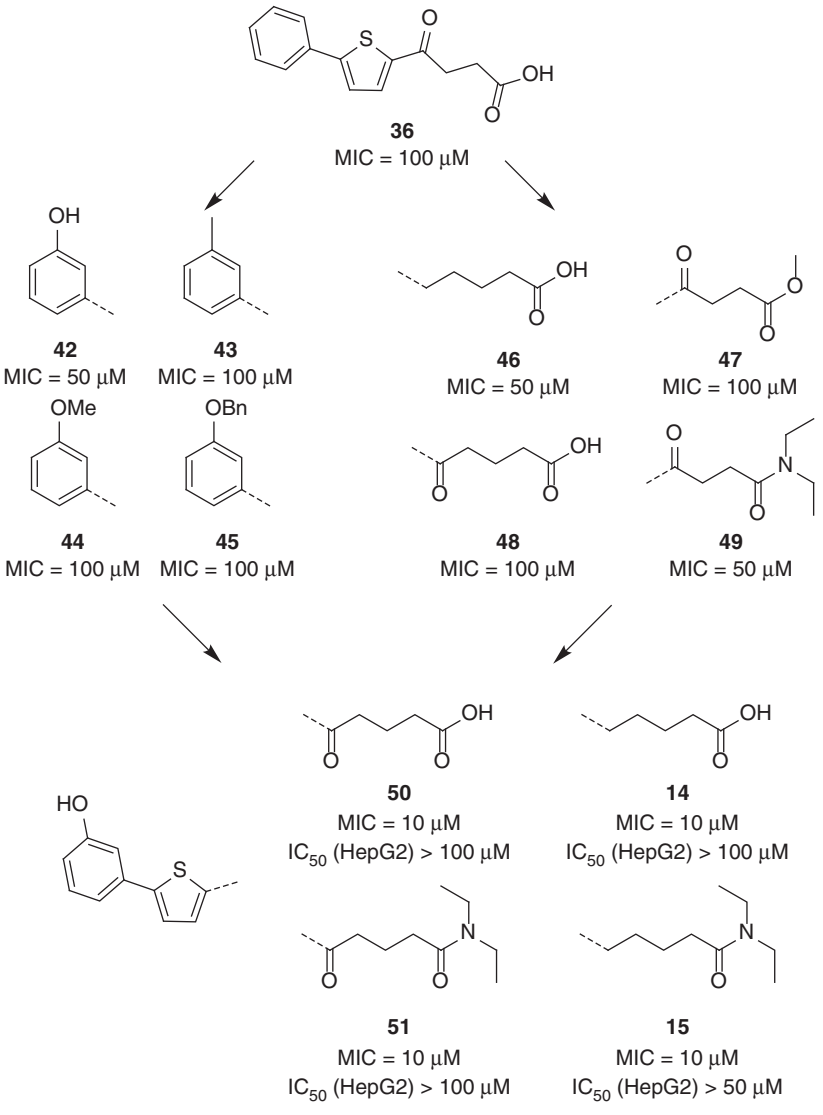


Figure 8.12 Summary of the development of BACA derivatives with *in vitro* activity against adult *S. mansoni*.

damage. Given that a polar residue with a negative inductive effect and a positive mesomeric effect was expected to yield an inhibitor (**42**) with good activity, a corresponding 3-aminophenyl derivative was also synthesized, but was inactive. This finding suggests that deprotonatable groups are tolerated, but not protonatable ones.

At the same time, derivatives of the 4-oxo-butanoic acid partial structure were investigated (Figure 8.12, Table 8.1). Of note, elongation of the alkyl spacer by introduction of one methylene unit (compound **48**) slightly improved activity.

Table 8.1 Overview of the effects of BACA derivatives on *S. mansoni* paired males and females *in vitro*.

		Pairing stability				Vitality				Egg production				Further phenotypes (BFM)			
Compound	**Time (h)\µM**	**100**	**50**	**25**	**10**	**100**	**50**	**25**	**10**	**100**	**50**	**25**	**10**	**100**	**50**	**25**	**10**
36	24	0	0	+	0	0	0	0	0	+++	+++	++	+	0	0	0	0
	48	0	0	0	0	+	0	0	0	+++	++	+	+	*	0	0	0
	72	0	+	0	0	0	0	0	0	+++	+++	+	+	*	0	0	0
42	24	+	0	0	0	+	+	0	0	+++	++	+	+	*	0	0	0
	48	+++	0	0	0	++	0	0	0	+++	+	+	+	***	0	0	0
	72	+++	0	0	0	++	+	0	0	+++	+	+	+	***	0	0	0
43	24	0	—	—	—	0	—	—	—	+++	—	—	—	0	—	—	—
	48	0	—	—	—	0	—	—	—	+++	—	—	—	0	—	—	—
	72	0	—	—	—	0	—	—	—	+++	—	—	—	0	—	—	—
44	24	0	—	—	—	0	—	—	—	++	—	—	—	0	—	—	—
	48	0	—	—	—	0	—	—	—	+	—	—	—	0	—	—	—
	72	+	—	—	—	0	—	—	—	+	—	—	—	0	—	—	—
45	24	0	—	—	—	0	—	—	—	+++	—	—	—	0	—	—	—
	48	0	—	—	—	0	—	—	—	+++	—	—	—	0	—	—	—
	72	+	—	—	—	+	—	—	—	+++	—	—	—	0	—	—	—
46	24	+	0	0	—	0	0	0	—	+	+	0	—	0	0	0	—
	48	0	0	0	—	0	0	0	—	++	+	0	—	*	0	0	—
	72	0	0	0	—	0	0	0	—	++	0	0	—	*	0	0	—
47	24	0	0	0	—	0	0	0	—	++	++	++	—	0	0	0	—
	48	0	0	0	—	+	0	0	—	++	+++	++	—	0	0	0	—
	72	+	0	0	—	0	0	0	—	++	++	++	—	0	0	0	—

Table 8.1 (Continued)

48	24	+	0	0	0	+	0	0	0	+++	+++	++	+	0	0	0	0
	48	+	+	0	0	+	0	0	0	+++	++	+	0	0	0	0	0
	72	++	0	0	0	+	0	0	0	+++	+++	+	+	0	0	0	0
49	24	+++	+++	0	0	+++	++	+	0	+++	+++	++	+	0	**	0	0
	48	+++	++	0	0	++	++	+	0	+++	+++	++	0	*	**	0	0
	72	+++	+++	0	0	++		+	0	+++		++	0	***	**	0	0
50	24	++	+++	+++	0	+++	+++	+++	++	+++	+	+	++	***	***	***	*
	48	—	+++	+++	++	—	+++	+++	++	—	+++	++	++	—	***	***	***
	72	—	—	—	++	—	—	—	++	—	—	—	++	—	—	—	***
14	24	+++	++	+++	0	+++	++	+++	++	++	++	++	++	**	***	***	**
	48	—	+++	+++	+	—	+++	+++	++	—	+++	++	++	—	***	***	**
	72	—	+++	+++	+++	—	+++	+++	++	—	+++	+++	++	—	***	****	**
51	24	++	+++	+++	++	+++	+++	+++	++	+	++	++	++	*	*	0	*
	48	++	+++	+++	+	+++	+++	+++	++	++	+++	++	++	***	**	*	**
	72	+++	+++	+++	++	+++	+++	+++	++	+++	+++	++	++	****	**	**	**
15	24	++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	++	*	**	*	0
	48	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	**	**	***	*
	72	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	***	***	***	*

List of the BACA derivatives that induced effects on pairing stability, vitality, egg production, and further phenotypic changes visible by bright-field microscopy. The number of + marks indicates the strength of the effects: + weak; ++ medium, +++ strong. +++ indicates that 100% of the couples were separated (pairing stability), the vast majority of worms (80–100%) were dead or nearly dead at the time point of observation (vitality), and/or most (80–100%) of the eggs were deformed or no egg production occurred at all; ++ indicates that about 50% of the worms showed the normal phenotype; + indicates that less than 50% of the worms showed the normal phenotype. The number of * marks indicates the quantity of additional phenotypes which occurred in some of the worms treated with the respective BACA derivatives. 0 no abnormality; — not determined.

This provoked the question of whether the carbonyl group of the alkyl spacer is essential for activity. Two derivatives lacking this moiety were synthesized and exhibited different activities. The activity of the pentanoic acid derivative **46** was slightly improved relative to that of the parental compound **36** (no eggs produced within 72 hours, only 2 of 10 couples were paired at 100 μM), but the butanoic acid derivative was less active. Two important insights have been inferred from this finding: a carbonyl group seems not to be essential and elongation of the linker chain increases activity. Due to the loss of the negatively charged carboxylate and the resulting superior membrane permeability, achievement of higher bioavailability was attempted by converting the carboxylic acid moiety to a methyl ester. The ester derivative **46** displayed lower activities than the carboxylic acid compound **36**. The ester moiety may not, or very slowly, be converted into its corresponding carboxylic acid by schistosomal esterases. However, because the free carboxylic acid is a potential interaction partner of the alpha-1-acid glycoprotein (AGP) and other serum proteins [146–148], derivation of the carboxylic acid to *N*,*N*-diethyl carboxamides (e.g. compound **49**) was the subsequent step that resulted in improving activity. At 100 μM, both compounds completely inhibited egg production and pairing stability, and greatly decreased motility, vitality, and gut peristalsis. The pentanoic acid amide **49** was more active than the butanoic acid amide as all treated worms were dead within 24 hours at 100 μM: tegumental damage and gut dilation occurred, in addition to the decrease of pairing stability and egg production. Both compounds were also tested at a lower concentration (50 μM) with comparable results.

Combining the most active residues strongly increased activity with the appearance of new phenotypes (Figure 8.12, Table 8.1). Lethal effects were observed up to 25 μM (the 5-oxo pentanoic acid derivative **50** caused lethality after 48 hours and the pentanoic acid derivative **14** after 72 hours). The phenotypes also included tegumental damage, the loss of internal structures, and decreases in egg production and pairing stability. Both compounds showed satisfactory activity up to 10 μM, although the pentanoic acid derivative **14** was slightly less potent. Again, tegumental damage including blebbing was noted as well as strongly reduced pairing stability, motility, vitality, egg production, and the absence of gut peristalsis. The carboxamides **15** and **51** also showed an improvement in phenotypic activity but were not lethal at any of the tested concentrations. Phenotypic effects included the loss of internal structures, anisocytosis, strong reductions in pairing stability, motility, vitality, and egg production, as well as the absence of gut peristalsis. It can be assumed that the effects on activity were additive.

8.10 Case Study 2: Arylmethylamino Steroids (AASs) as Antischistosomals

Lipophilic aminosteroids such as squalamine [149–152], its analogs, and cholesteryl amines are of therapeutic interest due to their intrinsic antimicrobial activity. In the context of designing novel therapeutics, preferentially

those with new mechanisms of action, steroid-derived hybrid compounds [153] represent a promising approach. In particular, the *de novo* synthesis of steroidal hybrids has come into focus [154–157], whereby the hydrophobic steroid carrier may pave the way for biological action. A screening program to identify compounds with antiprotozoal activity led to the discovery of 3β-amino-22,26-epiminocholest-5-ene **52** (sarachine, Figure 8.13a). This amino steroid naturally occurs in leaves of *Saracha punctata*, a plant species of the family *Solanaceaea* [158]. The spectrum of efficacy of sarachine included the malaria parasite, *P. falciparum*. Follow-up studies showed that modified amino steroids containing, e.g. a chloroquinoline moiety in the side chain contribute to the biological activity of this class of compounds [159]. These findings paved the way for the synthesis of hybrid compounds, with the intended advantage of making use of the hydrophobic steroid unit to improve bioavailability. Among these hybrid compounds are AASs that are active against human and murine malarial asexual blood stages, and the gametocytes of *P. falciparum in vitro*. *In vivo*, AASs were active against *Plasmodium berghei* in a mouse model and inhibited the transmission of parasites to mosquitoes [144].

Due to the dual lipid bilayer-like nature of the schistosome surface, a physiologically active tegument covered with transporters for metabolites like amino acids and glucose [160, 161], it was anticipated that AASs would easily penetrate these parasites. Indeed, *in vitro* studies with adult *S. mansoni* confirmed that AASs exert fatal effects on the morphology, physiology, and survival of female and male worms [144]. These effects were achieved at concentrations similar to PZQ, the gold standard for such comparisons. Among 60 synthesized AAS derivatives, compounds **53o**, **54o**, and **53c,** Figure 8.13b,d) greatly influenced pairing stability, egg production, and viability [144]. The most dramatic effects were observed with compound **54o**, which decreased motility and viability at 1 μM and killed the parasites within three days at 10 μM. Morphological analyses revealed tegumental invaginations and edema-like swellings of the body. By CLSM analyses, an enlarged gut lumen and degradation of the gastrodermis were observed. The latter led to the accumulation of degraded tissue that formed aggregates within the gut lumen (Figure 8.10). In addition, deleterious effects on the gonads were found. Oocytes appeared to be disorganized within the ovary of treated females and the sizes of the testicular lobes were reduced in treated males. Currently, detailed toxicological and pharmacokinetic studies are being carried out. These data will allow for the directed optimization of pharmacokinetic properties of the compounds and further preclinical evaluation.

8.11 Brief Summary of the Drug Development Pipeline

Given the desired TPP for new antischistosomal drugs (Section 8.2) and the limited data for experimental antischistosomal chemotypes, it is difficult to make a confident assessment of the likely success of either drug repurposing or the discovery of a new drug. For drug repurposing, the most promising entities appear to be the semisynthetic artemisinins and the ivermectin analog,

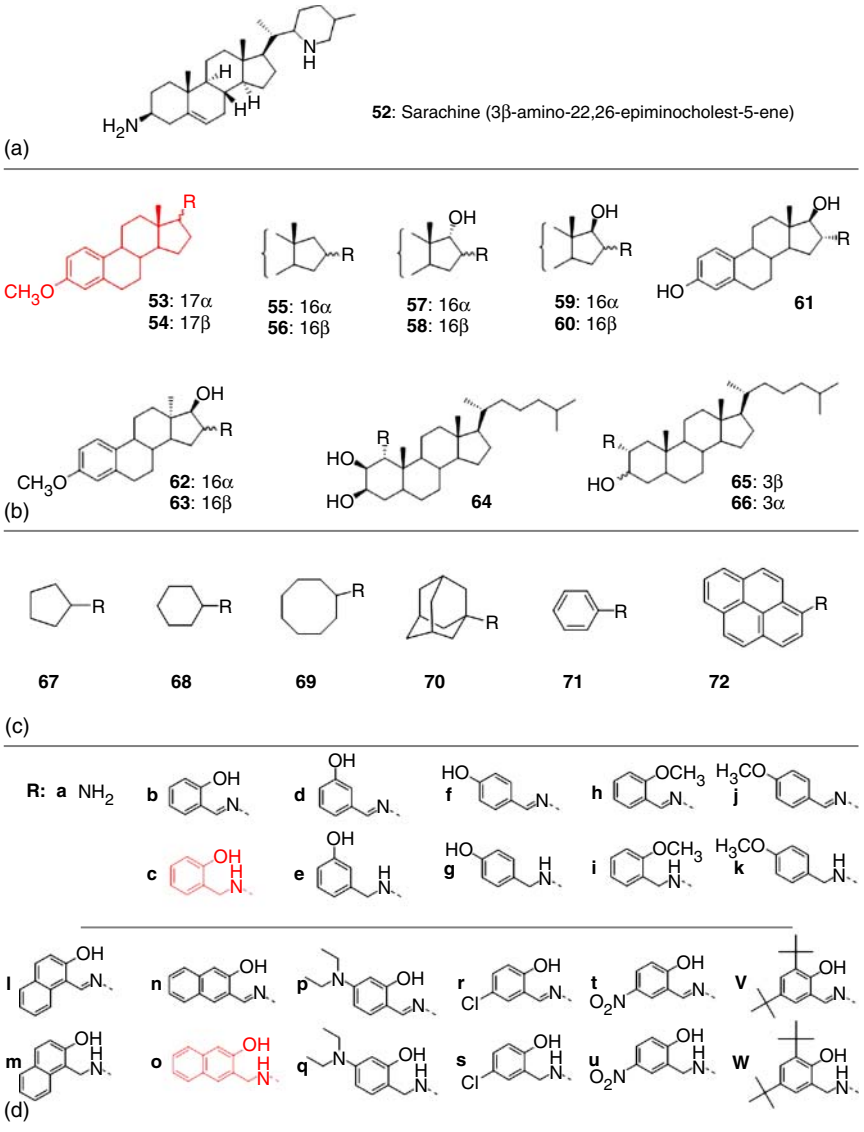


Figure 8.13 Steroids as antischistosomals. (a) Sarachine and (b) arylmethylamino steroid cores investigated, and (d) the formulae of the arylmethyl substituents R. Activity against various organisms depends crucially on the underlying gonane core, the position and configuration of R, and the substitution pattern of R. Current leads are highlighted in red. The ortho-hydroxy function is essential for high activity, suggesting a quinone methide–mediated action associated with metal catalytic effects. Aminosteroids (a) and azomethine precursors (b, d, …) as well as the nonsteroidal analogs (c, series **67**–**72**) were inactive.

doramectin. For new antischistosomal chemotypes, the aryl hydantoins, AASs, BACAs, and the triterpene furanolactone limonin seem to have good potential to move forward. As data regarding metabolism, pharmacokinetics, safety, and *in vivo* antischistosomal efficacy are generated for the most promising

antischistosomal compounds, we anticipate the identification of at least one drug development candidate within the near future.

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References

1 Hessien, M.H., El-Sharkawi, I.M., El-Barbary, A.A. et al. (2010). Non-invasive index of liver fibrosis induced by alcohol, thioacetamide and schistosomal infection in mice. *BMC Gastroenterol.* 10: 53.
2 Steinmann, P., Keiser, J., Bos, R. et al. (2006). Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *Lancet. Infect. Dis.* 6: 411–425.
3 Doenhoff, M.J., Cioli, D., and Utzinger, J. (2008). Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. *Curr. Opin. Infect. Dis.* 21: 659–667.
4 Colley, D.G., Bustinduy, A.L., Secor, W.E., and King, C.H. (2014). Human schistosomiasis. *Lancet* 383: 2253–2264.
5 Hotez, P.J. and Fenwick, A. (2009). Schistosomiasis in Africa: an emerging tragedy in our new global health decade. *PLoS Negl. Trop. Dis.* 3: e485.
6 Ukwandu, N.C. and Nmorsi, O.P. (2004). The perception, beliefs and practices toward genitourinary schistosomiasis by inhabitants of selected endemic areas (Edo/Delta States) in south-eastern Nigeria. *Rev. Inst. Med. Trop. Sao Paulo* 46: 209–216.
7 de Vlas, S.J., Danso-Appiah, A., van der Werf, M.J. et al. (2004). Quantitative evaluation of integrated schistosomiasis control: the example of passive case finding in Ghana. *Trop. Med. Int. Health* 9: A16–A21.
8 Danso-Appiah, A., De Vlas, S.J., Bosompem, K.M., and Habbema, J.D. (2004). Determinants of health-seeking behaviour for schistosomiasis-related symptoms in the context of integrating schistosomiasis control within the regular health services in Ghana. *Trop. Med. Int. Health* 9: 784–794.
9 King, C.H. and Dangerfield-Cha, M. (2008). The unacknowledged impact of chronic schistosomiasis. *Chronic Illness* 4: 65–79.
10 King, C.H., Dickman, K., and Tisch, D.J. (2005). Reassessment of the cost of chronic helmintic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. *Lancet* 365: 1561–1569.
11 Engels, D. and Savioli, L. (2006). Reconsidering the underestimated burden caused by neglected tropical diseases. *Trends Parasitol.* 22: 363–366.

12 Hotez, P.J. and Ferris, M.T. (2006). The antipoverty vaccines. *Vaccine* 24: 5787–5799.

13 Fenwick, A., Webster, J.P., Bosque-Oliva, E. et al. (2009). The Schistosomiasis Control Initiative (SCI): rationale, development and implementation from 2002-2008. *Parasitology* 136: 1719–17130.

14 Fenwick, A. and Webster, J.P. (2006). Schistosomiasis: challenges for control, treatment and drug resistance. *Curr. Opin. Infect. Dis.* 19: 577–582.

15 Pax, R., Bennett, J.L., and Fetterer, R. (1978). A benzodiazepine derivative and praziquantel: effects on musculature of *Schistosoma mansoni* and *Schistosoma japonicum*. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 304: 309–315.

16 You, H., McManus, D.P., Hu, W. et al. (2013). Transcriptional responses of *In vivo* praziquantel exposure in schistosomes identifies a functional role for calcium signalling pathway member CamKII. *PLoS Pathog.* 9: e1003254.

17 Chan, J.D., Cupit, P.M., Gunaratne, G.S. et al. (2017). The anthelmintic praziquantel is a human serotoninergic G-protein-coupled receptor ligand. *Nat. Commun.* 8: 1910.

18 Willcockson, W.S. and Hillman, G.R. (1984). Drug effects on the 5-HT response of *Schistosoma mansoni*. *Comp. Biochem. Physiol.* 77: 199–203.

19 Salvador-Recatala, V. and Greenberg, R.M. (2012). Calcium channels of schistosomes: unresolved questions and unexpected answers. *Wiley Interdiscip. Rev. Membr. Transp. Signal.* 1: 85–93.

20 Fallon, P.G., Cooper, R.O., Probert, A.J., and Doenhoff, M.J. (1992). Immune-dependent chemotherapy of schistosomiasis. *Parasitology* 105 (Suppl): S41–S48.

21 Chitsulo, L., Engels, D., Montresor, A., and Savioli, L. (2000). The global status of schistosomiasis and its control. *Acta Trop.* 77: 41–51.

22 Caffrey, C.R. (2007). Chemotherapy of schistosomiasis: present and future. *Curr. Opin. Chem. Biol.* 11: 433–439.

23 Caffrey, C.R. and Secor, W.E. (2011). Schistosomiasis: from drug deployment to drug development. *Curr. Opin. Infect. Dis.* 24: 410–417.

24 Botros, S.S. and Bennett, J.L. (2007). Praziquantel resistance. *Expert Opin. Drug Discovery* 2: S35–S40.

25 Meyer, T., Sekljic, H., Fuchs, S. et al. (2009). Taste, a new incentive to switch to (R)-praziquantel in schistosomiasis treatment. *PLoS Negl. Trop. Dis.* 3: e357.

26 Mduluza, T. and Mutapi, F. (2017). Putting the treatment of paediatric schistosomiasis into context. *Infect. Dis. Poverty* 6: 85.

27 Rollinson, D., Knopp, S., Levitz, S. et al. (2013). Time to set the agenda for schistosomiasis elimination. *Acta Trop.* 128: 423–440.

28 Bergquist, R., Utzinger, J., and Keiser, J. (2017). Controlling schistosomiasis with praziquantel: How much longer without a viable alternative? *Infect. Dis. Poverty* 6: 74.

29 Coeli, R., Baba, E.H., Araujo, N. et al. (2013). Praziquantel treatment decreases *Schistosoma mansoni* genetic diversity in experimental infections. *PLoS Negl. Trop. Dis.* 7: e2596.

30 Fallon, P.G. and Doenhoff, M.J. (1994). Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in Schistosoma mansoni in mice is drug specific. *Am. J. Trop. Med. Hyg.* 51: 83–88.

31 Ismail, M.M., Attia, M.M., el-Badawy, A.A. et al. (1994). Treatment of schistosomiasis with praziquantel among school children. *J. Egypt. Soc. Parasitol.* 24: 487–494.

32 Lotfy, W.M., Hishmat, M.G., El Nashar, A.S., and Abu El Einin, H.M. (2015). Evaluation of a method for induction of praziquantel resistance in *Schistosoma mansoni*. *Pharm. Biol.* 53: 1214–1219.

33 Ismail, M., Botros, S., Metwally, A. et al. (1999). Resistance to praziquantel: direct evidence from *Schistosoma mansoni* isolated from Egyptian villagers. *Am. J. Trop. Med. Hyg.* 60: 932–935.

34 Stelma, F.F., Talla, I., Sow, S. et al. (1995). Efficacy and side effects of praziquantel in an epidemic focus of *Schistosoma mansoni*. *Am. J. Trop. Med. Hyg.* 53: 167–170.

35 Hotez, P.J., Brindley, P.J., Bethony, J.M. et al. (2008). Helminth infections: the great neglected tropical diseases. *J. Clin. Invest.* 118: 1311–1321.

36 Cioli, D., Pica-Mattoccia, L., Basso, A., and Guidi, A. (2014). Schistosomiasis control: praziquantel forever? *Mol. Biochem. Parasitol.* 195: 23–29.

37 Panic, G., Flores, D., Ingram-Sieber, K., and Keiser, J. (2015). Fluorescence/luminescence-based markers for the assessment of *Schistosoma mansoni* schistosomula drug assays. *Parasit. Vectors* 8: 624.

38 Long, T., Rojo-Arreola, L., Shi, D. et al. (2017). Phenotypic, chemical and functional characterization of cyclic nucleotide phosphodiesterase 4 (PDE4) as a potential anthelmintic drug target. *PLoS Negl. Trop. Dis.* 11: e0005680.

39 Abdulla, M.H., Ruelas, D.S., Wolff, B. et al. (2009). Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening. *PLoS Negl. Trop. Dis.* 3: e478.

40 Spivak, A.Y., Keiser, J., Vargas, M. et al. (2014). Synthesis and activity of new triphenylphosphonium derivatives of betulin and betulinic acid against *Schistosoma mansoni in vitro* and *in vivo*. *Bioorg. Med. Chem.* 22: 6297–6304.

41 Medicines for Malaria Venture. https://www.mmv.org/research-development/open-source-research/open-access-malaria-box (accessed 30 August 2018).

42 The Pathogen Box. https://www.pathogenbox.org/ (accessed 30 August 2017).

43 Berriman, M., Haas, B.J., LoVerde, P.T. et al. (2009). The genome of the blood fluke *Schistosoma mansoni*. *Nature* 460: 352–358.

44 Protasio, A.V., Tsai, I.J., Babbage, A. et al. (2012). A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* 6: e1455.

45 Zhou, Y., Zheng, H., Chen, X. et al. (2009). The *Schistosoma japonicum* genome reveals features of host-parasite interplay. *Nature* 460: 345–351.

46 Young, N.D., Jex, A.R., Li, B. et al. (2012). Whole-genome sequence of *Schistosoma haematobium*. *Nat. Genet.* 44: 221–225.

47 Rojo-Arreola, L., Long, T., Asarnow, D. et al. (2014). Chemical and genetic validation of the statin drug target to treat the helminth disease, schistosomiasis. *PLoS One* 9: e87594.

48 Stefanic, S., Dvorak, J., Horn, M. et al. (2010). RNA interference in *Schistosoma mansoni* schistosomula: selectivity, sensitivity and operation for larger-scale screening. *PLoS Negl. Trop. Dis.* 4: e850.

49 Patocka, N., Sharma, N., Rashid, M., and Ribeiro, P. (2014). Serotonin signaling in *Schistosoma mansoni*: a serotonin-activated G protein-coupled receptor controls parasite movement. *PLoS Pathog.* 10: e1003878.

50 MacDonald, K., Kimber, M.J., Day, T.A., and Ribeiro, P. (2015). A constitutively active G protein-coupled acetylcholine receptor regulates motility of larval *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 202: 29–37.

51 Ittiprasert, W., Mann, V.H., Karinshak, S.E. et al. (2019). Programmed genome editing of the omega-1 ribonuclease of the blood fluke, *Schistosoma mansoni*. *Elife* 15: e41337.

52 Jurberg, A.D. and Brindley, P.J. (2015). Gene function in schistosomes: recent advances toward a cure. *Front. Genetics* 6: 144.

53 Mann, V.H., Suttiprapa, S., Skinner, D.E. et al. (2014). Pseudotyped murine leukemia virus for schistosome transgenesis: approaches, methods and perspectives. *Transgenic Res.* 23: 539–556.

54 Williams, D.L., Bonilla, M., Gladyshev, V.N., and Salinas, G. (2013). Thioredoxin glutathione reductase-dependent redox networks in platyhelminth parasites. *Antioxid. Redox Signaling* 19: 735–745.

55 Keiser, J. and Utzinger, J. (2012). Antimalarials in the treatment of schistosomiasis. *Curr. Pharm. Des.* 18: 3531–3538.

56 Abdulla, M.H., Lim, K.C., Sajid, M. et al. (2007). Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. *PLoS Med.* 4: e14.

57 Chen, G.Z., Foster, L., and Bennett, J.L. (1991). Purification and characterization of 3-hydroxymethylglutaryl-coenzyme A reductase of *Schistosoma mansoni*: regulation of parasite enzyme activity differs from mammalian host. *Exp. Parasitol.* 73: 82–92.

58 Pierce, R.J., Dubois-Abdesselem, F., Lancelot, J. et al. (2012). Targeting schistosome histone modifying enzymes for drug development. *Curr. Pharm. Des.* 18: 3567–3578.

59 Wheeler, N.J., Agbedanu, P.N., Kimber, M.J. et al. (2015). Functional analysis of *Girardia tigrina* transcriptome seeds pipeline for anthelmintic target discovery. *Parasit. Vectors* 8: 34.

60 Sanchez Alvarado, A., Newmark, P.A., Robb, S.M., and Juste, R. (2002). The *Schmidtea mediterranea* database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development* 129: 5659–5665.

61 Caffrey, C.R. (2015). Schistosomiasis and its treatment. *Future Med. Chem.* 7: 675–676.

62 Thetiot-Laurent, S.A., Boissier, J., Robert, A., and Meunier, B. (2013). Schistosomiasis chemotherapy. *Angew. Chem. Int. Ed. Engl.* 52: 7936–7956.

63 da Rocha Pitta, M.G., da Rocha Pitta, M.G., de Melo Rego, M.J., and Galdino, S.L. (2013). The evolution of drugs on schistosoma treatment: looking to the past to improve the future. *Mini Rev. Med. Chem.* 13: 493–508.

64 Njoroge, M., Njuguna, N.M., Mutai, P. et al. (2014). Recent approaches to chemical discovery and development against malaria and the neglected tropical diseases human African trypanosomiasis and schistosomiasis. *Chem. Rev.* 114: 11138–11163.

65 de Moraes, J. (2015). Natural products with antischistosomal activity. *Future Med. Chem.* 7: 801–820.

66 Hess, J., Keiser, J., and Gasser, G. (2015). Toward organometallic antischistosomal drug candidates. *Future Med. Chem.* 7: 821–830.

67 Neves, B.J., Andrade, C.H., and Cravo, P.V. (2015). Natural products as leads in schistosome drug discovery. *Molecules* 20: 1872–1903.

68 Mafud, A.C., Ferreira, L.G., Mascarenhas, Y.P. et al. (2016). Discovery of novel antischistosomal agents by molecular modeling approaches. *Trends Parasitol.* 32: 874–886.

69 Liu, L.X., Li-Li, J., Qiong, C., and Xiao-Lin, F. (2017). Recent advances in the synthesis of antischistosomal drugs and agents. *Mini Rev. Med. Chem.* 17: 467–484.

70 da Silva, V.B.R., Campos, B., de Oliveira, J.F. et al. (2017). Medicinal chemistry of antischistosomal drugs: praziquantel and oxamniquine. *Bioorg. Med. Chem.* 25: 3259–3277.

71 Ramamoorthi, R., Graef, K.M., and Dent, J. (2015). Repurposing pharma assets: an accelerated mechanism for strengthening the schistosomiasis drug development pipeline. *Future Med. Chem.* 7: 727–735.

72 Ferreira, L.G., Oliva, G., and Andricopulo, A.D. (2015). Target-based molecular modeling strategies for schistosomiasis drug discovery. *Future Med. Chem.* 7: 753–764.

73 Keiser, J., Ingram, K., Vargas, M. et al. (2012). *In vivo* activity of aryl ozonides against *Schistosoma* species. *Antimicrob. Agents Chemother.* 56: 1090–1092.

74 Zhang, X.G., Li, G.X., Zhao, S.S. et al. (2014). A review of dihydroartemisinin as another gift from traditional Chinese medicine not only for malaria control but also for schistosomiasis control. *Parasitol. Res.* 113: 1769–1773.

75 Saeed, M.E.M., Krishna, S., Greten, H.J. et al. (2016). Antischistosomal activity of artemisinin derivatives *in vivo* and in patients. *Pharmacol. Res.* 110: 216–226.

76 Xue, J., Wang, X., Dong, Y. et al. (2014). Effect of ozonide OZ418 against *Schistosoma japonicum* harbored in mice. *Parasitol. Res.* 113: 3259–3266.

77 Leonidova, A., Vargas, M., Huwyler, J., and Keiser, J. (2016). Pharmacokinetics of the antischistosomal lead ozonide OZ418 in uninfected mice determined by liquid chromatography-tandem mass spectrometry. *Antimicrob. Agents Chemother.* 60: 7364–7371.

78 Mossallam, S.F., Amer, E.I., and El-Faham, M.H. (2015). Efficacy of Synriam, a new antimalarial combination of OZ277 and piperaquine, against different developmental stages of *Schistosoma mansoni*. *Acta Trop.* 143: 36–46.

79 Barda, B., Coulibaly, J.T., Puchkov, M. et al. (2016). Efficacy and safety of moxidectin, synriam, synriam-praziquantel versus praziquantel against *Schistosoma haematobium* and *S. mansoni* infections: a randomized, exploratory phase 2 trial. *PLoS Negl. Trop. Dis.* 10: e0005008.

80 Cowan, N., Datwyler, P., Ernst, B. et al. (2015). Activities of *N,N′*-diarylurea MMV665852 analogs against *Schistosoma mansoni*. *Antimicrob. Agents Chemother.* 59: 1935–1941.

81 Portela, J., Boissier, J., Gourbal, B. et al. (2012). Antischistosomal activity of trioxaquines: *in vivo* efficacy and mechanism of action on *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* 6: e1474.

82 Ingram-Sieber, K., Cowan, N., Panic, G. et al. (2014). Orally active antischistosomal early leads identified from the open access malaria box. *PLoS Negl. Trop. Dis.* 8: e2610.

83 Neves, B.J., Braga, R.C., Bezerra, J.C. et al. (2015). *In silico* repositioning-chemogenomics strategy identifies new drugs with potential activity against multiple life stages of *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* 9: e3435.

84 Neves, B., Dantas, R., Senger, M. et al. (2016). The antidepressant drug paroxetine as a new lead candidate in schistosome drug discovery. *MedChemComm.* 7: 1176–1182.

85 Whitworth, J.A., Morgan, D., Maude, G.H. et al. (1991). A field study of the effect of ivermectin on intestinal helminths in man. *Trans. R. Soc. Trop. Med. Hyg.* 85: 232–234.

86 Eissa, M.M., Mossallam, S.F., Amer, E.I. et al. (2017). Repositioning of chlorambucil as a potential anti-schistosomal agent. *Acta Trop.* 166: 58–66.

87 Beckmann, S. and Grevelding, C.G. (2010). Imatinib has a fatal impact on morphology, pairing stability and survival of adult Schistosoma mansoni in vitro. *Int. J. Parasitol.* 40: 521–526.

88 Cowan, N. and Keiser, J. (2015). Repurposing of anticancer drugs: *in vitro* and *in vivo* activities against *Schistosoma mansoni*. *Parasit. Vectors* 8: 417.

89 Dissous, C. and Grevelding, C.G. (2011). Piggy-backing the concept of cancer drugs for schistosomiasis treatment: a tangible perspective? *Trends Parasitol.* 27: 59–66.

90 Gelmedin, V., Dissous, C., and Grevelding, C.G. (2015). Re-positioning protein-kinase inhibitors against schistosomiasis. *Future Med. Chem.* 7: 737–752.

91 Sayed, A.A., Simeonov, A., Thomas, C.J. et al. (2008). Identification of oxadiazoles as new drug leads for the control of schistosomiasis. *Nat. Med.* 14: 407–412.

92 Neves, B.J., Dantas, R.F., Senger, M.R. et al. (2016). Discovery of new anti-schistosomal hits by integration of QSAR-based virtual screening and high content screening. *J. Med. Chem.* 59: 7075–7088.

93 Wang, W.-L., Song, L.-J., Hu, B.-C. et al. (2017). Decoquinate derivatives: a new class of potent antischistosomal agents against *Schistosoma japonicum*. *Chin. Chem. Lett.* 28: 1547–1552.

94 Okombo, J., Singh, K., Mayoka, G. et al. (2017). Antischistosomal activity of pyrido[1,2-a]benzimidazole derivatives and correlation with inhibition of beta-hematin formation. *ACS Infect. Dis.* 3: 411–420.

95 Johann, L., Belorgey, D., Huang, H.H. et al. (2015). Synthesis and evaluation of 1,4-naphthoquinone ether derivatives as SmTGR inhibitors and new anti-schistosomal drugs. *Febs J.* 282: 3199–3217.

96 Heimburg, T., Chakrabarti, A., Lancelot, J. et al. (2016). Structure-based design and synthesis of novel inhibitors targeting HDAC8 from *Schistosoma mansoni* for the treatment of schistosomiasis. *J. Med. Chem.* 59: 2423–2435.

97 Jiang, L., Peng, X., Huang, P. et al. (2017). TEMPO-catalyzed oxidative dimerization and cyanation of indoles for the synthesis of 2-(1*H*-indol-3-yl)-3-oxoindoline-2-carbonitriles. *Tetrahedron* 73: 1389–1396.

98 Mäder, P., Blohm, A.S., Quack, T. et al. (2016). Biarylalkyl carboxylic acid derivatives as novel antischistosomal agents. *ChemMedChem* 11: 1459–1468.

99 de Brito, M.R.M., Freitas, R.M., Pelaez, W.J. et al. (2017). Cyclohexene-fused 1,3-oxazines with selective antibacterial and antiparasitic action and low cytotoxic effects. *Toxicol. in Vitro* 44: 273–279.

100 Pereira, A.C., Silva, M.L., Souza, J.M. et al. (2015). *In vitro* and *in vivo* anthelmintic activity of (-)-6,6′-dinitrohinokinin against schistosomula and juvenile and adult worms of *Schistosoma mansoni*. *Acta Trop.* 149: 195–201.

101 Link, H. and Stohler, H.R. (1984). 3-Arylhydantoine, eine substanzklasse mit schistosomizider wirkung. *Eur. J. Med. Chem. – Chim. Ther.* 19: 261–265.

102 Glaser, J., Schurigt, U., Suzuki, B.M. et al. (2015). Anti-schistosomal activity of cinnamic acid esters: eugenyl and thymyl cinnamate induce cytoplasmic vacuoles and death in schistosomula of *Schistosoma mansoni*. *Molecules* 20: 10873–10883.

103 Magalhães, L.G., Lima, T.C., de Paula, R.G. et al. (2016). Effects of (−)-6,6′-dinitrohinokinin on adult worms of Schistosoma mansoni: a proteomic analyses. *Rev. Bras. Farmacogn.* 26: 334–341.

104 Schebb, N.H., Inceoglu, B., Ahn, K.C. et al. (2011). Investigation of human exposure to triclocarban after showering and preliminary evaluation of its biological effects. *Environ. Sci. Technol.* 45: 3109–3115.

105 Yao, H., Liu, F., Chen, J. et al. (2016). Antischistosomal activity of *N,N′*-arylurea analogs against *Schistosoma japonicum*. *Bioorg. Med. Chem. Lett.* 26: 1386–1390.

106 Keiser, J., Panic, G., Vargas, M. et al. (2015). Aryl hydantoin Ro 13-3978, a broad-spectrum antischistosomal. *J. Antimicrob. Chemother.* 70: 1788–1797.

107 Keiser, J., Vargas, M., and Vennerstrom, J.L. (2010). Activity of antiandrogens against juvenile and adult *Schistosoma mansoni* in mice. *J. Antimicrob. Chemother.* 65: 1991–1995.

108 de Mendonça, R.L., Escrivá, H., Bouton, D. et al. (2000). Hormones and nuclear receptors in schistosome development. *Parasitol. Today* 16: 233–240.

109 Wang, C., Zhao, Q., Min, J. et al. (2014). Antischistosomal versus antiandrogenic properties of aryl hydantoin Ro 13-3978. *Am. J. Trop. Med. Hyg.* 90: 1156–1158.

110 Wang, C., Zhao, Q., Vargas, M. et al. (2016). Revisiting the SAR of the antischistosomal aryl hydantoin (Ro 13–3978). *J. Med. Chem.* 59.

111 Khan, M.O., Keiser, J., Amoyaw, P.N. et al. (2016). Discovery of antischistosomal drug leads based on tetraazamacrocyclic derivatives and their metal complexes. *Antimicrob. Agents Chemother.* 60: 5331–5336.

112 de Moraes, J., Dario, B.S., Couto, R.A. et al. (2015). Antischistosomal activity of oxindolimine-metal complexes. *Antimicrob. Agents Chemother.* 59: 6648–6652.

113 Hess, J., Panic, G., Patra, M. et al. (2017). Ferrocenyl, ruthenocenyl, and benzyl oxamniquine derivatives with cross-species activity against *Schistosoma mansoni* and *Schistosoma haematobium*. *ACS Infect. Dis.* 3: 645–652.

114 Silva, M.P., de Oliveira, R.N., Mengarda, A.C. et al. (2017). Antiparasitic activity of nerolidol in a mouse model of schistosomiasis. *Int. J. Antimicrob. Agents* 50: 467–472.

115 Eraky, M.A., Aly, N.S., Selem, R.F. et al. (2016). *In Vitro* Schistosomicidal activity of phytol and tegumental alterations induced in Juvenile and adult stages of *Schistosoma haematobium*. *Korean J. Parasitol.* 54: 477–484.

116 Viegas, F.P.D., de Castro, A.T., Castro, A.P. et al. (2017). *In vitro* schistosomicidal activity of the crude extract, fractions and Primin, the major active benzoquinone constituent from the leaves of *Miconia willdenowii* (*Melastomaceae*). *S. Afr. J. Bot.* 111: 365–370.

117 Silva, A.P., Silva, M.P., Oliveira, C.G. et al. (2015). Garcinielliptone FC: antiparasitic activity without cytotoxicity to mammalian cells. *Toxicol. in Vitro* 29: 681–687.

118 Kang, N.X., Zhu, Y.J., Zhao, J.P. et al. (2017). Antischistosomal activity of hederacochiside C against *Schistosoma japonicum* harbored in experimentally infected animals. *J. Asian Nat. Prod. Res.* 19: 402–415.

119 Mafud, A.C., Silva, M.P., Monteiro, D.C. et al. (2016). Structural parameters, molecular properties, and biological evaluation of some terpenes targeting *Schistosoma mansoni* parasite. *Chem. Biol. Interact.* 244: 129–139.

120 Roskoski, R. FDA approved protein kinase inhibitors. http://www.brimr.org/PKI/PKIs.htm (accessed 6 September 2017).

121 Gamo, F.-J., Sanz, L.M., Vidal, J. et al. (2010). Thousands of chemical starting points for antimalarial lead identification. *Nature* 465: 305.

122 Doerig, C. and Grevelding, C.G. (2015). Targeting kinases in Plasmodium and Schistosoma: same goals, different challenges. *BBA* 1854: 1637–1643.

123 Urbaniak, M.D., Mathieson, T., Bantscheff, M. et al. (2012). Chemical proteomic analysis reveals the drugability of the kinome of *Trypanosoma brucei*. *ACS Chem. Biol.* 7: 1858–1865.

124 Andrade, L.F., Nahum, L.A., Avelar, L.G. et al. (2011). Eukaryotic protein kinases (ePKs) of the helminth parasite *Schistosoma mansoni*. *BMC Genom.* 12: 215.

125 Grevelding, C.G., Langner, S., and Dissous, C. (2018). Kinases: molecular stage directors for schistosome development and differentiation. *Trends Parasitol.*

126 de Andrade, L.F., Mourão, M.D.M., Geraldo, J.A. et al. (2014). Regulation of *Schistosoma mansoni* development and Reproduction by the mitogen-activated protein kinase signaling pathway. *PLoS Negl. Trop. Dis.* 8: e2949.

127 Drewry, D.H., Wells, C.I., Andrews, D.M. et al. (2017). Progress towards a public chemogenomic set for protein kinases and a call for contributions. *PLoS One* 12: e0181585.

128 Stroehlein, A.J., Young, N.D., Jex, A.R. et al. (2015). Defining the *Schistosoma haematobium* kinome enables the prediction of essential kinases as anti-schistosome drug targets. *Sci. Rep.* 5: 17759.

129 Beckmann, S., Quack, T., Burmeister, C. et al. (2010). Schistosoma mansoni: signal transduction processes during the development of the reproductive organs. *Parasitology* 137: 497–520.

130 Buro, C., Beckmann, S., Oliveira, K.C. et al. (2014). Imatinib treatment causes substantial transcriptional changes in adult *Schistosoma mansoni in vitro* exhibiting pleiotropic effects. *PLoS Negl. Trop. Dis.* 8: e2923.

131 Vanderstraete, M., Gouignard, N., Cailliau, K. et al. (2013). Dual targeting of insulin and venus kinase Receptors of *Schistosoma mansoni* for novel anti-schistosome therapy. *PLoS Negl. Trop. Dis.* 7: e2226.

132 Long, T., Cailliau, K., Beckmann, S. et al. (2010). *Schistosoma mansoni* Polo-like kinase 1: a mitotic kinase with key functions in parasite reproduction. *Int. J. Parasitol.* 40: 1075–1086.

133 Long, T., Neitz, R.J., Beasley, R. et al. (2016). Structure-bioactivity relationship for benzimidazole thiophene inhibitors of Polo-Like kinase 1 (PLK1), a potential drug target in *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* 10: e0004356.

134 Shaw, D.L. (2017). Is open science the future of drug development? *Yale J. Biol. Med.* 90: 147–151.

135 Lee, W.H. (2015). Open access target validation is a more efficient way to accelerate drug discovery. *PLoS Biol.* 13: e1002164.

136 Eisenmann, M., Steuber, H., Zentgraf, M. et al. (2009). Structure-based optimization of aldose reductase inhibitors originating from virtual screening. *ChemMedChem* 4: 809–819.

137 Fournel-Gigleux, S., Shepherd, S.R., Carre, M.C. et al. (1989). Novel inhibitors and substrates of bilirubin: UDP-glucuronosyltransferase. Arylalkylcarboxylic acids. *Eur. J. Biochem.* 183: 653–659.

138 Landmann, H., Proia, D.A., He, S. et al. (2014). UDP glucuronosyltransferase 1A expression levels determine the response of colorectal cancer cells to the heat shock protein 90 inhibitor ganetespib. *Cell Death Dis.* 5: e1411.

139 Kang, E.S., Iwata, K., Ikami, K. et al. (2011). Aldose reductase in keratinocytes attenuates cellular apoptosis and senescence induced by UV radiation. *Free Radical Biol. Med.* 50: 680–688.

140 Lu, Z., Sessler, F., Holroyd, N. et al. (2016). Schistosome sex matters: a deep view into gonad-specific and pairing-dependent transcriptomes reveals a complex gender interplay. *Sci. Rep.* 6: 31150.

141 Lu, Z., Sessler, F., Holroyd, N. et al. (2017). A gene expression atlas of adult *Schistosoma mansoni* and their gonads. *Sci. Data* 4: 170118.

142 Knobloch, J., Beckmann, S., Burmeister, C. et al. (2007). Tyrosine kinase and cooperative TGFbeta signaling in the reproductive organs of *Schistosoma mansoni*. *Exp. Parasitol.* 117: 318–336.

143 Beckmann, S., Leutner, S., Gouignard, N. et al. (2012). Protein kinases as potential targets for novel anti-schistosomal strategies. *Curr. Pharm. Des.* 18: 3579–3594.

144 Krieg, R., Jortzik, E., Goetz, A.A. et al. (2017). Arylmethylamino steroids as antiparasitic agents. *Nat. Commun.* 8: 14478.

145 Liu, J., Dyer, D.H., Cheng, J. et al. (2013). Aldose reductase from *Schistosoma japonicum*: crystallization and structure-based inhibitor screening for discovering antischistosomal lead compounds. *Parasit. Vectors* 6: 162.

146 Min, J., Xie, M., Dong, Z. et al. (2004). Xing, C, Spectroscopic studies on the interaction of cinnamic acid and its derivatives with human serum albumin. *J. Mol. Struct.* 692: 71–80.

147 Sudlow, G., Birkett, D.J., and Wade, D.N. (1975). The characterization of two specific drug binding sites on human serum albumin. *Mol. Pharmacol.* 11: 824–832.

148 Israili, Z.H. and Dayton, P.G. (2001). Human alpha-1-glycoprotein and its interactions with drugs. *Drug Metab. Rev.* 33: 161–235.

149 Zasloff, M., Adams, A.P., Beckerman, B. et al. (2011). Squalamine as a broad-spectrum systemic antiviral agent with therapeutic potential. *Proc. Natl. Acad. Sci. U. S. A.* 108: 15978–15983.

150 Jones, S.R., Kinney, W.A., Zhang, X. et al. (1996). The synthesis and characterization of analogs of the antimicrobial compound squalamine: 6 beta-hydroxy-3-aminosterols synthesized from hyodeoxycholic acid. *Steroids* 61: 565–571.

151 Moore, K.S., Wehrli, S., Roder, H. et al. (1993). Squalamine: an aminosterol antibiotic from the shark. *Proc. Natl. Acad. Sci. U. S. A.* 90: 1354–1358.

152 Wehrli, S.L., Moore, K.S., Roder, H. et al. (1993). Structure of the novel steroidal antibiotic squalamine determined by two-dimensional NMR spectroscopy. *Steroids* 58: 370–378.

153 Tietze, L.F., Schneider, G., Wölfling, J. et al. (1998). Efficient synthesis of a novel estrone- talaromycin hybrid natural product. *Angew. Chem. Int. Ed.* 37: 2469–2470.

154 De Riccardis, F., Meo, D., Izzo, I. et al. (1998). Design and synthesis of estrarubicin: a novel class of estrogen-anthracenedione hybrids. *Eur. J. Org. Chem.* 1998: 1965–1970.

155 Ishiki, N., Onishi, H., and Machida, Y. (1997). Conversion characteristics of the conjugates of mitomycin C with estradiol and estradiol benzoate in various pH media. *Chem. Pharm. Bull.* 45: 1345–1349.

156 Siddiqui, A.U., Satyanarayana, Y., and Siddiqui, A.H. (1995). Synthesis of trans β-lactams of estrane series. *Collect. Czech. Chem. Commun.* 60: 1186–1190.

157 Scherlitz-Hofmann, I., Dubs, M., Krieg, R. et al. (1997). Hybridnaturstoffe: synthese cyclischer Hydroxamsäuren der estra-1,3,5(10)-trien-reihe. *Helv. Chim. Acta* 80: 2345–2351.

158 Moretti, C., Sauvain, M., Lavaud, C. et al. (1998). A novel antiprotozoal aminosteroid from *Saracha punctata*. *J. Nat. Prod.* 61: 1390–1393.

159 Sharma, U., Srivastava, K., Puri, S.K., and Singh, C. (2008). Amino steroids as antimalarial agents. *Med. Chem. Res.* 17: 326–334.
160 Wilson, R.A. and Barnes, P.E. (1974). The tegument of *Schistosoma mansoni*: observations on the formation, structure and composition of cytoplasmic inclusions in relation to tegument function. *Parasitology* 68: 239–258.
161 Da'dara, A., Krautz-Peterson, G., Faghiri, Z., and Skelly, P.J. (2012). Metabolite movement across the schistosome surface. *J. Helminthol.* 86: 141–147.

9

Soil-transmitted Helminthiasis – Challenges with Discovery of Novel Anthelmintics

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9.1 Current Therapies and Unmet Needs for Soil-transmitted Helminthiases (STHs)

Soil-transmitted helminthiases (STHs) are responsible for many millions of disability-adjusted life-years (DALYs) in the developing world. The global strategy to control morbidity due to STH infections is preventive chemotherapy with one of the two benzimidazole drugs (albendazole and mebendazole using single 400 and 500 mg doses for adults and children above two years) being periodically administered to at-risk populations [1]. Albendazole and mebendazole were originally developed and licensed as veterinary drugs [2]. In the early 1970s, the first clinical trials were conducted with mebendazole in hookworm-infected patients [3]. Studies with albendazole followed a decade later [4]. Albendazole and mebendazole are donated by the pharmaceutical industry to the World Health Organization (WHO), which is responsible for coordination and overview of country programs [1]. In 2015, coverage of preventive chemotherapy in populations at risk for soil-transmitted helminth infections was almost 60% [5]. In 2015, 711 million (150 million preschool-aged children, 417 million school-aged children, and 144 million women of childbearing age) were treated for STH infections [6]. Both benzimidazoles are generally safe and well tolerated when used appropriately. Albendazole achieves high cure rates and egg reduction rates against infections with *Ascaris lumbricoides* (95.7% and 98.5%) and hookworm infections (79.5% and 89.6%). Lower efficacy is observed against *Trichuris trichiura* infections with a cure rate and an egg reduction rate of 30.7% and 49.9%, respectively. Mebendazole treatment results in high cure rate and egg reduction rate against *A. lumbricoides* (96.2%; 98.0%). Efficacy against *T. trichiura* (cure rate: 42.1%; egg reduction rate: 66%) and hookworm (cure rate: 32.5%; egg reduction rate: 61%) is low to moderate [7].

Levamisole is included in the WHO Model List of Essential Medicines as an intestinal anthelmintic. However the drug was withdrawn from the US

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and Canadian markets in 2000 and 2003, respectively, because of reports of agranulocytosis [8]. Currently, it is primarily used as an anthelmintic medication in veterinary sciences and not used in preventive chemotherapy programs [9]. Levamisole has excellent activity against *A. lumbricoides* but low efficacy against *T. trichiura* and hookworm infections [7].

Pyrantel is another drug on the Essential Medicine List for the treatment of human soil-transmitted helminthiasis. It is a tetrahydropyrimidine derivative first used in humans in the late 1960s [10]. Oxantel is a meta-oxyphenyl analog of pyrantel that was discovered in 1972 by Pfizer as treatment for veterinary helminthiasis and a few years later studied in humans [11]. Both drugs are used as pamoate salts and are very poorly absorbed, hence they act exclusively against intraluminal gastrointestinal nematodes. When used at single oral dosages of 10 mg/kg, pyrantel pamoate achieves low cure rates against hookworm and *T. trichiura* of 49.8% and 20.2%, respectively, while a good cure rate of 92.6% was calculated for the treatment of *A. lumbricoides* [7]. The activity of oxantel pamoate is confined to *T. trichiura*, and recent randomized controlled trials revealed excellent cure and egg reduction rates [12, 13]. However, oxantel pamoate is currently no longer marketed for human health and would need to undergo registration at a stringent regulatory health authority before it could be used in preventive chemotherapy programs.

It is very obvious that the current treatment situation for STH infections is not satisfactory, since only a few drugs (albendazole, mebendazole, and pyrantel pamoate) are available, which have shortcomings in their efficacy profiles. Moreover, drug resistance, widely observed in veterinary medicine, is a threat [7]. To overcome this unsatisfactory situation and to bridge a gap until eventually a new drug would be developed, drug coadministrations are on the forefront for the treatment of STH infections. In early 2017, the combination of albendazole-ivermectin was approved for the treatment of STH infections. Coadministration of albendazole-ivermectin, a treatment widely used for lymphatic filariasis [14], would broaden the spectrum of activity, covering also *T. trichiura* infections. Ivermectin was developed in the early 1980s as a veterinary drug [15] and was repurposed within the Special Programme for Research and Training in Tropical Diseases (TDR), in partnership with Merck Pharmaceuticals, for the treatment of human onchocerciasis following promising results in initial studies [16]. Ivermectin, administered as a single dose of 200 μg/kg, however has also good activity against *T. trichiura* infections and the combination albendazole-ivermectin revealed higher activity against STH infections than treatment with benzimidazole monotherapy [12].

Two alternative treatments that could be used for the treatment of human STH infections as monotherapy or combination chemotherapy in the future are tribendimidine and moxidectin. Tribendimidine was approved for human use by the Chinese Food and Drug Administration in 2004 as an anthelmintic agent against intestinal nematodes with a broad spectrum of activity [17]. It is not yet approved in the United States or Europe; however, efforts are ongoing to achieve Federal Drug Administration (FDA) registration. Tribendimidine is given to children younger than 15 and adults at doses of 200 and 400 mg, respectively. Tribendimidine shows a high cure rate (92–95%) and egg reduction rate (76–98%) against *A. lumbricoides*, moderate to good efficacy against

hookworm (cure rate: 52–88%; egg reduction rate 74–98%), and low efficacy against *T. trichiura* [18, 19].

Moxidectin was first approved for commercial veterinary use as an injectable formulation for cattle in Argentina in 1989. Today, it is produced and marketed in most parts of the world in oral, injectable, and topical forms for animal use [20]. The drug is currently under development as an oral formulation for humans for the treatment of onchocerciasis by Medicines Development for Global Health based in Australia. Preliminary data show a high efficacy against infections with *A. lumbricoides* and *T. trichiura* [21], while hookworm infections are only moderately affected [22].

In summary, millions of people are affected by STH infections, yet the available drugs can be counted on one hand. Of the registered drugs available for the treatment of human STH infections, only albendazole and ivermectin reveal satisfactory efficacy for the treatment of hookworm and *T. trichiura* infections, respectively. Moxidectin, oxantel pamoate, and tribendimidine hold promise as combination chemotherapy drug partners once the drugs have been registered for human use in the United States or in Europe.

9.2 Anthelmintic Research and Development in Animal Health: Value Drivers

As outlined in Section 9.1, there is a major unmet need for new anthelmintics to manage STHs in human health. Unfortunately, the current climate is little changed from the historical situation, where there was no value driver to support investment in research to manage these diseases in humans. This resulted in most modern anthelmintic drugs being leveraged from the animal health industry [23]; for example, the development and subsequent distribution of ivermectin for the management of onchocerciasis, and albendazole for the treatment of lymphatic filariasis and STHs [24, 25].

In 2006, when the Helminth Initiative [26] was first initiated to identify approaches to addressing these challenges, it was clear that investment in research for new treatments, diagnostics and vaccines for human helminth diseases was low when compared to diseases such as malaria and tuberculosis. The Helminth Initiative helped focus academic institutions, companies, and funding organizations on the unmet needs for helminth control, with the aim of focusing research efforts. The recommendation was to have an emphasis on management of schistosomiasis, lymphatic filariasis, and onchocerciasis, leading to a more structured approach to identifying and progressing preclinical leads for these diseases. Recent investments in antifilarial drug discovery by the Gates Foundation, through DND*i* (*Drugs for Neglected Diseases initiative*), outlined in Chapter 7, are another positive step in recognizing and addressing the major impact of helminth diseases in the developing world. However, for STHs, there are still no focused efforts to replace existing drugs, so it is expedient to evaluate whether there are opportunities to leverage from animal health.

To enable the development of new anthelmintics for animal health, there needs to be a value driver, which is unfortunately lacking in human health. The companion animal antiparasitic market is one of the largest therapeutic areas in animal

health, with a market size of $4.2 billion (CEESA[1] MAT [Moving Annual Total] 1st Quarter 2017). Although the majority of these sales are for ectoparasiticides, anthelmintic sales are still respectable, with $1.8 billion of these sales for endo- and endectoparasiticides. The livestock market is driven by ruminants (primarily cattle); in a $1 billion cattle parasiticide market, $704 000 sales are for endo- and endectoparasiticides (CEESA 4Q2013-2016). Recognizing that CEESA only reports sales through the veterinary channel, overall sales are significantly higher.

The economic impact of helminth infections on livestock, especially cattle and sheep, production is well documented [27, 28]. In companion animals, helminth infections are mainly a concern for younger animals, with highest occurrence in dogs under six months old and cats less than 18 months old [29], with prevalence from 5% to 70% globally [30]. Clinically, symptoms can vary from zero to critical (emaciation, anemia, death), and the zoonotic risks associated with some helminths are an additional concern. Heartworm infections are a major concern globally, including all 50 states in the United States, where year-round heartworm protection is recommended [31], to avoid the lengthy and potentially risky treatment of adult heartworm infections.

Since the discovery of ivermectin and subsequent introduction of other related macrocyclic lactones (MLs) in the 1980s and 1990s [32], this "wonder drug" has dominated the market for 30 years [33] for both livestock and companion animal endectoparasite control. The unfortunate downside to this was that expectations for long-acting parasiticides, with the ability to control multiple species of internal and external parasites, became the standard and investment in novel classes of anthelmintic was reduced, or halted. Exacerbating this was the fact that, although multidrug resistance in sheep and goats has been a problem for many years, there was little evidence for resistance in cattle emerging in parallel. Early reports of nematode resistance to MLs in cattle in New Zealand [34] was not considered to be a significant issue until it became clear that resistance was appearing in cattle nematodes in major markets [35–39]. More recently, increasing reports of refractory canine heartworm isolates in the Mississippi Delta in the United States, has been confirmed as resistance to MLs [40–42]. This is a major concern to veterinarians and pet owners, as the only heartworm preventives currently available in major markets are MLs. This increasing anthelmintic resistance in both livestock and companion animal helminths is important in stimulating research into new anthelmintic classes in animal health. However, to contextualize this, we need to recognize that, although antiparasitic pharmaceuticals are the major market for animal health, only a few companies are investing in research, of which, only a small percentage is invested in anthelmintic research. In 2007, there were only nine major animal health companies with significant investment in research; further consolidation in the following years has only reduced this number further (Figure 9.1).

This review highlights that the relatively low investment in anthelmintic research is reflected in the limited number of new anthelmintics marketed since the advent of the MLs. In previous reviews [23, 43, 44] we outlined

1 CEESA is a nonprofit international association based in Belgium that collects sales data on the animal health market.

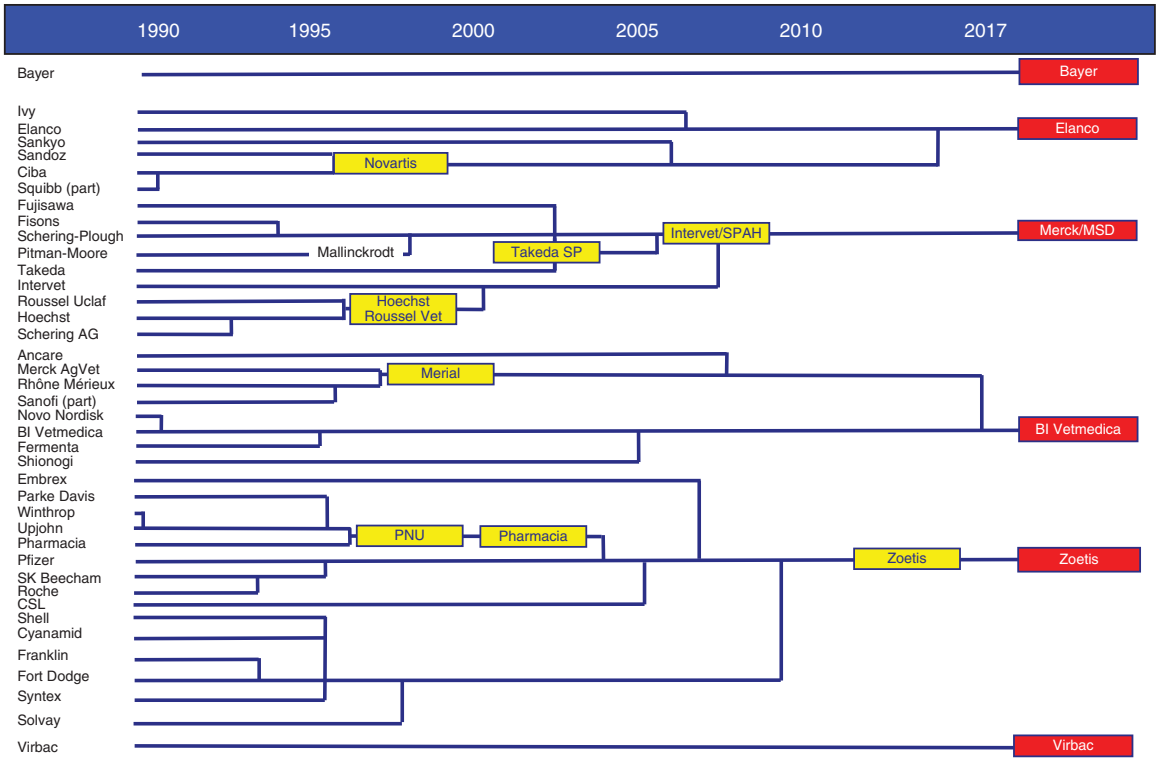


Figure 9.1 Consolidation of animal health companies over time (not to scale and not anchored in years).

some of the challenges of research and development (R&D) for antiparasitic pharmaceuticals, including anthelmintics. Ten years ago, only nitazoxanide [45] and emodepside [46] had been marketed as new anthelmintics in the previous couple of decades. Nitazoxanide has not been widely used, perhaps due to spectrum limitations [47]. Emodepside is marketed in a topical combination with praziquantel, by Bayer, for the control of gut intestinal (GI) nematodes and tapeworms in dogs and cats (discussed further in Section 9.3.2.3). Subsequently, two new anthelmintics were launched for control of GI nematodes in sheep, monepantel (Zolvix® from Bayer) [48, 49] and a combination of the novel anthelmintic, derquantel, with abamectin (Startect® from Zoetis) [50, 51]. Although it was promising to see these new anthelmintics launched, no new anthelmintic classes for Animal Health have been commercialized since 2010.

9.3 Anthelmintic Discovery: State of the Art (2005–2017)

In this section, we aim to update earlier reviews [23, 44] and provide a review of the state of the art in anthelmintic drug discovery from 2005 onwards, with a particular focus on medicinal chemistry approaches, highlighting recent advances in the progression of three of these novel anthelmintic classes. The focus of this review is on the potential of leveraging compounds discovered by industry, but there are also substantial efforts ongoing in academia [52].

9.3.1 New Molecules from the Patent Literature

In Table 9.1, the number of patents disclosing new molecules that claim anthelmintic activity, from 2005 to 2016, are listed (note that the methodology

Table 9.1 Patent landscape: 2005–2017.

Year of priority filing	Anthelmintic patents containing new molecules
2005	3
2006	1
2007	9
2008	5
2009	7
2010	9
2011	8
2012	2
2013	16
2014	15
2015	6
2016	1

for this patent review is different from that utilized for the evaluation of the patent landscape in the Woods et al. 2007 paper [23], which included all members of a family in all countries, versus each individual family).

There are four chemical classes that stand out as having been most heavily patented during the 2005 to 2017 period. There were seven aminoacetonitrile derivative (AAD) patents, seven "multicyclic" patents, and five patents for both the biarylethylamides (BAEAs) and carboxamides published during the 2005 to 2017 time period. There was a peak in the number of patents filed in 2013–2014, with four multicyclic patents appearing in 2013, with four BAEA patents being published in 2014. The multicyclics are discussed in Section 9.3.2.1. Structures of representative example molecules from each of the AAD and BAEA patents are shown in Figure 9.2.

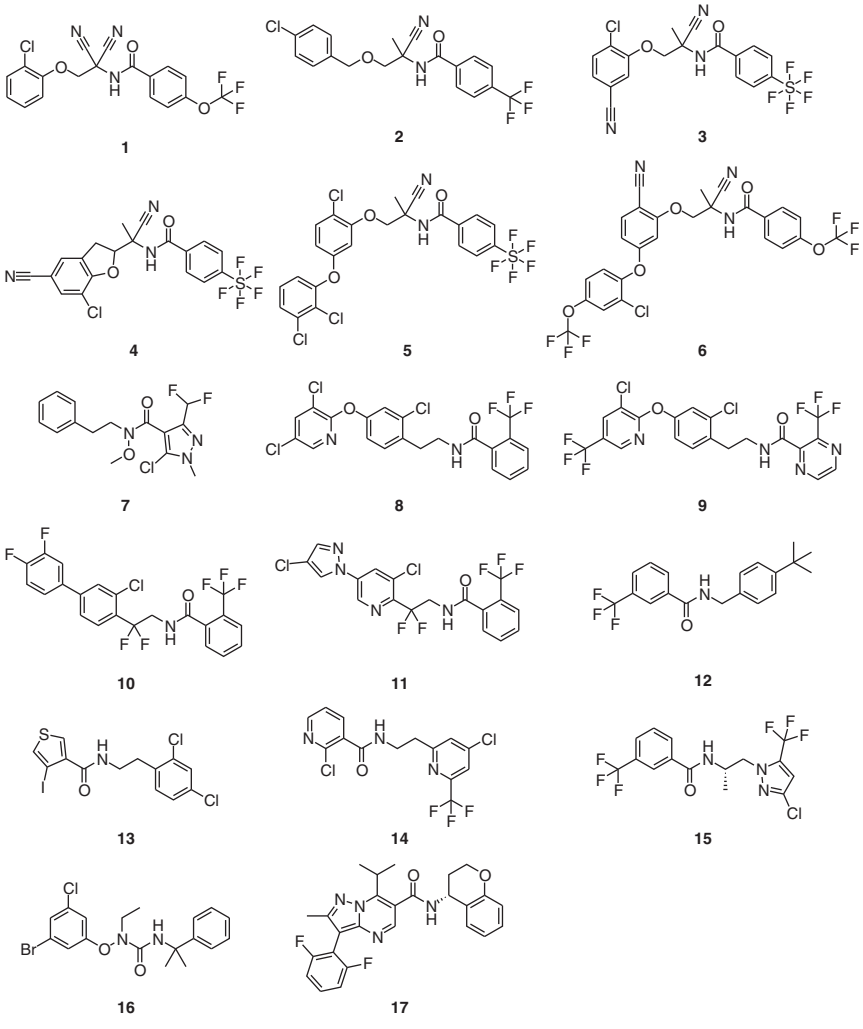


Figure 9.2 Structures of recently patented anthelmintic molecules.

The AADs consist of molecules containing either an aminomalononitrile moiety or aminoacetonitrile moiety and are structurally related to the Novartis marketed anthelmintic monepantel [48]. In 2007, Novartis disclosed anthelmintic activity of AADs [53], where malononitrile **1** was reported to have *in vivo* activity against *Trichostrongylus colubriformis* and *Haemonchus contortus* in Mongolian gerbils (jirds), giving greater than 80% efficacy at 32 mg/kg when administered orally. A follow-up patent from Novartis [54] focused on the key structural change from an amino-malononitrile linker to an amino-acetonitrile linker. Novartis' AAD patent detailed both *in vitro* and *in vivo* activity against *T. colubriformis* and *Haemonchus contortus* from compounds such as amino-acetonitrile **2**. In 2008, Zoetis filed follow-on patents, where pentafluoroaryl AAD **3** and benzofuran AAD **4** exhibited *in vitro* activity against *H. contortus* showing a minimum effective dose (MED) = 1 μg/ml and MED < 1 μg/ml, respectively [55]. In 2010, Novartis filed patents incorporating an oxydibenzene moiety where a phenoxy moiety had been appended as an aryl substituent. Both oxydibenzene AADs **5** and **6** showed >90% efficacy against *T. colubriformis* and *H. contortus* at 0.1 ppm [56].

In 2014, a Bayer patent published containing pyrazole BAEA **7**, which exhibited a minimum lethal dose (MLD) of 80% at 20 ppm against *H. contortus* [57]. In 2016, BAEAs **8** and **9** from the University of Tokyo were patented [58]; both compounds had *in vitro* activity against *H. contortus* at 1–10 ppm. In 2016, Bayer reported [59] that compound **10** had *in vitro* activity against *Cooperia curticei*, *H. contortus*, and *Dirofilaria immitis*. *In vivo* efficacy of >90% against *T. colubriformis* and *H. contortus* in gerbils at 10 mg/kg subcutaneously was also observed. In the same year, Bayer reported [60] that the pyrazole BAEA **11** showed *in vitro* activity against *C. curticei*, *H. contortus*, and *Nippostrongylus brasiliensis* and *in vivo* efficacy >90% against *T. colubriformis* and *H. contortus* at 5 mg/kg when administered subcutaneously in gerbils.

The University of Tokyo was listed assignee on a patent application that appeared in 2014 [61] containing carboxamides such as **12**, which exhibited *in vitro* activity against *H. contortus* L3 stage of between 40% and 69% migration inhibition at 1 ppm. In 2013, Bayer disclosed a patent [62] that detailed *in vitro* activity against *N. brasiliensis, Heligmosomoides polygyrus*, *H. contortus*, and *C. curticei. In vivo* efficacy against *H. polygyrus* in mice of 100% at 100 mg/kg and against *H. contortus* in sheep of 81% at 20 mg/kg was observed with thiophene **13**. In 2014, the University of Tokyo patented the representative carboxamide **14** [63], which was found to have *in vitro* activity against *H. contortus,* with an EC_{50} of 0.5 ppm. Another patent from the University of Tokyo in 2015 [64] also showed *in vitro* activity against *H. contortus* with EC_{50} values of 0.5 ppm or lower; a representative example is the pyrazole **15**. In 2017, Nippon Soda filed a patent application [65] disclosing compounds such as urea **16**, which had *in vitro* activity against *Ascaridia galli* and *Oesophagostomum dentatum* with MEDs of less than 50 μM, in addition to having *in vivo* efficacy of >80% against *H. contortus* in jirds at 10 mg/kg.

A patent application recently published from Bayer [66] described pyrazolopyrimide derivatives such as **17**, which exhibited *in vitro* activity against *Ni. brasiliensis, C. curticei, H. contortus, Litomosoides sigmodontis*, both the L1

and L4 stages of *D. immitis*, and had *in vivo* efficacy against *H. contortus* and *T. colubriformis* in jirds at oral doses ≤50 mg/kg.

9.3.2 Medicinal Chemistry Approaches to New Molecules

9.3.2.1 Intervet Multicyclics

Scientists at Merck recently reported the discovery of drug candidate **18** [67] (Figure 9.3), which showed anthelmintic efficacy against *H. contortus* when given orally to sheep, with a 96% reduction in *H. contortus* worm burden at a dose of 50 mg/kg. Compound **18** was discovered by a lead optimization campaign using high-throughput screening lead **19** as a starting point.

A high-throughput screen of approximately 160 000 compounds against *A. galli* L3 stage at a single concentration identified around 8000 primary hits, which was then narrowed to 960 secondary hits after a single concentration screen against *A. galli* L3 stage and *O. dentatum* L4 stage. Finally, a dose titration of the 960 hits against *A. galli* L3 stage and *O. dentatum* L3 and L4 stages resulted in 480 tertiary hits representing 0.3% of the original 160 000 library. After removal of compounds with known toxicity or high reactivity, the tertiary hits were then classified into 61 structural classes, 171 singleton compounds, with 13 distinct phenotypes within the different larvae of *A. galli, O. dentatum*, and *H. contortus*. The lead compound **19** was selected from the hits and a medicinal chemistry program initiated.

Compound **19** was split into five constituent parts (Figure 9.4): the terminal aromatic rings **a** and **e**; the diamines **b** and **d**, and the central dicarboxylic acid **c**. Compounds that were as structurally diverse as possible from the lead were synthesized first in order to rapidly determine the pharmacophore. Changing the central aromatic linker **c** (as shown in structure **20**) to alternative aliphatic linkers such as 1,5-pentanedioic acid or 1,3-cyclohexanedioic acid gave more active compounds when compared to 1,3-benzenedioic acid derivatives, whereas *trans*-1,4-butenedioic acid linked derivatives were less active.

Desymmetrization of compound **19** could be achieved by breaking bonds in rings **b** or **d** giving a generic structure **21**, or by excluding one of the two carboxamide carbonyl groups in **19**. The Merck scientists showed that by combining two different aromatic amines as rings **b** and **d** with a monoacid replacing a diacid in linker **c**, the orientation of the remaining carbonyl group triggered functional activity. An example of this was that compounds such as monocarboxamide **22** were, on average, more active than compounds derived from monocarboxamide **23** (Figure 9.5). Other changes such as substitution of the left-hand side diamine

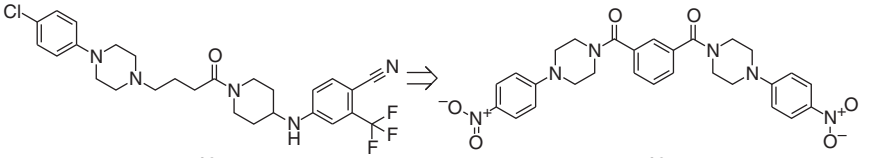


Figure 9.3 Discovery of drug candidate **18** from lead **19**.

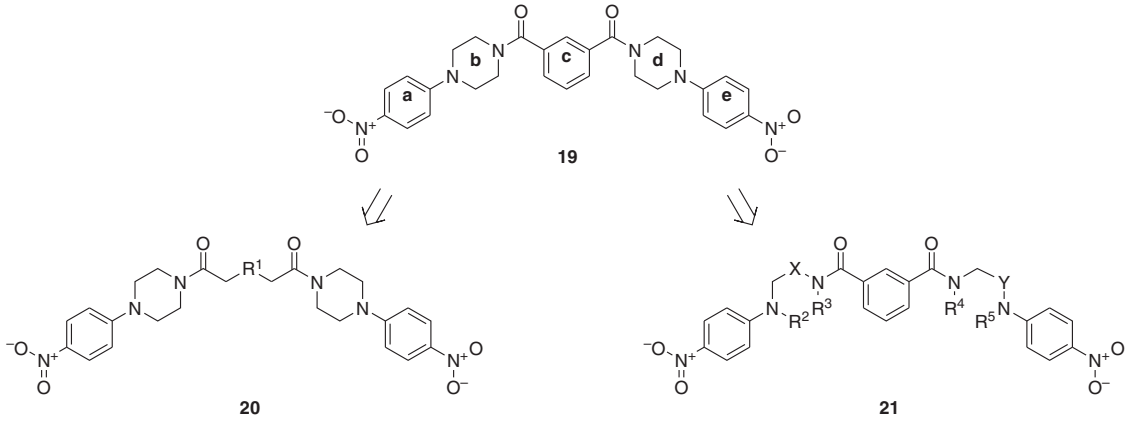
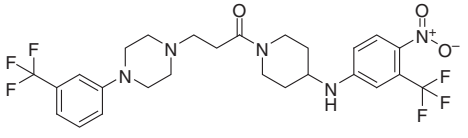


Figure 9.4 Optimization of lead **19**.



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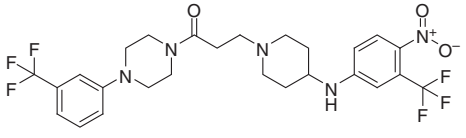

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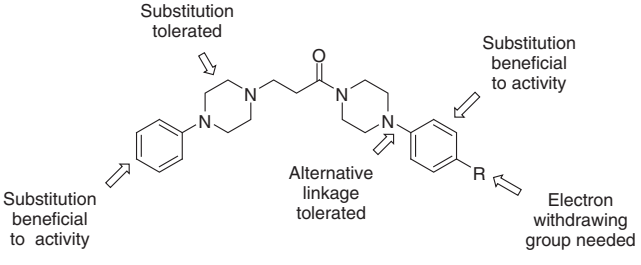

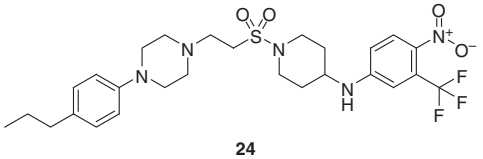

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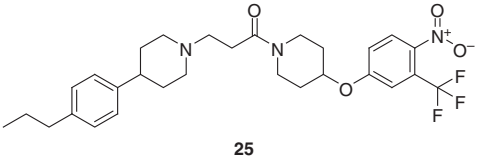

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Figure 9.5 Multicyclic structure–activity relationships.

to the attached aromatic ring were tolerated, but had no significant influence on the functional activity. Additional modifications to the linker and ring moieties resulted in compounds such as **24** and **25**, with excellent anthelmintic activity in all species.

In 2014, Merial filed follow-on patents in the multicyclic area [68]. Compounds such as multicyclic **26** (Figure 9.6), where a fused ring system provided novelty over Intervet's earlier patents, were described. *In vitro* activity against resistant isolates of *H. contortus*, *in vitro* activity against *Cooperia oncophora*, and *in vitro* activity against *D. immitis* were reported. *In vivo* activity against *H. contortus* in jirds giving >90% reduction at 30 mg/kg via oral gavage was observed. Permeability of compounds such as **26** in the Caco-2/TC7 *in vitro* model for permeability showed increased permeability over chosen Intervet compounds. The same year Novartis also filed a follow-on patent in the multicyclic area [69]. Pyrimidine **27** is a representative example, with the six-membered heterocyclic central ring being the structural feature that distinguishes the Novartis contribution over the **c** ring from the linkers bridging rings **b** and **d** in Intervet compounds **19**–**25**, i.e. the prior art. Compounds such as pyrimidine **27** exhibited *in vitro* activity above 50% at 30 ppm against *D. immitis* microfilaria, and compound **28** showed >90% efficacy against *Acanthocheilonema viteae* in gerbils at 3 mg/kg orally. The following year, Novartis filed a patent application [70] that detailed Markush structures such as **29**, with a six-membered heterocycle along with a carbonyl linker being the point of novelty. *In vitro* activity against both *H. contortus, T. colubriformis* and *D. immitis* were observed, and in the case of pyrimidine **30**, *in vivo* efficacy against *A. viteae* in gerbils of >80% at 3 mg/kg when administered subcutaneously. In 2016, Merial filed a patent application [71] on compounds such as pyridine **31**, with the **c** ring linker being pyridine and the piperazine R substituent being amide, heteroaryl, carbonate, alkyl, urea, thiourea, or saturated heterocycle. *In vitro* activity against both *H.contortus* and *D. immitis* was exemplified, in addition to activity in the Caco-2/TC7 *in vitro* model for permeability. Compound **32** showed *in vivo* efficacy of 99% against *T. colubriformis* and *H. contortus* in jirds when administered at a dose of 5 mg/kg by oral gavage. In the same year, Intervet published a patent application [72] that described how compounds disclosed in earlier applications and references [66, 73–75] had activity against both the L1 and L4 stages of *D. immitis*, with compounds such as amide **33** being active in against the L4 stage of *D. immitis* of MIC100 < 5 μM (concentration at which 100% of nematodes were killed).

9.3.2.2 Vesicular Acetylcholine Transporter (VAChT) Inhibitors

In 2012, Syngenta Crop Protection identified spiroindolines (Figure 9.7) as a new class of potential insecticides and anthelmintics [76]. This class of molecules acts through inhibition of neuromuscular vesicular acetylcholine transporters (VAChT), thereby possessing a different mechanism of action (MOA) than existing insecticide and anthelminthic drugs such as the isoxazolines [77, 78], MLs [79], or aminoacetonitrile derivatives (AADs) [49].

The Syngenta spiroindolines were initially identified through high-throughput screening, with compound **34** found to have significant insecticidal activity against *Drosophila melanogaster*, *Heliothis virescens* (tobacco budworm), and

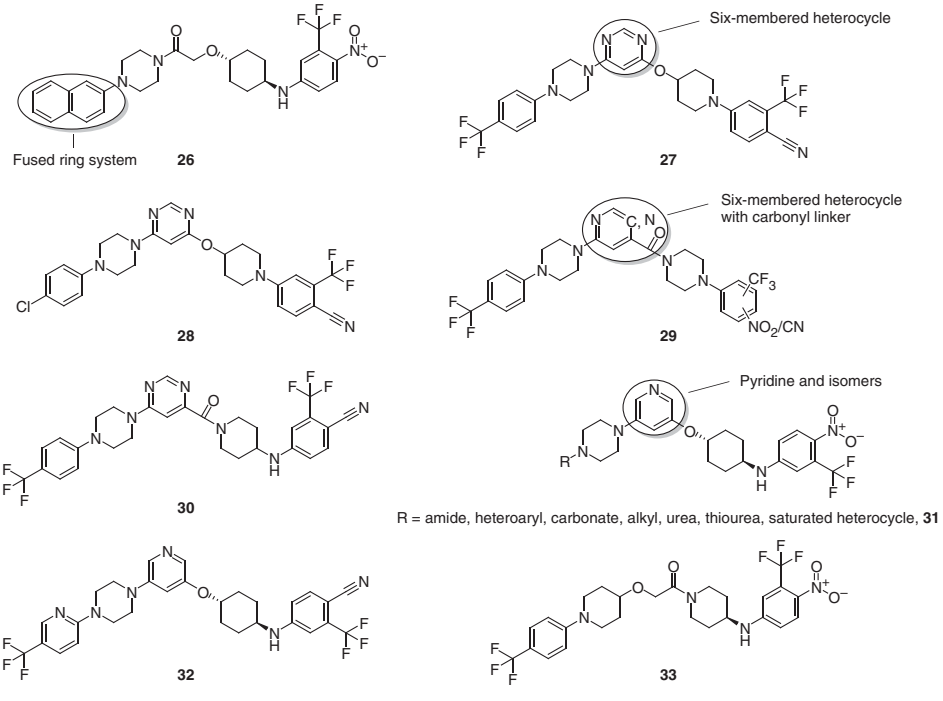


Figure 9.6 Representative multicyclic follow-on structures from the patent literature.

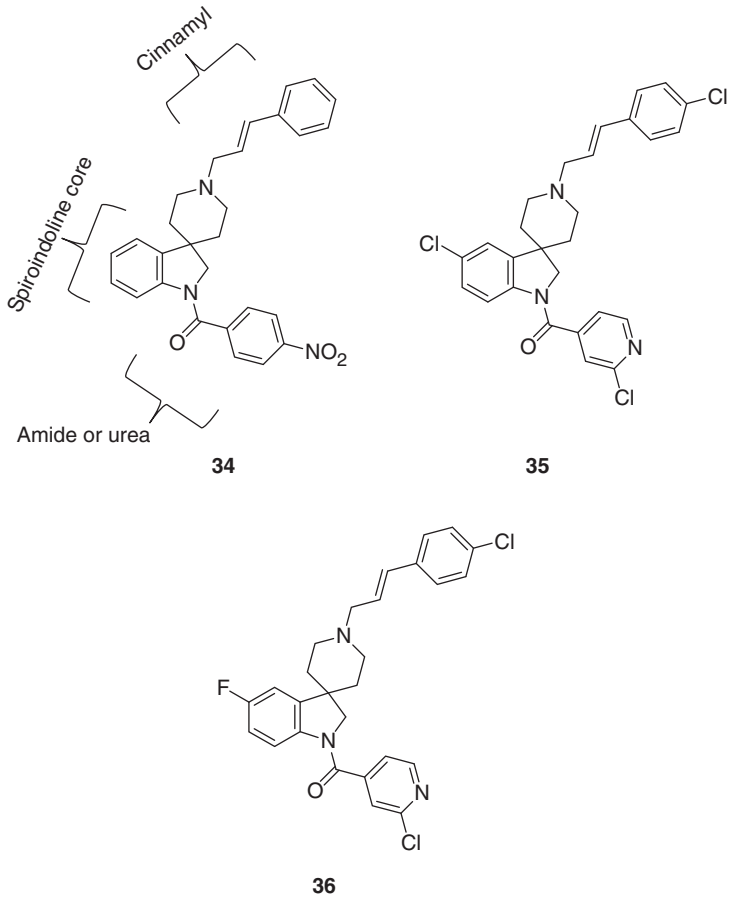
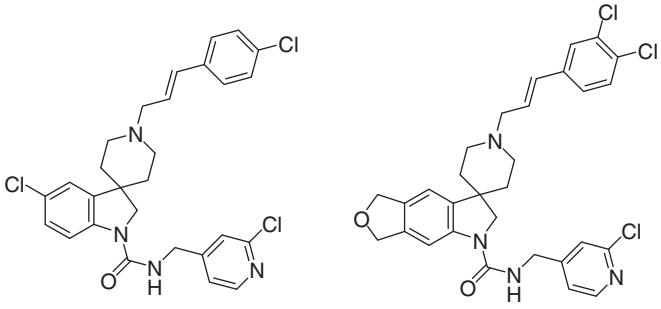


Figure 9.7 Structures of Syngenta spiroindolines.

Plutella xylostella (diamond back moth). Synthetic structural modifications by Syngenta chemists resulted in more potent and selective lead compounds (**35** and **36**). First, the nitro group on the amide portion of compound **34** was replaced by an isonicitinoyl moiety, giving increased *in vitro* potency and photostability. A second structural modification was the addition of halogen atoms (F or Cl) to the phenyl rings of the cinnamyl portion and spiroindoline core of the molecule. This also resulted in enhanced insecticidal potency, thought to be from increased metabolic stability. Lead compound **36** was progressed to field studies and exhibited insecticidal activities comparable to the commercial standard spinosad. In addition, lead spiroindolines were shown to have anthelmintic activity against *Caenorhabditis elegans*, a roundworm found in temperate soil environments, and demonstrated favorable acute oral toxicity in rats ($MLD_{50} > 200$ mg/kg).

In 2015 and 2016, Zoetis submitted patent applications of spiroindolines and γ-carbolines in this class. The first Zoetis patent application included bicyclic and monocyclic spiroindolines with appended urea moieties [80]. Although a



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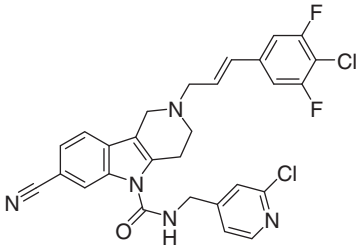

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Figure 9.8 Structures of Syngenta and Zoetis spiropiperidine urea and γ-carboline.

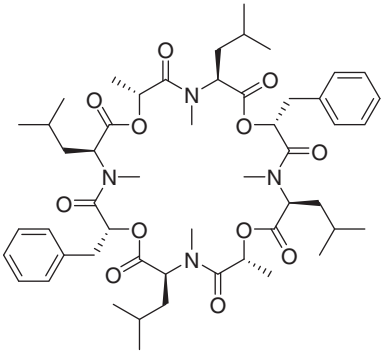
few mono-spiropiperidine ureas were previously published by Syngenta, the Zoetis patent application claimed new urea compounds possessing VAChT selectivity in nematode versus bovine species, with examples ranging from 4- to 6000-fold selectivity in radioligand assays. Since inhibition of the VAChT protein results in neuromuscular paralysis and ultimate toxicity, nematode-selective molecules would likely translate to safer molecules in target species. Compound **38**, an example of a bicyclic spiropiperidine urea from Zoetis, has a bovine Ki of 70 nM versus 1.4 nM for the Syngenta urea **37** (Figure 9.8).

Compound **38** also is reported to have efficacy against *H. contortus*, L3 stage, in a motility assay at 1 μM. It appears that addition of steric bulk to the spiroindoline core and cinnamyl portion of the molecule are responsible for subtle differences in species selectivity. Zoetis also reported γ-carbolines as a new series of VAChT inhibitors with parasiticidal activity in 2016 [81]. Compound **39**, a carboline with a cyano moiety at the 7-position of the carboline core, is reported to have sub-micromolar (0.2 μM) *H. contortus* L3 activity and exhibits 100% efficacy in an *in vivo* infection model against *T. colubriformis* at 10 mg/kg in jirds. Although structurally similar to the spiroindolines, the piperidine moiety of the γ-carboline has a less basic nitrogen ($pK_a = 7.6$ versus 8.8[2]), which could result in different pharmacokinetic properties between the two series. Also, it appears that substitutions on the phenyl ring of the carboline core and cinnamyl portion are again important to anthelmintic activity, as seen in the spiroindoline examples.

2 Calculated property using ChemBioDraw Ultra v.14.0.

9.3.2.3 Cyclooctadepsipeptides

PF1022A **40** (Figure 9.9), an active metabolite of the fungus *Mycelia sterilia*, was identified in the early 1990s, as a broad-spectrum anthelmintic with low toxicity in animals [82]. This 24-membered cyclooctadepsipeptide (CODP) inhibits locomotion by binding to calcium-activated potassium channels (*slo*-1) of parasitic nematodes [83]. In a study published in 1995, PF1022A was shown to be efficacious in a jird model against *H. contortus, T. colubriformis, and Ostertagia ostertagi* [84]. PF1022A also demonstrated efficacy against *H*. contortus and *T. colubriformis* in sheep with oral dosing of 5 and 10 mg/kg, respectively [46]. Several additional naturally occurring CODPs, such as bassianolide and verticilide, have also been identified and display anthelmintic activity; however, PF1022A remains the most active isolate from fermented *Mycelia* fungal strains [85]. Due to its promising anthelmintic activity in a variety of mammalian species, and unique MOA, PF1022A has been a lead candidate for anthelmintic lead optimization since the 1990s. Although very interesting from a biological perspective, reports of synthetic derivatization of the naturally occurring PF1022A (to produce new anthelmintic drug candidates) are limited. In 1993, Fujisawa submitted a patent application which included emodepside, a semisynthetic derivative of PF1022A with two morpholine moieties appended at the para position of the phenyllactic acids [86]. Emodepside **41** is currently marketed by Bayer as a companion animal combination product with praziquantel,



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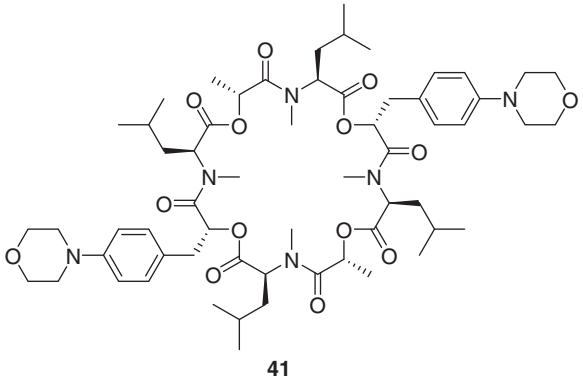

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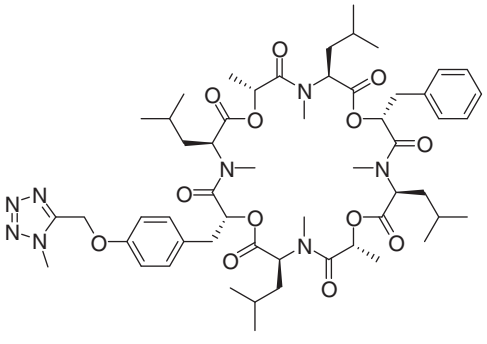
Figure 9.9 Structures of PF1022A and emodepside.

Profender®, for the treatment and control of roundworms, hookworms, and tapeworms. It remains the only CODP approved for anthelmintic use to date.

Bayer published work in 2005, in collaboration with Fuji and Meiji, using the natural products PF1022A and PF1022E as precursors to new semisynthetic CODP analogs [87]. The synthetic modifications consist of mono- and di-substitution on the phenyl ring(s) of PF1022A and E. The resulting asymmetric and symmetric compounds showed promising anthelmintic activity against *H. contortus* and *T. colubriformis* in sheep. Later in 2011, Meiji published additional semisynthetic analogs to PF1022A, PF1022E, and PF1022H with reported anthelmintic activity against the parasitic nematode *A. galli* in chickens [88]. Structure–activity relationships (SARs) were examined in para-substituted analogs of the phenyllactic acid moiety of PF1022A. It was found that physicochemical properties such as lipophilicity and polar surface area were especially important to *in vivo* activity. Several mono-substituted analogs, such as examples **42** and **43** (Figure 9.10), were found to have optimal physicochemical properties and anthelmintic activity better than that of PF1022A. The authors postulated that perhaps only one portion of the CODP is responsible for receptor binding, making mono-substitution a viable option for SAR due to resulting lower molecular weight and better membrane permeability.

More recently, Sivanathan et al. [89, 90] published work on semisynthetic analogs of PF1022A and PF1022H, citing the need for new CODPs with



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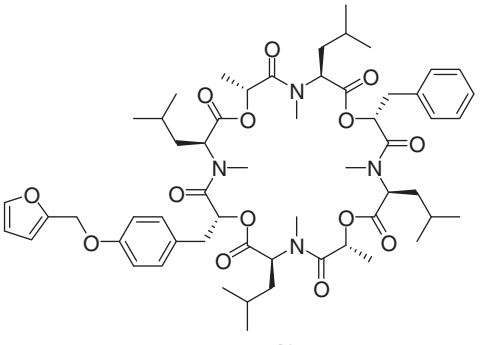

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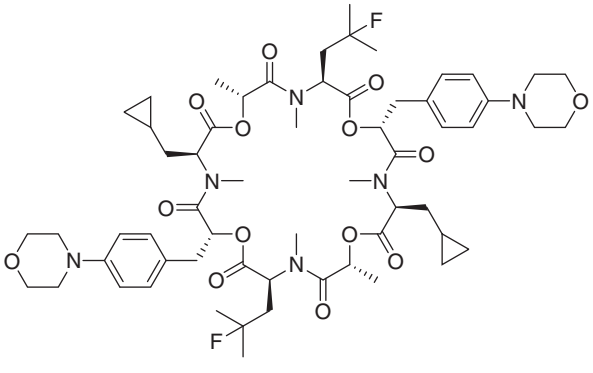
Figure 9.10 Structures of semisynthetic Meiji analogs of PF-1022A.

improved activity against human worm infections; however, no new *in vitro* or *in vivo* data were disclosed.

In addition to isolation of PF1022A from fermentation broths, several reports on the total synthesis of PF1022A derivatives have been published [91, 92]. Most recently, Merial (now Boehringer Ingelheim Vetmedica) published new total synthesis patent applications, containing data showing efficacy against heartworms [93, 94]. It has been known for a number of years that emodepside is a potent *in vitro* antifilarial (WHO-Pfizer Animal Health, unpublished data); and DND*i* is working on evaluating the potential of emodepside for treatment of onchocerciasis, but this is the first patent data showing efficacy against heartworms, both *in vitro* and *in vivo*. Unlike earlier semisynthetic reports where structural modifications were mainly limited to the phenyl rings of PF1022A, as well as other naturally occurring fermentation products, the latest Merial patent applications are totally synthetic in nature and start from modified amino acid building blocks (Figure 9.11). The resulting CODP analogs (**44**, **45**) have structural changes to the backbone region, as well as the phenyl moiety of the original PF1022A scaffold. In addition to having heartworm efficacy, these new analogs are reported to have sub-micromolar activity in *H. contortus*, L1 stage larval assays.

It is somewhat unusual to see animal health companies invest in a chemical route of more than 10 synthetic steps; in fact, several of the new Merial



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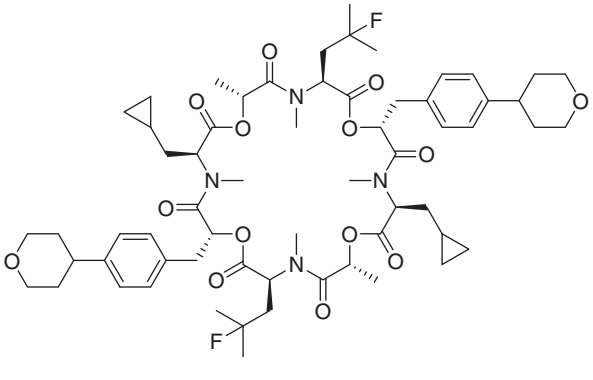

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Figure 9.11 Structures of fully synthetic Merial analogs of PF-1022A.

compounds require 15 or more synthetic steps to complete. However, this effort only serves to highlight the scarcity of new anthelmintic substrate and the need for further investigation.

9.4 Discussion

It is a major concern with STHs that there is limited research focused on identifying new classes to better manage these serious and impactful infections.

Although we were somewhat pessimistic in the introduction about the investment in anthelmintic research in Animal Health, there is some optimism from the fact that ML resistance in cattle gut intestinal nematodes and canine heartworms is clearly driving research into the identification of novel anthelmintic classes and/or optimization of older classes to address emerging issues. The importance of crop protection research in supplying substrate to support animal health anthelmintic research is still very evident with the Syngenta VAChT inhibitors and the Meiji-discovered cyclooctadepsipeptides; and a close relationship between these industries continues to nurture these synergies.

As new classes are discovered and developed for animal health helminth infections, there needs to be a concerted will from NGOs, governments, and industry to develop these for use in humans; to continue the WHO efforts in large-scale deworming into the future [95].

Acknowledgment

Many thanks to Valerie Vaillancourt for her invaluable help with evaluating the anthelmintic patent literature.

References

1 World Health Organisation (2006). *Preventive Chemotherapy in Human Helminthiasis: Coordinated Use of Anthelminthic Drugs in Control Interventions: A Manual for Health Professionals and Programme Managers*. Geneva: World Health Organization.

2 Horton, J. (2000). Albendazole: a review of anthelmintic efficacy and safety in humans. *Parasitology* 121: S113–S132.

3 Banerjee, D., Mandal, A., and Prakash, O. (1971). Mebendazole (R-17,635): a new anthelmintic in the development of hookworms. *Trans. R. Soc. Trop. Med. Hyg.* 65 (5): 685–686.

4 Pene, P., Mojon, M., Garin, J.P. et al. (1982). Albendazole: a new broad spectrum anthelmintic. Double-blind multicenter clinical trial. *Am. J. Trop. Med. Hyg.* 31 (2): 263–266.

5 World Health Organisation (2017). *Preventive Chemotherapy to Control Soil-Transmitted Helminth Infections in at-Risk Population Groups*. Geneva: WHO.

6 World Health Organisation (2016). Schistosomiasis and soil-transmitted helminthiases: number of people treated in 2015. *Wkly Epidemiol. Rec.* 91 (49-50): 585–595.

7 Moser, W., Schindler, C., and Keiser, J. (2017). Efficacy of recommended drugs against soil transmitted helminths: systematic review and network meta-analysis. *BMJ* 358: j4307.

8 Brunt, T.M., van den Berg, J., Pennings, E., and Venhuis, B. (2017). Adverse effects of levamisole in cocaine users: a review and risk assessment. *Arch. Toxicol.* 91 (6): 2303–2313.

9 Williams, J.C. (1997). Anthelmintic treatment strategies: current status and future. *Vet. Parasitol.* 72 (3-4): 461–470.

10 Bumbalo, T.S., Fugazzotto, D.J., and Wyczalek, J.V. (1969). Treatment of enterobiasis with pyrantel pamoate. *Am. J. Trop. Med. Hyg.* 18 (1): 50–52.

11 Rim, H.J., Won, C.Y., Lee, S.I., and Lim, J.K. (1975). Anthelmintic effect of oxantel pamoate and pyrantel pamoate suspension against intestinal nematode infestations. *Kisaengchunghak Chapchi* 13 (2): 97–101.

12 Speich, B., Ali, S.M., Ame, S.M. et al. (2015). Efficacy and safety of albendazole plus ivermectin, albendazole plus mebendazole, albendazole plus oxantel pamoate, and mebendazole alone against Trichuris trichiura and concomitant soil-transmitted helminth infections: a four-arm, randomised controlled trial. *Lancet Infect. Dis.* 15 (3): 277–284.

13 Speich, B., Ame, S.M., Ali, S.M. et al. (2014). Oxantel pamoate–albendazole for Trichuris trichiura infection. *NEJM* 370 (7): 610–620.

14 Rebollo, M.P. and Bockarie, M.J. (2013). Toward the elimination of lymphatic filariasis by 2020: treatment update and impact assessment for the endgame. *Expert Rev. Anti Infect. Ther.* 11 (7): 723–731.

15 Chabala, J.C., Mrozik, H., Tolman, R.L. et al. (1980). Ivermectin, a new broad-spectrum antiparasitic agent. *J. Med. Chem.* 23 (10): 1134–1136.

16 Aziz, M.A., Diallo, S., Diop, I.M. et al. (1982). Efficacy and tolerance of ivermectin in human onchocerciasis. *Lancet* 2 (8291): 171–173.

17 Xiao, S.H., Utzinger, J., Tanner, M. et al. (2013). Advances with the Chinese anthelminthic drug tribendimidine in clinical trials and laboratory investigations. *Acta Trop.* 126 (2): 115–126.

18 Zhang, J.H., Xiao, S.H., Wu, Z.X. et al. (2008). Tribendimidine enteric coated tablet in treatment of 1,292 cases with intestinal nematode infection--a phase IV clinical trial. *Chin. J. Parasitol. Parasit. Dis.* 26 (1): 6–9.

19 Steinmann, P., Zhou, X.N., Du, Z.W. et al. (2008). Tribendimidine and albendazole for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp.: open-label randomized trial. *PLoS Negl. Trop. Dis.* 2 (10): e322.

20 Nolan, T.J. and Lok, J.B. (2012). Macrocyclic lactones in the treatment and control of parasitism in small companion animals. *Curr. Pharm. Biotechnol.* 13 (6): 1078–1094.

21 Attah S., Kataliko K., Mutro M. et al. (2014): Effect of a single dose of 8 mg moxidectin or 150 μg/kg ivermectin on intestinal helminths in participants of a clinical trial conducted in Northeast DRC, Liberia and Ghana. *Conference of the American Society of Tropical medicine and Hygiene*.

22 Barda, B., Sayasone, S., Phongluxa, K. et al. (2017). Efficacy of moxidectin versus ivermectin against *Strongyloides stercoralis* infections: a randomized, controlled noninferiority trial. *Clin. Infect. Dis.* 65 (2): 276–281.
23 Woods, D.J., Lauret, C., and Geary, T.G. (2007). Anthelmintic discovery and development in the animal health industry. *Expert Opin. Drug Discovery* 2 (S1): S25–S33.
24 Gustavsen, K.M., Bradley, M.H., and Wright, A.L. (2009). GlaxoSmithKline and Merck: private-sector collaboration for the elimination of lymphatic filariasis. *Ann. Trop. Med. Parasitol.* 103 (S1): 11–15.
25 Vercruysse, J., Behnke, J.M., Albonico, M. et al. (2011). Assessment of the anthelmintic efficacy of albendazole in school children in seven countries where soil-transmitted helminths are endemic. *PLoS Negl.Trop. Dis.* 5 (3): e948.
26 WHO/TDR & Japanese Parasitology Society (2007). Summary report from the helminth drug initiative consultation meetings: informal consultation of experts to advise TDR on the proposed initiative. *Expert Opin. Drug Discovery* 2 (S1): S99–S103.
27 Charlier, J., van der Voort, M., Kenyon, F. et al. (2014). Chasing helminths and their economic impact on farmed ruminants. *Trends Parasitol.* 30: 361–367.
28 Mavrot, F., Hertzberg, H., and Torgerson, P. (2015). Effect of gastro-intestinal nematode infection on sheep performance: a systematic review and meta-analysis. *Parasit. Vectors* 8: 557.
29 Gates, M.C. and Nolan, T.J. (2009). Endoparasite prevalence and recurrence across different age groups of dogs and cats. *Vet. Parasitol.* 166: 153–158.
30 Stull, J.W., Carr, A.P., Chomel, B.B. et al. (2007). Small animal deworming protocols, client education, and veterinarian perception of zoonotic parasites in western Canada. *Can. Vet. J.* 48: 269–276.
31 https://www.heartwormsociety.org/veterinary-resources/american-heartworm-society-guidelines (accessed 13 November 2017).
32 Vercruysse, J. and Rew, R.S. (eds.) (2002). *Macrocyclic Lactones in Antiparasitic Therapy*. CABI.
33 Geary, T.G. (2005). Ivermectin 20 years on: maturation of a wonder drug. *Trends Parasitol.* 21 (11): 530–532.
34 Waghorn, T.S., Leathwick, D.M., Rhodes, A.P. et al. (2006). Prevalence of anthelmintic resistance on sheep farms in New Zealand. *N. Z. Vet. J.* 54 (6): 271–277.
35 Gasbarre, L.C., Smith, L.L., Hoberg, E., and Pilitt, P.A. (2009). Further characterization of a cattle nematode population with demonstrated resistance to current anthelmintics. *Vet. Parasitol.* 166: 275–280.
36 Gasbarre, L.C., Smith, L.L., Lichtenfels, J.R., and Pilitt, P.A. (2009). The identification of cattle nematode parasites resistant to multiple classes of anthelmintics in a commercial cattle population in the US. *Vet. Parasitol.* 166: 281–285.
37 Gasbarre, L.C. (2014). Anthelmintic resistance in cattle nematodes in the US. *Vet. Parasitol.* 204: 3–11.

38 Sutherland, I.A. and Leathwick, D.M. (2011). Anthelmintic resistance in nematode parasites of cattle: a global issue? *Trends Parasitol.* 27 (4): 1471–4922.

39 Geurden, T., Chartier, C., Fanke, J. et al. (2015). Anthelmintic resistance to ivermectin and moxidectin in gastrointestinal nematodes of cattle in Europe. *Int. J. Parasitol. Drugs. Drug Resist.* 5: 163–171.

40 Geary T., Pulaski C., Ballesteros C. et al. (2015). Correlating genotype with phenotypic response to a macrocyclic lactone in *Dirofilaria immitis*: a status update. *American Heartworm Society, Triennial Symposium,* New Orleans, LA (22–25 September) (abstract published).

41 Blagburn, B.L., Arther, R.G., Dillon, A.R. et al. (2016). Efficacy of four commercially available heartworm preventive products against the JYD-34 laboratory strain of *Dirofilaria immitis*. *Parasit. Vectors* 9: 191.

42 Prichard, R.K., McTier, T., Woods, D.J. et al. (2017). Genetic profile of seven *Dirofilaria immitis* isolates with variable response profiles to heartworm preventives. *Parasit. Vectors* 10 (Suppl. 2): 485.

43 Woods, D.J. and Knauer, C.S. (2010). Discovery of veterinary antiparasitic agents in the 21st century: a view from industry. *Int. J. Parasitol.* 40: 1177–1181.

44 Woods, D.J., Vaillancourt, V.A., Wendt, J.A., and Meeus, P.F. (2011). Discovery and development of veterinary antiparasitic drugs: past, present and future. *Future Med. Chem.* 3 (7): 887–896.

45 Hemphill, A., Mueller, J., and Esposito, M. (2006). Nitazoxanide, a broad-spectrum thiazolide anti-infective agent for the treatment of gastrointestinal infections. *Expert Opin. Pharmacother.* 7: 953–964.

46 Harder, A. and von Samson-Himmelstjerna, G. (2002). Cyclooctadepsipeptides – a new class of anthelmintically active compounds. *Parasitol. Res.* 88: 481–488.

47 Speich, B., Ame, S.M., Ali, S.M. et al. (2012). Efficacy and safety of nitazoxanide, albendazole, and nitazoxanide-albendazole against *Trichuris trichiura* infection: a randomized controlled trial. *PLoS Negl. Trop. Dis.* 6 (6): e1685.

48 Kaminsky, R., Gauvry, N., Schorderet Weber, S. et al. (2008). Identification of the amino-acetonitrile derivative monepantel (AAD 1566) as a new anthelmintic drug development candidate. *Parasitol. Res.* 103 (4): 931–939.

49 Rufener, L., Mäser, P., Roditi, I., and Kaminsky, R. (2009). *Haemonchus contortus* acetylcholine receptors of the DEG-3 subfamily and their role in sensitivity to monepantel. *PLoS Pathog.* 5 (4): 1–11.

50 Little, P.R., Hodge, A., Maeder, S.J. et al. (2011). Efficacy of a combined oral formulation of derquantel-abamectin against the adult and larval stages of nematodes in sheep, including anthelmintic-resistant strains. *Vet. Parasitol.* 181: 180–193.

51 Woods, D.J., Maeder, S.J., Robertson, A.P. et al. (2012). Discovery, mode of action, and commercialization of derquantel. In: *Parasitic Helminths: Targets, Screens, Drugs and Vaccines* (ed. C.R. Caffrey). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA https://doi.org/10.1002/9783527652969.ch18.

52 Preston, S., Jiao, Y., Baell, J.B. et al. (2017). Screening of the 'Open Scaffolds' collection from Compounds Australia identifies a new chemical entity with anthelmintic activities against different developmental stages of the barber's pole worm and other parasitic nematodes. *Int. J. Parasitol. Drugs. Drug Resist.* 7 (3): 286–294.

53 Ducray, P., Fruchtel, J., Gauvry, N., Schorderet Weber, S. (2007). Amidonitrile compounds. PCT Int. Appl., WO017088 A1.

54 Goebel, T., Ducray, P., Bouvier, J., Schorderet Weber, S. (2008). The use of aminoacetonitrile compounds in the control of endoparasites in warm blook animals. PCT Int. Appl., WO062005 A1.

55 (a) Comlay, S.N., Hannam, J.C., Howson, W. et al. (2008). Antiparasitic agents. PCT Int. Appl., WO096231 A1; (b) Gibson, S.P. and Lauret, C. (2008). Benzofuran Antiparasitic Agents. PCT Int. Appl., WO102232 A1.

56 Goebel, T., Gauvry, N., Sager, H. (2011). Amidoacetonitrile compounds having pesticidal activity. PCT Int. Appl., WO061326 A1.

57 Décor, A., Greul, J.N., Heilmann, E.K. et al. (2014). Nematicidal N-substituted phenethylcarboxamides WO177514 A1.

58 Kita, K., Suwa, A., Oda, M. (2016). Endoparasite control agent. US Pat. Appl., US0367532 A1.

59 Welz, C., Kohler, A., Borngen, K. et al. (2016). Compounds for use in anthelminthic treatment WO066574 A1.

60 Welz, C., Kohler, A., Borngen, K. et al. (2016). Compounds for use in anthelminthic treatment WO012485 A1.

61 Kita, K. and Suwa, A. (2014). Endoparasite control agent. US Pat. Appl., US0088157 A1.

62 Schwarz, H-G., Trautwien, A., Wilims, L. et al. (2013). Use of aryl and hetaryl carboxamides as endoparasiticides. WO076230 A1.

63 Kita, K., Akiyuki, S., Oda, M., Koji, T. (2015). Endoparasite control agent. US Pat. Appl., US0266853 A1.

64 Kita, K., Suwa, A., Odad, M., Koji, T. (2015). Endoparasite control agent and method for using the same. US Pat. Appl., US0259322 A1.

65 Iwasa, T. (2017). Parasite control agent for parasiticide. US Pat. Appl., US0035711 A1.

66 Kohler, A., Welz, C., Borngen, K. et al. (2017). Pyrazolopyrimidine derivatives. PCT Int. Appl., WO178416 A1.

67 Rohwer, A., Lutz, J., Chassaing, C. et al. (2012). Identification and profiling of nematicidal compounds in veterinary parasitology. In: *Parasitic Helminths: Targets, Screens, Drugs and Vaccines* (ed. C. Caffrey). Weinheim: Wiley-VCH.

68 Meng, C.Q., Long, A., Huber, S. et al. (2014). Anthelmintic compounds and compositions and method or using thereof WO081697 A2.

69 Gauvry, N. and Pautrat, F. (2014). Substituted azines as pesticides WO023723 A1.

70 Gauvry, N. and Pautrat, F. (2015). New compounds WO071417 A1.

71 Long, A. and Wilkinson, D.E. (2016). Anthelmintic compounds, compositions and method of suing thereof WO118638 A1.

72 Chassaing, C.P.A., Lutz, J., Hecker-Oth, A.R. (2016). Use of anthelmintic agents against *Dirofilaria immitis* WO005577.

73 Chassaing, C.P.A., Schroder, J., Ilg, T.S. et al. (2009). Anthelmintic agents and their use WO077527 A1.

74 Chassaing, C.P.A. and Meyer, T. (2010). Anthelmintic agents and their use WO115688 A1.

75 Chassaing, C.P.A. and Meyer, T. (2010). Anthelmintic agents and their use WO146083 A1.

76 Sluder, A., Shah, S., Cassayre, J. et al. (2012). Spiroindolines identify the vesicular acetylcholine transporter as a novel target for insecticide action. *PLoS One* 7: e34712.

77 Curtis, M.P., Vaillancourt, V., Goodwin, R.M. et al. (2016). Design and synthesis of sarolaner, a novel, once-a-month, oral isoxazoline for the control of fleas and ticks on dogs. *Bioorg. Med. Chem. Lett.* 26: 1831–1835.

78 McTier, T.L., Chubb, N., Curtis, M.P. et al. (2016). Discovery of sarolaner: a novel, orally administered, broad-spectrum, isoxazoline ectoparasiticide for dogs. *Vet. Parasitol.* 222: 3–11.

79 Wolstenholme, A.J. and Rogers, A.T. (2005). Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitology* 131: S85–S95.

80 Wendt, J., Cox, M., Sheehan, S.M.K. et al. (2015). Preparation of spiroindoline antiparasitic derivatives. PCT Int. Appl. WO 2015100232 A2.

81 Subramanian, G., Curtis, M.P., Deng, J. et al. (2016). Carbolines as antiparasitics and their preparation. PCT Int. Appl., WO 2016209635 A1.

82 Sasaki, T., Takagi, T., Yaguchi, T. et al. (1992). A new anthelmintic cyclodepsipeptide, PF1022A. *J. Antibiot.* 45: 692–697.

83 Guest, M., Bull, K., Walker, R.J. et al. (2007). The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in Caenorhabditis elegans. *Int. J. Parasitol.* 37: 1577–1588.

84 Conder, G.A., Johnson, S.S., Nowakowski, D.S. et al. (1995). Anthelmintic profile of the cyclodepsipeptide PF1022A in in vitro and in vivo models. *J. Antibiot.* 48: 820–823.

85 Sivanathan, S. and Scherkenbeck, J. (2014). Cyclodepsipeptides: a rich source of biologically active compounds for drug research. *Molecules* 19: 12368–12420.

86 Nishiyama, H., Ohgaki, M., Yamanishi, R., Hara, T. (1993). Preparation of depsipeptides derivatives as anthelmintics. PCT Int. Appl., WO 9319053 A1.

87 Jeschke, R., Iinuma, K., Harder, A. et al. (2005). Influence of the cyclooctadepsipeptides PF1022A and PF1022E as natural products on the design of semi-synthetic anthelmintics such as emodepside. *Parasitol. Res.* 97 (Suppl. 1): S11–S16.

88 Ohyama, M., Okada, Y., Takahashi, M. et al. (2011). Structure-activity relationship of anthelmintic cyclooctadepsipeptides. *Biosci. Biotechnol., Biochem.* 75: 1354–1363.

89 Sivanathan, S., Koerber, F., and Scherkenbeck, J. (2016). Semisynthetic routes to PF1022H-A precursor for new derivatives of the anthelmintic cyclooctadepsipeptide PF1022A. *Bioorg. Med. Chem.* 24: 873–876.

90 Sivanathan, S., Vollrodt, J., and Scherkenbeck, J. (2016). New heteroaromatic derivatives of the anthelmintic cyclooctadepsipeptide PF1022A by Click chemistry. *Lett. Org. Chem.* 13: 441–445.

91 Lee, B.H. (1997). Solid-phase synthesis of cyclooctadepsipeptide PF1022A analogs using a cyclization-cleavage method with oxime resin. *Tetrahedron Lett.* 38: 757–760.

92 Luettenberg, S., Sondermann, F., and Scherkenbeck, J. (2012). Anthelmintic PF1022A: stepwise solid-phase synthesis of a cyclodepsipeptide containing *N*-methyl amino acids. *Tetrahedron* 68: 2068–2073.

93 De Fallois, L.L., Pacofsky, G., Long, A. et al. (2016). Preparation of cyclooctadepsipeptides as anthelmintics. PCT Int. Appl., WO 2016187534 A1.

94 De Fallois, L.L., Pacofsky, G., Long, A. et al. (2017). Preparation of cyclic depsipeptides as anthelmintics. PCT Int. Appl., WO 2017116702 A1.

95 Preston, S. and Gasser, R.B. (2018). Working towards new drugs against parasitic worms in a public-development partnership. *Trends Parasitol.* 34 (1): 4–6.

10

Drug Discovery and Development for the Treatment of Echinococcosis, Caused by the Tapeworms *Echinococcus granulosus* and *Echinococcus multilocularis*

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10.1 *Echinococcus* and Echinococcosis

Echinococcosis, caused by larval stages of the genus *Echinococcus* (Cestoda, Platyhelminthes), is a life-threatening disease affecting humans and livestock. The two most prominent members of the genus *Echinococcus* are *Echinococcus granulosus sensu lato* and *Echinococcus multilocularis*. The taxonomy of *E. granulosus sensu lato* is rather complex, as it includes *E. granulosus sensu stricto, Echinococcus equinus, Echinococcus ortleppi, Echinococcus canadensis, Echinococcus intermedius,* and *Echinococcus felidis* [1–4], all of which cause cystic echinococcosis (CE or hydatid disease) with different host specificities worldwide. For simplification, we collectively refer to these species as *E. granulosus. E. multilocularis* is the causative agent of alveolar echinococcosis (AE) and it occurs in the Northern Hemisphere. Other species include *Echinococcus oligarthrus*, causing unicystic echinococcosis in South America; *Echinococcus vogeli*, causing polycystic echinococcosis in South America; and *Echinococcus shiquicus* that was identified as a sister species to *E. multilocularis* more recently [2, 4, 5].

E. multilocularis, commonly known as the small fox tapeworm, is found to be highly endemic in regions such as Central and Western China, Russia, Western-Central Europe (classically Switzerland, Southern Germany, Eastern France, and Western Austria), Eastern Europe including the Baltic countries, and in Alaska (Northern America) and Hokkaido (Japan) [6]. Ninety-one percent of human AE cases are located in the Tibetan plateau of Western China [7]. Recently, AE has become an increasing health problem, in particular in Kyrgyzstan [6]. In Western-Central Europe, 0.3 to 3 per 1 000 000 inhabitants get infected with *E. multilocularis* annually [8]. AE also affects other species, such as dogs, monkeys, pigs, horses, beavers, and others [9].

E. granulosus (the small dog tapeworm) occurs globally, and mostly in the Mediterranean area, Central Europe, South America, Africa, and Central Asia. In addition, CE exists as an imported disease in Western Europe and in the United

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States [10]. In terms of case numbers and distribution, CE by far outnumbers AE; however, AE is a much more severe disease and more difficult to treat.

CE, but to some extent also AE, occurs predominantly in resource-poor communities. For AE, although also present in higher developed countries, the number of patients is most likely underestimated by a factor of 3–5 [11]. This means that investments in the development of new drugs against echinococcosis will have a low market return, and thus the pharmaceutical industry will not be compelled to develop novel drugs against echinococcosis. However, the habitats of *E. multilocularis* and *E. granulosus* have continuously expanded, and both AE and CE are now found in regions which were previously free of disease. Thus, emergence (or reemergence), especially in developing countries, is likely, with an increasing economic impact due to the necessity of livelong treatments. The global impact of CE and AE is comparable to other neglected tropical diseases such as trypanosomiasis, Chagas disease, and schistosomiasis [12].

10.2 The Biological Features of *E. granulosus* and *E. multilocularis*: Similar, but Different

Both *Echinococcus* species undergo a typical predator–prey life cycle and humans represent accidental intermediate hosts. The hermaphroditic adult stage lives in the intestine of the respective definitive hosts, where it undergoes sexual development. Definitive hosts for *E. multilocularis* are the red fox and arctic fox, coyote, raccoon dog, wolf, domestic dog, and the cat [13]. The main definitive host of *E. granulosus* is the domestic dog, but other canine carnivores can also get involved. The adult worms produce infective eggs, which are released into the environment via fecal shedding. Eggs contain an oncosphere (the first larval stage) and are orally infective for a wide range of intermediate hosts. Typical intermediate hosts for *E. granulosus* are cattle, sheep, goats, pigs, and camels, while those of *E. multilocularis* are predominantly voles (such as *Microtus arvalis* or *Arvicola terrestris*), but also other small mammals, mostly small rodents, as well as dogs and sheep. Once eggs are ingested, the oncospheres get activated during the stomach passage, will be set free in the intestine, penetrate the intestinal tissue and reach blood and lymphatic vessels, and eventually reach the target organ. There it develops into a second larval stage, the metacestode. Metacestodes are characterized by continuous, potentially unlimited growth, and the differentiation into protoscoleces, namely, precursors of newly formed tapeworm heads, takes place. Once intermediate hosts or tissues containing metacestodes and protoscoleces are ingested by a carnivorous definitive host, protoscoleces evaginate and attach to the intestinal epithelium and develop into adult tapeworms, thus concluding the life cycle.

Humans represent an aberrant intermediate host for these parasites. The most affected organs in humans are the liver for *E. multilocularis,* and the liver, lung, and other sites in the case of *E. granulosus*. Metacestodes at these sites are the target of chemotherapeutical and surgical treatment approaches. Protoscolece development in humans infected with *E. multilocularis* has only rarely been

described, while it is more commonly observed in individuals infected with *E. granulosus*.

Echinococcus metacestodes resemble fluid-filled vesicles, which in both species exhibit a range of common features (Figure 10.1). The wall of these vesicles is separated into an inner germinal layer representing the living and metabolically active parasite tissue, and an outer, acellular and carbohydrate-rich compartment known as laminated layer, mediating the direct physical contact with host immune and nonimmune cells [14]. In terms of thickness, the laminated layer is much more prominent in *E. granulosus* metacestodes. The distal part of the germinal layer, the tegument, is directly associated with the inner surface of the laminated layer, and is characterized by microvilli-like extensions termed microtriches. The germinal layer itself is built up by muscle cells, nerve cells (serving possibly a neuroendocrine function), glycogen storage cells, connective tissue cells, and totipotent stem cells (also called germinative cells or neoblasts) [15–17]. These stem cells make up 20–25% of all cells in the germinal layer. They are responsible for the high regenerative potential of the parasite, and they are thought to be responsible for metastasis formation [18–20]. The germinal layer secretes vesicle fluid into the interior of the metacestodes, and vesicle fluid plays a role in nutrition and in exchange of metabolites within the parasite. For *E. granulosus*, the vesicle fluid is also termed hydatid fluid. *E. granulosus* metacestodes are, in addition, surrounded by a prominent host-derived adventitial layer, which is largely composed of collagenous fibers.

E. granulosus metacestodes grow by expansion rather than by proliferation. Multiplication takes place internally, resulting in septated, multichambered cysts. The entire parasite mass progressively grows in size and compresses the neighboring tissues. Metastasis formation can occur upon rupture of such cysts and leakage of hydatid fluid containing protoscoleces, which themselves can then differentiate into new metacestodes in peripheral tissues. In contrast, *E. multilocularis* metacestodes represent multivesicular organisms that reproduce asexually, by exogenous formation, and budding of daughter vesicles. This process is often referred to as "progressive infiltrative tumor-like growth," and leads to the formation of a large and heterogeneous parasitic mass. This mass consists mostly of peripheral, actively proliferating, sites, and, in many cases, centrally located necrotic tissue, all intermingled with host connective tissue. Metastasis formation can occur in neighboring organs such as the gall bladder, abdominal lymph nodes, pancreas, diaphragm, and peritoneum, and in more distant regions (lungs, bones, brain, etc.) and lead to severe complications in treatment [7].

10.3 Clinical Hallmarks, Diagnosis, and Prevention and Control of CE and AE

The clinical presentation of CE is highly variable, and is dependent on several features such as the involved organ and the location of the cyst within the organ, cyst dimension, and the interaction with surrounding structures. In addition, different genotypes exhibit different growth characteristics, which will also

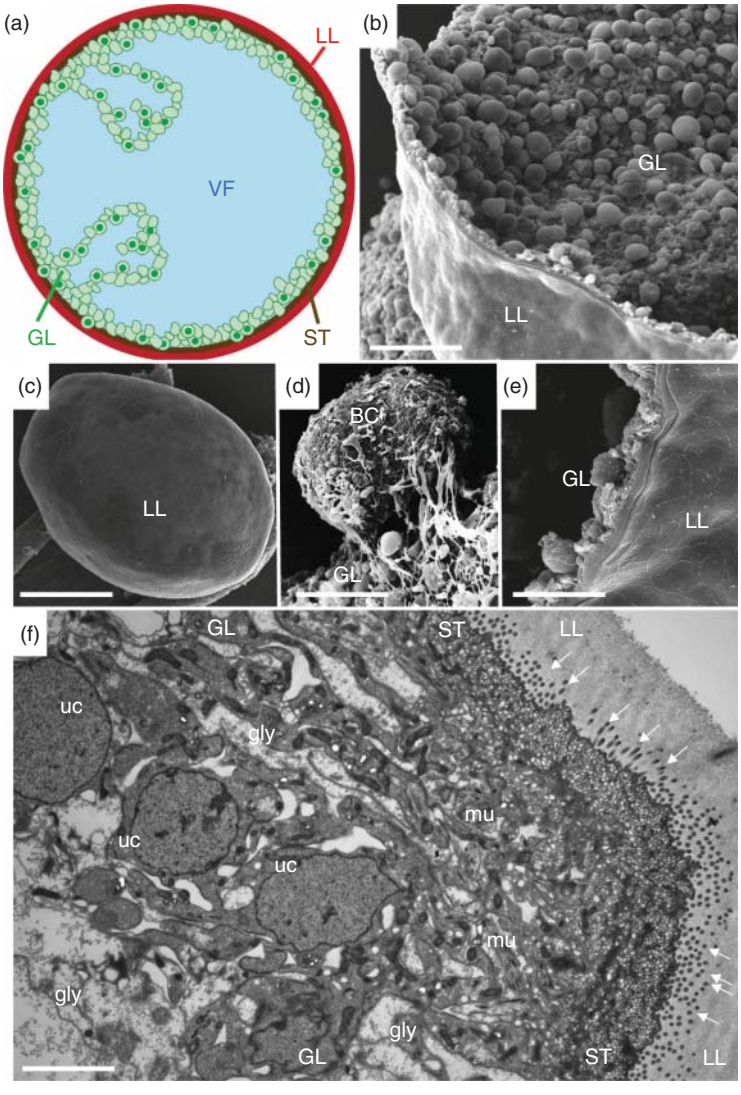

Figure 10.1 Structure of *E. multilocularis* metacestodes. Part (a) depicts a schematical view of a metacestode vesicle. The main components are indicated as color-coded: the laminated layer (LL, red); the syncytial tegument (ST, brown); the germinal layer (GL, green), the vesicle fluid (VF, blue). Parts (b)—(e) are scanning electron micrographs (SEMs) of *E. multilocularis* metacestodes. Part (b) allows a view into the interior of a metacestode, showing the germinal layer (GL) and the outer laminated layer (LL). Part (c) is an intact metacestode, with only the LL exposed, and (d) is a developing brood capsule (BC) still attached to the germinal layer (GL). A higher magnification SEM image of the vesicle wall is shown in (e). Part (f) is a section that was cut through the vesicle wall, shown by transmission electron microscopy (TEM). Note the outer laminated layer (LL), the syncytial tegument (ST) with microtriches protruding outwards into the LL (arrows), and the complex germinal layer (GL), containing undifferentiated cells (uc), muscle cells (mu), glycogen storing cells (gly), and also connective tissue. Bars in b = 330 μm; c = 1200 μm; d = 360 μm; e = 280 μm; f = 4.1 μm. (From Ref [166].)

impact on the clinical features [14]. Most patients (up to 80% of all cases) have a single cystic lesion located in a single organ. The liver is affected in 70% of the cases, the right lobe more commonly than the left, followed by the lungs in about 20% of the cases. Cysts can localize in any other organ and structure, such as abdominal or pleural cavities, kidney, spleen, bone, brain, vertebral column, ovary, breast, axillary region, and heart [21]. Rare immune-mediated reactions such as urticaria, asthma, membranous nephropathy, and anaphylaxis have also been well described [14]. Clinical signs may occur after a highly variable incubation period of several months or years, but hepatic cysts may remain asymptomatic for periods of up to 10–12 years. They can cause pain in the upper abdominal region, cholestasis, hepatomegaly, biliary cirrhosis, portal hypertension, ascites, and a variety of other manifestations [14]. Infection with *E. multilocularis,* affecting the liver in the vast majority of cases, is largely asymptomatic and remains mostly undiagnosed until a progressive state is reached. This time span can reach 10–15 years. At this stage, nonspecific symptoms such as abdominal pain, jaundice, cholestasis, hepatomegaly, fever, anemia, weight loss, and pleural pain can occur [7, 10].

For both AE and CE, it is, in many instances, noninvasive imaging techniques such as ultrasound, computed tomography, or magnetic resonance imaging that give the first, often coincidental, morphological indications of the disease. Ultrasound examination is the most widely used technique for screening purposes or examining individuals, and also for monitoring of postoperative and peri-therapeutic cyst development [22]. For screening purposes and confirmation of imaging findings, immunodiagnosis (ELISA and/or Western blot) is performed on the basis of a variety of native and/or recombinant antigens [23]. Biopsy retrieval and direct detection of parasite DNA by PCR or of parasite tissue by histology/immunohistochemistry is less common due to the risk of metastases formation, but can be used for confirming the diagnosis postoperatively [23]. For AE, information on the viability status of parasite lesions can be gained by F18-fluorodeoxyglucose-positron emission tomography (FDG-PET), which highlights peri-parasitic inflammation as a response to the parasite lesion, contrast-enhanced ultrasound (CEUS), and serological follow-up or RT-PCR [24–26].

10.4 Currently Applied Benzimidazole Treatments for CE and AE

Surgery and chemotherapy based on the benzimidazole carbamate derivatives albendazole (ABZ, **1**) and mebendazole (MBZ, **2**) (Figure 10.2) are the two treatment options for CE and AE that are currently applied. For CE, surgery was considered the gold standard treatment until the 1980s. However, invasive surgery may be impractical, or even not feasible, in many cases, such as in patients with multiple cysts in several organs, or in patients living in regions that lack adequate facilities for advanced surgery [27]. PAIR (puncture, aspiration, injection, reaspiration) can be considered as an alternative to conventional surgery [28, 29]. PAIR

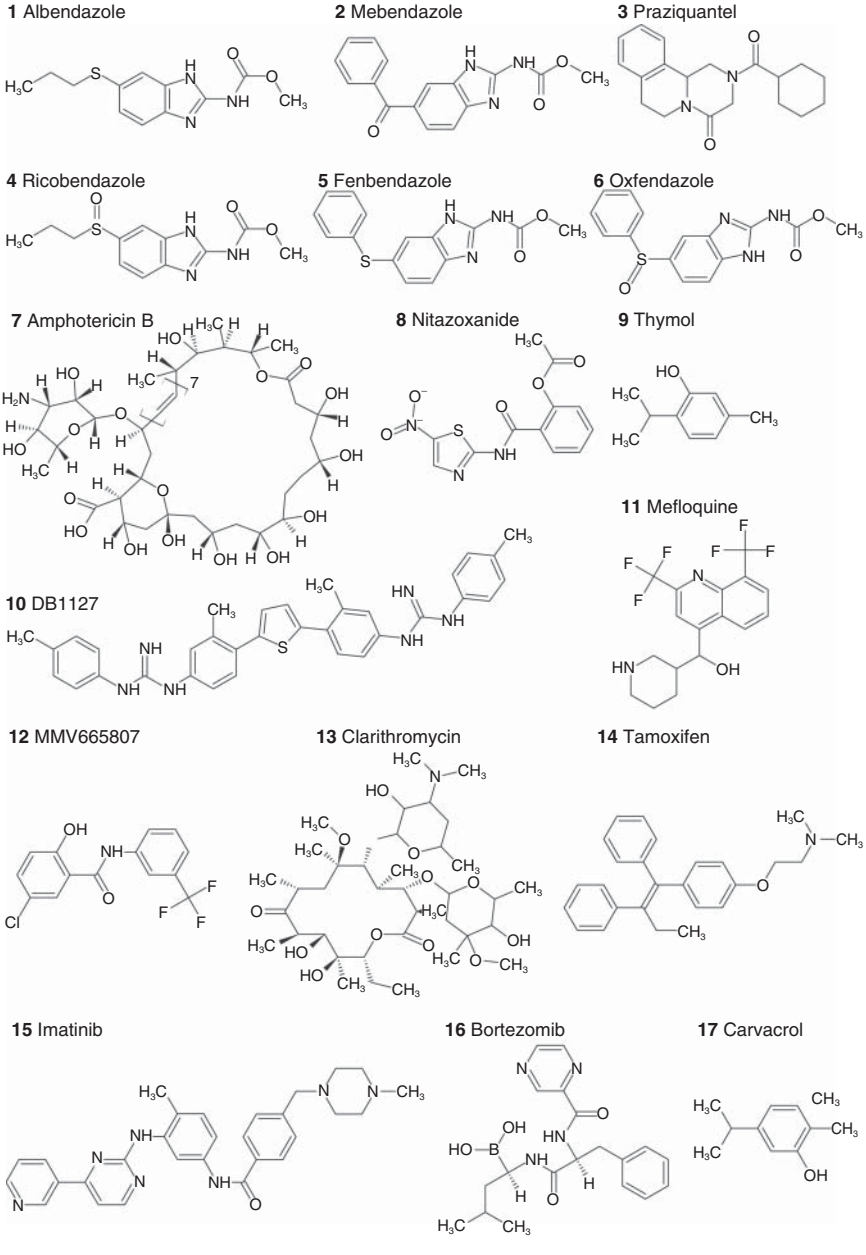

Figure 10.2 Chemical structures of selected compounds with activity against *Echinococcus*. Structures were prepared in ACD/ChemSketch 2017.1.2.

is minimally invasive, and includes (i) percutaneous puncture of the cyst under ultrasonographic guidance, (ii) aspiration of the cyst fluid, (iii) injection of a parasiticidal solution, and (iv) reaspiration of the fluid content after five minutes. Although hypertonic NaCl solution (20% final concentration in the cyst fluid) is not optimally effective, it is currently the most-used scolicidal solution in PAIR, as its application induces the lowest adverse side effects.

For inoperable cases, chemotherapy with the benzimidazoles ABZ and MBZ remains the only option. In the case of CE, a combined therapy using ABZ and the heterocyclic pyrazinoisoquinoline derivative praziquantel (PZQ, **3**) (Figure 10.2) has been suggested. PZQ exhibited promising efficacy against protoscoleces and metacestodes in animal experiments [30, 31] and was proposed to be applied during the month prior to surgery alongside with ABZ, since this increased the number of human patients with nonviable protoscoleces as compared to therapy with ABZ alone [32, 33]. Benzimidazoles must be taken over extended periods of time, often lifelong. However, under long-term benzimidazole therapy, adverse reactions such as hepatotoxicity may occur. Adverse effects can be avoided by introducing regular monitoring of drug serum levels and, if necessary, adjustment of the dosage, but this is highly dependent on a health service with a functional infrastructure, which does not exist in many countries. In general terms, benzimidazole therapy has significantly improved the life expectancy and the quality of life of many affected patients. A study carried out with 3282 patients with echinococcosis treated with ABZ showed that gastrointestinal tract problems represent the most common adverse events, but no fatal cases were described [34]. MBZ and ABZ may induce embryotoxic or teratogenic effects [35]. Of the over 2000 well-documented inoperable cases of CE treated with benzimidazoles evaluated up to 12 months after initiation of chemotherapy, 30% of patients showed cyst disappearance, 50–70% exhibited cyst degeneration, and in 20–30% of patients *E. granulosus* metacestodes did not respond to chemotherapy [36].

Unfortunately, the prognosis is less favorable for patients with AE. The only curative treatment is invasive surgical resection of the entire parasite lesion including a safety margin, and this is applied in 20–50% of all cases [7, 28]. However, radical surgery can often not be performed as most cases are diagnosed at a late stage and the parasite grows highly invasive. According to a long-term cohort study in Germany, complete surgery could only be performed in 16.1% and 36.1% of all patients with AE (referring to cases described before and after the year 2000, respectively) [37]. If surgery is carried out, it is always accompanied by benzimidazole chemotherapy, for at least two years thereafter, and monitoring of patients should be continued for 10 years [33]. Inoperable cases of AE must undergo long-term/mostly lifelong MBZ or ABZ chemotherapy. Nevertheless, clinical studies have shown that chemotherapy has significantly increased the 10-year survival rate of inoperable or non-radically operated patients with AE from 6–25% to 80–85% [10, 37]. A major setback of the current benzimidazole therapy is that ABZ and MBZ exhibit a parasitostatic rather than a parasiticidal effect *in vivo* [38]. Therefore, recurrence rates after treatment interruption are relatively high, especially in those patients not followed up with appropriate prognostic tools [26, 38]. Disease progression due to treatment failure was

described in up to 16% of all AE cases [39]. In countries with well-developed health care systems, where access to treatment and drug level monitoring is secured, an improved clinical management of AE can be achieved. However, the costs for treating one patient with AE amount to 108 762 Euros annually [40]. Thus, AE is still a lethal disease in less-developed countries with low, or no, financial resources [7]. A long-term study carried out in Germany showed that 54.5% of all patients experienced mild side effects, and 6.9% of the patients experienced life-threatening adverse effects such as hepatotoxicity that led to treatment discontinuation [37]. With increasing numbers of patients and no alternative to benzimidazoles developed so far, new and better treatment options are urgently needed.

One possible explanation for the parasitostatic, but not parasiticidal, effects of ABZ can be found at the molecular level. Upon oral uptake, ABZ is rapidly converted into ABZ-sulfoxide (also called ricobendazole, **4**; Figure 10.2), and at a later stage ABZ-sulfoxide is metabolized to ABZ-sulfone. The major metabolite ABZ-sulfoxide is known to bind to a distinct site on β-tubulin subunit of the tubulin dimer, and thus interferes in the polymerization of microtubules, thereby blocking many cellular functions and impairing uptake of nutrients and parasite growth [41]. In the *E. multilocularis* genome, there are three β-tubulin genes, *tub1, tub2,* and *tub3*. The stem cells that develop in the germinal layer of *E. multilocularis* express mainly the Tub-2 isoform. The ABZ-sulfoxide binding site on the Tub-2 protein is altered, and does not bind to ABZ-sulfoxide; thus, Tub-2 is resistant to the dosages of benzimidazoles used in standard treatment [42]. Other factors such as the limited half-life and uptake of benzimidazoles by the parasite could also account for the failure in parasite killing. In addition to beta-tubulin, several metabolic enzymes were described as targets of benzimidazoles [43], including the fumarate reductase system, which constitutes the malate dismutation pathway in many helminths including *Echinococcus* [44, 45], but these findings have not been further followed up. Electron microscopical studies have shown that benzimidazole treatment of *E. multilocularis* metacestodes has a rapid effect on the structural integrity of tegumental microtriches. This was shown not only for ABZ and its metabolites [46] but also for fenbendazole (**5**) and oxfendazole (fenbendazole-sulfoxide, **6**; see also Figure 10.2). However, microtriches do not contain any microtubules [47]. Therefore, additional targets of benzimidazoles in *Echinococcus* remain to be identified. A very recent study has shown that ABZ treatment increases the host immune response against the parasite [48]. To what extent this has an impact on the efficacy of the drug is not clear, but the crosstalk between chemotherapy and immunity should be further investigated.

Besides benzimidazoles, only two other compounds have reached clinical application against AE or CE. The antifungal agent amphotericin B (**7**, Figure 10.2) was applied as a salvage treatment, but it did not exert parasiticidal activity, and induced nephrotoxicity under long-term usage [49]. Nitazoxanide (**8**, Figure 10.2), a broad-spectrum anti-infective thiazolide, did also not fulfill the hopes that were put into that compound: despite promising activities in mouse studies, nitazoxanide failed to be active against human AE [49, 50]. However, a few studies suggested that nitazoxanide may be an effective treatment option

in CE, particularly in patients with progressive disease who are receiving conventional therapy [51–53].

10.5 *In vitro* and *in vivo* Models to Study Drug Efficacy and Drug Targets in *Echinococcus*

The well-established, easy-to-handle, and standardized *in vitro* culture of the *E. multilocularis* metacestode stage [54], the public availability of its genome sequence and transcriptome information [55], established stem cell culture, and the development of methods for genetic manipulation [56] have rendered *E. multilocularis* the prime model for the study of diseases inflicted by cestodes in humans. This includes not only AE but also CE as well as cysticercosis, caused by metacestodes (cysticerci) of the closely related *Taenia solium*. The genome and transcriptome of the closely related *E. granulosus* has also been published [57–59], and comparative genomics has revealed surprisingly little differences in genome structure and content between *E. granulosus* and *E. multilocularis*.

In vitro culture of *E. multilocularis* metacestodes has been reported as early as 1957 [60]. Other methods developed later [61, 62] did not result in efficient production of metacestodes that would allow large-scale *in vitro* drug efficacy studies. In addition, the earlier drug studies relied solely on morphological observations rather than on objective assays for viability assessment (reviewed in Hemphill et al. [63]; [33]). In 2004, Spiliotis and Brehm published a revolutionary culture method that allows generating large amounts of metacestodes in coculture with hepatoma feeder cells [54]. This provided the basis for long-term culture and proliferation of metacestodes, axenic metacestode culture without feeder cells, and also allowed to isolate the cells of the germinal layer ("primary cells"), of which up to 82% were stem cells, which will, upon *in vitro* culture, again form infective metacestodes [19, 54, 64]. Based on this metacestode and stem cell culture techniques, Stadelmann et al. established a screening cascade (Figure 10.3) that allows medium-throughput drug screening based on objective criteria [65]. As a first step, the physical impairment of *E. multilocularis* metacestodes is assessed quantitatively by measuring phosphoglucose isomerase (PGI) in the culture supernatant. This enzyme is an abundant vesicle fluid component, which is released upon physical impairment of the metacestode vesicle due to drug treatment [66]. This quantitative assay allows determination of EC_{50} values and analyses of structure–activity relationships of tested compounds. Similar approaches were also applied for related species such as *T. solium* or *E. granulosus* [67, 68]. Cytotoxicity of the same drugs to mammalian cells is measured by conventional alamar blue assay to explore a potential therapeutic window. The effects of selected compounds on germinal layer cell (and stem cell) viability can be assessed by measuring ATP production by commercially available kits [65]. Finally, a test based on alamar blue assay that measures the viability of germinal layer cells within intact metacestodes (metacestode viability assay) was developed [65].

For the most promising compounds, morphological effects are frequently visualized by electron microscopy, which can already indicate a potential mode of

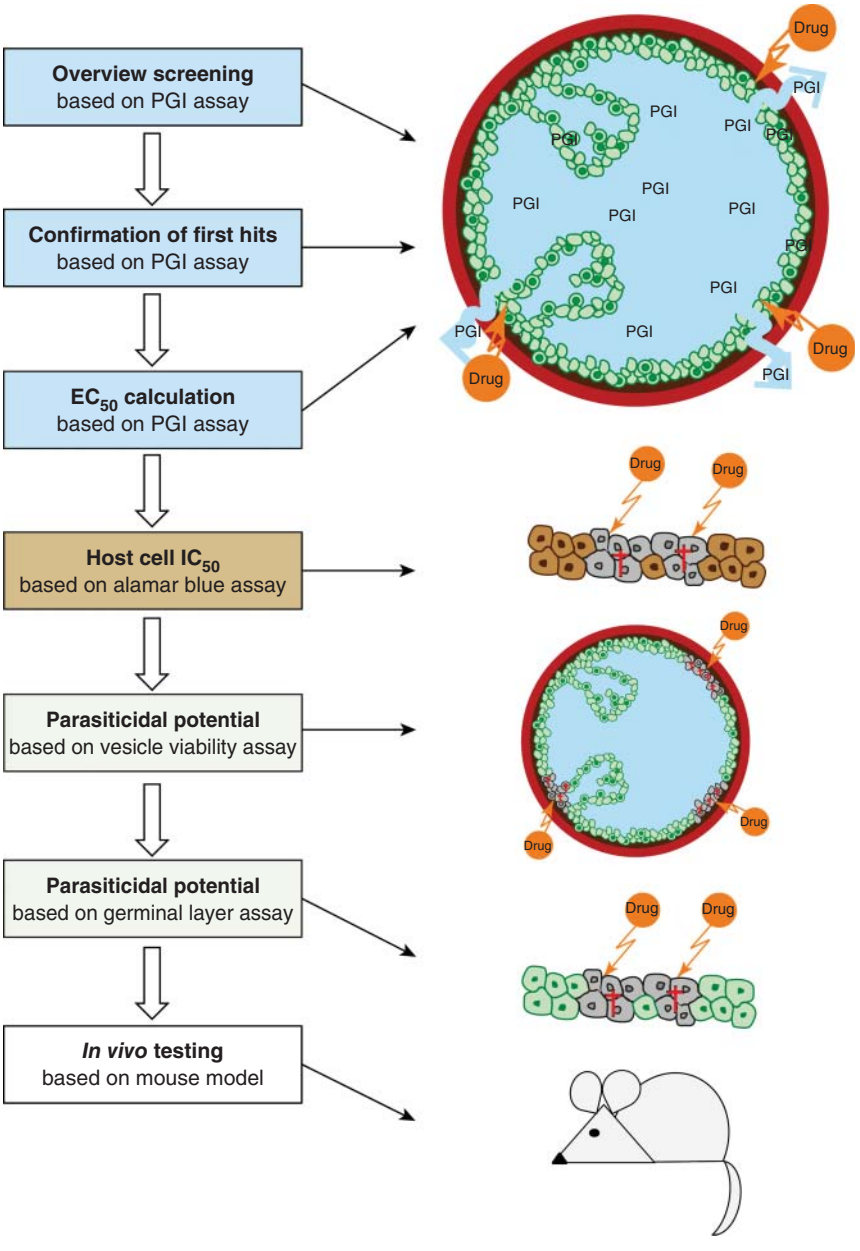

Figure 10.3 *In vitro* screening cascade of compounds against *E. multilocularis*. The three first steps of the screening are based on the PGI assay that detects metacestode damage. It is followed by host cell toxicity assessments; and only if a potential therapeutic window can be seen, further tests on potential parasiticidal activity are included. Parasite cells are depicted in green, host cells in brown, dead cells in gray, and drugs in orange. (From Ref [166].)

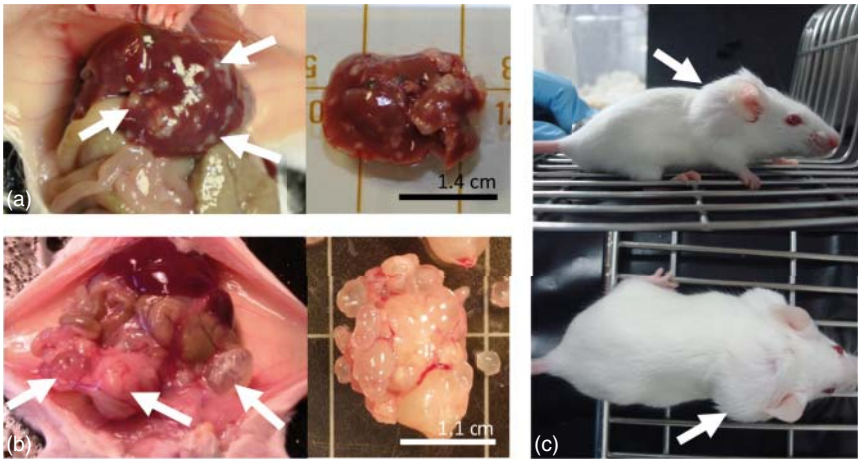

Figure 10.4 Different *in vivo* models for AE in mice. Peroral infection with *E. multilocularis* eggs resulting in liver lesions (a), intraperitoneal infection with metacestode material resulting in peritoneal lesions (b) and subcutaneous lesions visible from the outside (c, from [73]). Growing parasites are indicated by arrows. Source: PLOS, https://doi.org/10.1371/journal.pntd.0002235. Public domain.

action or target organelle. Molecular targets can be identified by pull-down studies of immobilized drugs on a matrix, through which parasite extract is passed [69], or by comparing the changes in the transcriptome or proteome of parasites treated with specific drugs. Also, metabolomic studies based on NMR were applied in the past to get further insights into the mode of action of drugs [46]. Reverse genetic approaches based on RNAi are applied to validate targets of interest in protoscoleces and stem cells [70, 71].

Once a compound with promising *in vitro* efficacy and selective toxicity is identified, it can be evaluated *in vivo*. Mice, as the natural intermediate host of *E. multilocularis,* represent an ideal experimental model. Two infection models can be distinguished: (i) a secondary infection model, in which mice are either intraperitoneally [72] or subcutaneously [73] infected with *E. multilocularis* vesicle suspension; (ii) a primary egg infection model [74], in which mice are orally infected by applying *E. multilocularis* eggs by gavage, thus representing the natural infection mode (Figure 10.4). In both models, treatments with drugs are initiated four to six weeks postinfection, and can last up to eight weeks or even longer, depending on compound properties and application mode. Ideally, compounds are applied orally, since multiple injections raise ethical concerns with regard to animal experimentation. Critical issues are compound formulation, mode of administration, frequency, and dosage. The formulation of drugs in honey was proposed by Küster et al. to make laboratory mice voluntarily ingest the compounds [75], but this approach is not feasible for all compounds (own observations). The *in vivo* mouse model for drug testing has been improved and standardized over the past years [73, 76, 77]. At the end of treatment, the final parasite mass is assessed upon necropsy and compared to placebo-treated

animals. Determination of the parasite weight is the most accurate method of choice and gives a clear readout of *in vivo* drug efficacy against AE [76].

For *E. granulosus*, respective *in vitro* culture of the metacestode stage is achieved by either culture of protoscoleces for several months until they differentiate into small metacestodes or the metacestodes are removed from the peritoneal cavity of an experimentally infected rodent and maintained *in vitro* without proliferation. Until very recently, the only assays to assess viability of protoscoleces were based on microscopical inspection of specimens subjected to a dye (e.g. eosin, trypan blue) that stains nonviable, but not viable, parasites, and corresponding assessments are not purely objective. A newly developed technique, based on an automated assessment of drug effects by monitoring of protoscolex movement, was introduced by Ritler et al. [78]. This assay was developed for *E. multilocularis,* but is surely also applicable to *E. granulosus* protoscoleces, and allows an improved objective and higher throughput assessment of drug effects compared to microscopical inspection. To investigate the impact of drugs on the viability of *E. granulosus* metacestodes *in vitro*, trypan blue incorporation into the detached germinal layer has also been assessed in most publications, but this method is rather subjective; and for the future, more quantitative assays such as the PGI assay [66] or the motility assay [78] should be employed. The time span between infection of rodents with *E. granulosus* protoscoleces and metacestode development can take 6–10 months, as opposed to 2–3 months for rodents secondarily infected with *E. multilocularis* metacestodes. Although experimental chemotherapy with *E. granulosus* is more time-consuming, numerous interesting studies have been carried out. In many instances, *in vitro* studies on compounds active against *E. granulosus* have employed protoscoleces, since these can be obtained in a local abattoir, and it was shown that several compounds that exhibit protoscolicidal activities are not active against the metacestode stage [63]. Conversely, compounds that exhibit profound activities against *E. multilocularis* metacestodes are most likely also efficacious against *E. granulosus* metacestodes [63].

10.6 Drug Repurposing as the Only Strategy for Discovering Novel Compounds to Treat Echinococcosis

Realistically, it is unlikely that the pharmaceutical industry will invest in the development of a novel compound for the treatment of echinococcosis, since these investments will not result in a relevant market return. In addition, echinococcosis is a disease that is difficult to diagnose, and rather time-consuming to treat, and monitoring of treatment success is a complex undertaking, since success or failure will become evident only after months or several years of treatment. Thus, echinococcosis is also not high on the list of those foundations and private public partnership organizations that are devoted to providing better health care for neglected tropical diseases, but at the same time want (or are obliged) to deliver success stories in a timely manner. Thus, additional compounds with promising

anti-*Echinococcus* activities can only be identified and implemented by exploiting the plethora of drugs that are currently on the market or being developed for other indications. Therefore, drug screening efforts have so far largely focused on already existing drugs or compound classes from other research areas. These include mostly broad-spectrum anti-infective drugs, and drugs that inhibit cellular proliferation such as anticancer compounds, but also natural products. In most studies, however, only relatively small numbers of compounds could be evaluated within a given time frame. This still stands true for *E. granulosus* (see Section 10.5); but for *E. multilocularis,* with the establishment of optimized *in vitro* culture approaches and a standardized screening cascade, medium-sized drug libraries could be screened.

10.6.1 Drug Repurposing for the Discovery of Novel Compounds to Treat AE

The search for novel treatment options against AE has focused on two major areas: (i) anti-infective drugs, and (ii) anticancer drugs. The first area is obvious: *E. multilocularis* metacestodes exhibit invasive growth, are highly adapted to a parasitic lifestyle, and exert considerable immunomodulation. These features are comparable to other pathogens, and therefore drugs that affect other pathogens could also be efficacious against *E. multilocularis*. The focus on anticancer drugs can be explained by the fact that *E. multilocularis* metacestodes and malignant tumors share some distinct features: they have an unlimited proliferative capacity, express similar cell cycle regulators such as 14-3-3 protein, modulate the immune response, secrete proteolytic enzymes to reach their target sites or organs, induce angiogenesis, and exhibit the capacity to form metastases. These similarities suggested early on that antiproliferative compounds could also affect *E. multilocularis* metacestodes. In addition, it is not surprising that many compounds that exhibit broad-spectrum anti-infective activities (including artemisinins, mefloquine, nitazoxanide-derivatives and benzimidazoles) show clearly elevated toxicity in proliferating cells [69, 79].

10.6.1.1 Anti-Infective Agents

Earlier animal experimentation studies in rodents demonstrated parasitostatic effects against experimental echincoccosis of mytomicin C, piperazine, quinacrine hydrochloride, chloroquine phosphate, alkylaminoethers, and propargylic alcohols, either at a lower level or comparable to benzimidazoles (reviewed by Siles-Lucas and Hemphill [72]). *In vivo* treatments with praziquantel were shown to enhance growth of the parasite rather than diminish it [80], and therapy employing α-difluoromethylornithine against secondary AE was ineffective [81]. The impact of other anti-infective compounds was studied *in vitro,* including ABZ and its metabolites ABZ-sulfoxide and ABZ-sulfone [46]. Conversely to what was previously thought, ABZ-sulfone exhibited anti-metacestode activity similar to that of ABZ and its sulfoxide. Other anti-infective agents such as nitazoxanide [82], artemether, caspofungin, itraconazole, ivermectin, methiazole, miltefosine, rifampicin, and trimethoprim/sulfamethoxazole were assessed *in vitro* [83]. ABZ and its two metabolites along with itraconazole,

methiazole, and nitazoxanide effectively destroyed parasite vesicles *in vitro*, but regrowth of vesicles was noted once these compounds were removed. Thus, only parasitostatic effects were demonstrated. Fenbendazole was equally active as was ABZ when applied in experimentally infected mice [47]. This is not surprising, since benzimidazoles appear to act with an identical mode of action on microtubules, and thus inhibit a variety of cellular functions related to the integrity of the cytoskeleton. In support of this notion, molecular genetics revealed that sensitivity to benzimidazoles in evolutionary distant organisms such as fungi, nematodes, platyhelminthes, and various protozoa was correlated with the presence of specific alleles of β-tubulin genes (reviewed in Hemphill and Müller [84]). Like other benzimidazoles, fenbendazole and its metabolites are believed to interfere with microtubule formation by binding to free β-tubulin of the parasite, thus interfering with microtubule-dependent uptake of glucose [41]. Improved *in vivo* efficacy could probably be achieved by employing the prodrug febantel, which is much better absorbed, and this would result in a prolonged half-life of the active oxfendazole. *In vivo*, febantel undergoes cyclization to fenbendazole, which is interconvertible with oxfendazole [47].

Nitazoxanide is a broad-spectrum antiparasitic drug belonging to the thiazolide family, with reported antiparasitic, antibacterial, and antiviral activities [85]. Besides being effective against *E. multilocularis* metacestodes and *E. granulosus* protoscoleces and metacestodes *in vitro* [52, 82], nitazoxanide, applied orally by gavage, was also effective in experimentally infected mice against CE and AE. Furthermore, an ABZ-nitazoxanide combination treatment was shown to be more effective than ABZ alone [74]. However, this effect was not caused by a synergistic mode of action, but both compounds are most likely metabolized by the same Cyp450 enzyme, resulting in a delay of metabolic ABZ-sulfoxide conversion to ABZ-sulfone due to the competition of nitazoxanide and causing a prolonged presence of ABZ-sulfoxide in the serum [74]. In human patients with AE, neither nitazoxanide monotherapy nor ABZ-nitazoxanide combination therapies were effective [49, 50]. Besides nitazoxanide, 29 nitazoxanide derivatives were assessed for anti-*E. multilocularis* metacestode activity *in vitro* using the PGI assay [66]. Nitro compounds, similar to nitazoxanide, but also halogenated molecules with halogenations on the thiazole moiety and also on the salicyl moiety of the thiazole scaffold exhibited enhanced PGI activity values, and extensive morphological damage was noted already after five days of treatment [66]. Thus, thiazolides are a promising class of compounds. Unfortunately, Romark LC (www.romark.com), which brought nitazoxanide on the market, was not willing to invest in a neglected tropical disease that would not bring substantial market return, and the development of thiazolides for treatment of AE was halted.

Another compound showing promising efficacy against murine AE in combination with ABZ is thymol (**9**, Figure 10.2). Thymol and a combination of thymol with ABZ exhibited promising efficacy against protoscoleces and metacestodes *in vitro* [86]. In experimentally secondary infected mice, combined ABZ/thymol treatment for 20 days starting at seven weeks after infection resulted in a considerably reduced parasite weight compared to ABZ or thymol treatments alone [87]. However, we have not been able to confirm these *in vitro* efficacy results in

our own laboratory employing the *E. multilocularis* protoscolex movement assay [78] and PGI assay [66] (own unpublished findings).

The antifungal compound amphotericin B desoxycholate (cAMB) was shown to inhibit the growth of *E. multilocularis* metacestodes *in vitro*, and in human patients *in vivo* [88, 89]. A major limitation of cAMB is the intravenous application mode, which makes it unsuitable for prolonged use, except for salvage treatment. Also, the effect of cAMB is only parasitostatic and the drug is nephrotoxic. Nevertheless, prolonged application of cAMB for months to years may be feasible in some cases, as side effects are mild and serious organ damage does not appear to occur [89].

Earlier studies had shown that pentamidine, belonging to the class of di-cationic compounds developed against intracellular and extracellular protozoan parasites such as *Leishmania, Trypanosoma*, and *Plasmodium* (for review, see [90]), did not have an effect in mice and jirds experimentally infected with *E. granulosus* [91]. A small panel of di-*N*-aryl-diguanidino compounds was screened for efficacy against *E. multilocularis* metacestodes *in vitro*. Only those with a thiophene core group were active against metacestodes, while furans were not [92, 93]. The most active compound, DB1127 (**10**, Figure 10.2), was further assessed in mice. DB1127 was effective against AE when administered intraperitoneally but not when applied orally [92]. Thus, thiophene-diguanidino derivatives with improved oral bioavailability should be further developed.

A major focus in the search for anti-echinococcal activities has been antimalarial compounds. Artemisinin and artemisinin peroxides (ozonids), and mefloquine (**11**, Figure 10.2) and its enantiomers, were initially evaluated using *in vitro* cultured metacestodes [94, 95]. *In vitro* treatment of *E. multilocularis* metacestodes with the antimalarials dihydroartemisinin and artesunate exhibited promising results. However, six weeks of *in vivo* treatment of mice infected with *E. multilocularis* metacestodes with these compounds had no effect. Combination treatments of both drugs with ABZ led to a measurable, but statistically nonsignificant, reduction in parasite weight compared to results with ABZ alone [95]. Further *in vitro* assessments of artemisinin derivatives using a series of amino-ozonids were carried out [94]. Three ozonids, namely OZ401, OZ455, and OZ491 containing an aminopropylether substructure, were the most potent, with IC_{50} values ranging from 11 to 14 μM. Cytotoxicity in different mammalian cell lines was observed only at higher concentrations. Transmission electron microscopy demonstrated complete destruction of the germinal layer after five days of drug exposure. Amino-ozonids have not been further pursued, since the concentration required for anti-echinococcal activity is not achieved in animals or humans. However, this class of compounds could be a valuable addition to the currently very limited arsenal of anti-echinococcal drugs, provided they are modified to increase bioavailability and pharmacokinetics to obtain increased exposure in the infected host.

Mefloquine, also widely used as an antimalarial, exhibits highly interesting *in vitro* and *in vivo* activities against *E. multilocularis* metacestodes. Previously, *in vitro* and *in vivo* studies demonstrated promising activities of mefloquine and mefloquine enantiomers in mice infected with young or adult stages of *Schistosoma mansoni* or *Schistosoma japonicum* [96, 97]. Mefloquine was also shown to

be active against *Opisthorchis viverrini in vitro* and in infected hamsters [98] and against larval and adult stages of *Brugia patei* and *Brugia malayi in vitro* [99]. *In vitro* treatment of *E. multilocularis* metacestodes resulted in detachment of large parts of the germinal layer from the inner surface of the laminated layer within a few hours [100]. Intraperitoneal application of mefloquine in secondarily infected mice (25 mg/kg, twice a week) resulted in a reduction in parasite weight that was similar to what was obtained with orally applied ABZ (200 mg/kg/d) [100]. More recent studies have shown that the success of oral application of mefloquine in mice is dose-dependent, and at a higher dosage (100 mg/kg/twice per week for 12 weeks), results in a reduction in parasite weight comparable to what is achieved by ABZ treatment (200 mg/kg /d) [101]. In the same study, two *Echinococcus* mefloquine-binding proteins were identified by affinity chromatography using mefloquine coupled to epoxy-activated Sepharose®, followed by SDS-PAGE and in-gel digestion LC–MS/MS. This resulted in the identification of *E. multilocularis* ferritin and cystatin as MEF-binding proteins. In contrast, affinity chromatography of human fibroblast extracts on mefloquine-sepharose matrices resulted in the identification of nicotinamide phosphoribosyl transferase. This indicates that mefloquine could potentially interact with different proteins in parasites and human cells [101].

Other antimalarial drugs with various degrees of efficacy against *E. multilocularis* metacestodes were identified more recently by repurposing the Medicines for Malaria Venture (MMV) Malaria Box [65]. This open-source library contains 400 commercially available chemicals that show *in vitro* activity against *Plasmodium falciparum*, and was provided by MMV free of charge. Primary PGI-assay-based screening was carried out at 10 µM, yielding 24 potentially interesting compounds that cause physical damage to metacestodes. Seven compounds retained their activity at 1 µM, but dose–response experiments showed that only two compounds exhibited an $IC_{50} < 5$ µM. After cytotoxicity assessment, only MMV665807 (**12**, Figure 10.2) was further assessed, and was shown to exhibit parasiticidal activity against germinal layer cell cultures. Transmission electron microscopy showed that MMV665807 primarily affected the structural organization of the mitochondrial matrix in the germinal layer tissue, and also caused an increased release of microvesicles into the laminated layer at a later stage. MMV665807 is a salicylanilide derivative, similar to the already commercially available niclosamide, which is used against adult-stage cestodes [102]. Niclosamide was shown to be also efficacious against various cancer cells *in vivo* and *in vitro* [103]. Unfortunately, when assessed in experimentally infected mice, both oral (gavage) and intraperitoneal application of MMV665807 did not result in reduced parasite burden [65]. However, currently, different formulations of MMV665807, designed to achieve increased plasma levels, are being assessed *in vitro* and *in vivo*.

Mathis et al. [104] were the first and only ones to describe a target-based *in silico* approach for the identification of novel compounds for echinococcosis treatment. They reported on the anti-echinococcal properties of clarithromycin (**13**, Figure 10.2), a macrolide antibiotic. Clarithromycin inhibits protein synthesis in bacteria by binding to the nascent peptide exit tunnel on the ribosome near the peptidyltransferase center of the large subunit rRNA [105]. Higher

eukaryotes carry a guanine at position 2058 of both cytoplasmic and mitochondrial rRNAs, and the modification at this position had been demonstrated to confer the resistance of eukaryotic cells to macrolide antibiotics. In contrast, the mitochondrial rRNA of *E. multilocularis* carries an adenine at sequence position 2058, which predicts susceptibility as in bacteria [106], while the nucleus-encoded rRNA is characterized by a guanine at 2058. *In vitro* culture of *E. multilocularis* metacestodes in the presence of clarithromycin resulted in severely impaired growth and overall morphology of the germinal layer of these parasites. However, these results have not been followed up *in vivo*.

10.6.1.2 Anticancer Drugs

Doxorubicin, or hydroxydaunorubicin, a DNA-interacting drug used widely in the treatment of cancers, was one of the first anticancer drugs to be studied for its potential use against AE. Doxorubicin bound to polyisohexylcyanoacrylate nanoparticles (a colloidal biodegradable drug carrier) was applied in *E. mutlilocularis*-infected mice, which yielded a reduction of the parasite development in the liver and a reduced viability of the metacestode. In contrast, free doxorubicin or unbound nanoparticles had no antiparasitic activity [107]. However, due to the massive side effects that are generally encountered by doxorubicin, this treatment approach was not further pursued.

Isatin (1*H*-indole-2,3-dione) and its derivatives are responsible for a broad spectrum of biological activities. Among these, the cytotoxic and antineoplastic properties have been the most widely reported. The synthetic versatility of isatin has led to the generation of a large number of structurally diverse derivatives, due to its privileged scaffold. Several propargylic alcohols derived from isatin were synthesized and the drug-induced morphological alterations in *E. multilocularis*-infected *Meriones unguiculatus* were described, documenting interesting antiparasitic properties [108].

Isoflavonoids, another class of antitumor agents with proven antiparasitic activities, are formed by plant tissue in response to physiological stimuli such as infectious agents. The isoflavonoid genistein, a major component of soya, is active against breast, prostate, skin, and colon cancer cell lines. Genistein also stimulates the synthesis of TGF-β, which itself inhibits cancer cell proliferation [109]. Besides other targets, genistein acts on several signaling pathways, inhibiting the activity of several kinases (tyrosine kinase, MAP kinase, ribosomal S6 kinase). In addition, genistein acts as a ligand for the estrogen receptor-beta; and upon long-term treatment, this could exert unfavorable effects [110]. Naguleswaran et al. [111] showed that besides genistein, several genistein derivatives that do not bind to the estrogen receptor-beta were also effective against *E. multilocularis* metacestodes *in vitro*, as well as against *E. granulosus* metacestodes and protoscoleces. These compounds could interfere in signaling, for instance, by interfering in the tyrosine kinase activity associated with the epidermal growth factor receptor identified in *E. multilocularis* [112], but the molecular mechanisms have not been elucidated. The anti-echinococcal efficacy of isoflavonoids has not been assessed *in vivo* to date. In contrast, tamoxifen (**14**, Figure 10.2), an antagonist of the estrogen receptor-alpha and used for the treatment of primary breast cancer, was shown to be moderately active against

E. multilocularis in vitro [113], and to severely impair the growth of *E. granulosus* cysts in mice [114].

Another compound with antitumor effects, 2-methoxyestradiol (2-ME), an endogenous metabolite of estrogen, also induces severe and dose-dependent damage to *E. multilocularis* metacestodes *in vitro* [115]. Treatment of experimentally infected mice with 2-ME alone did not result in a reduction in parasite weight compared to the non-treated controls. The results achieved with the treatment of a combination of 2-ME and ABZ led to a substantial, but not statistically significant, increased reduction in parasite weight compared to ABZ treatment alone.

Protein kinases, especially serine/threonine and tyrosine kinases, activate a multitude of proteins and mediate signal transduction, cell growth, and differentiation. Kinases are known to play a crucial role in tumor progression, and they also are involved in the regulation of a plethora of cellular events in other diseases. From the list of the 20 most promising drug targets identified in the *E. multilocularis* genome [55], four are protein kinases. *E. multilocularis* metacestodes have been shown to express a wide range of signaling receptors including nuclear hormone receptor, TGF receptor, insulin receptor, epidermal growth factor receptor, and fetal growth factor receptor, which have been shown to be activated by either parasite- or host-derived ligands (reviewed in [116–118]). Pyridinyl imidazole compounds such as ML3403 and SB202190 are selective inhibitors of p38 mitogen-activated protein kinase (MAPK) *in vitro*, block pro-inflammatory cytokine production *in vivo*, and are implicated in the treatment of melanoma [119]. These pyridinyl imidazoles were identified to act as ATP-competitive inhibitors of MAPK of *E. multilocularis in vitro*, which resulted in the death of parasite vesicles at concentrations that did not affect cultured mammalian cells [120]. Other kinase inhibitors that were assessed in *E. multilocularis* vesicles were the Raf-inhibitor sorafenib and the MEK1/2 inhibitor PD184352 [121], which inhibited vesicle growth, but failed to exert parasticidal activity. The ABL-like kinase inhibitor imatinib (**15**, Figure 10.2), one of the first kinase inhibitors that was FDA approved as an anticancer drug, exhibited dose-dependent efficacy against *E. multilocularis* metacestodes, protoscoleces, and stem cell cultures *in vitro* [122]. *E. multilocularis* metacestodes also express a kinase with significant homology to the Plk1 subfamily of Polo-like kinases in higher eukaryotes. Addition of BI 2536, a Plk1 inhibitor that has been tested in clinical trials against cancer, at concentrations as low as 20 nM significantly blocked the formation of metacestode vesicles from *Echinococcus* germinal cell cultures [123]. In addition, low concentrations of BI 2536 eliminated the stem cell population from mature metacestode vesicles *in vitro*, yielding parasite tissue that was no longer capable of proliferation. Thus, a series of kinase inhibitors that are candidate drugs (or are in use) for cancer treatment exhibit profound inhibitory properties on *E. multilocularis*. However, to the best of our knowledge, none of these findings has been reproduced in an animal model to date.

Cytostatic drugs can also exhibit effects that lead to increased proliferation and growth of *E. multilocularis* metacestodes *in vivo* [77]. It was shown that *in vitro* exposure of metacestodes to methotrexate and subsequent infection of mice with

treated parasites led to massive growth and enhanced parasite proliferation, while navelbine and vincristine treatment had a slight negative impact on parasite proliferation.

Nitazoxanide, previously introduced as an anti-infective drug (see Section 10.6.1.1), also inhibits the proliferation of colon cancer cells *in vitro*, and Müller et al. [69] have shown that this happens by interfering with, and inhibiting, the activity of glutathione-*S*-transferase (GST) class π, an isoform overexpressed in many proliferating cells. In *E. granulosus* and *E. multilocularis*, the only GSTs characterized so far, have some sequence homologies to the mammalian class μ [124, 125]. The catalytical properties of recombinant GST of *E. multilocularis* had, however, exhibited higher similarities to mammalian classes α and π, with, especially, a high conjugating activity on ethacrynic acid, another anticancer drug. In principle, GSTs may have two opposite effects on drugs, namely, by inactivating drugs or by activating ineffective prodrugs. Especially, the latter effects have been employed as an anticancer drug strategy and may be further developed against AE. In addition, these studies suggest that *Echinococcus* GST should be further investigated, and validated, as a potential drug target.

Another class of anticancer compounds that has attracted increasing attention in the past years are metallo-organic ruthenium complexes. Metallo-drugs were also shown to exhibit interesting antimicrobial properties, including activities against bacteria, trypanosomatids, *Toxoplasma*, and *Plasmodium* [126–129]. Two series of *η*6-areneruthenium(II) phosphite complexes were evaluated *in vitro* for their toxic potential against *E. multilocularis* metacestodes [130]. This screening identified several hydrolytically stable ruthenium complexes with *in vitro* toxicity for metacestodes in the range of nitazoxanide, also high cytotoxicity against rat hepatoma cells, but little toxicity for Vero cells and human fibroblasts. This indicates a certain potential for ruthenium compounds, but corresponding *in vivo* studies are still pending.

The first screening of a commercially available drug library was performed by Stadelmann et al. [113] by evaluating the efficacy of 426 compounds contained in an FDA-approved drug library. This library is composed of drugs against a diverse range of diseases, many of which are against viral infections and/or cancer. Initial screening at 20 μM revealed that seven drugs induced considerable metacestode damage, and further dose–response studies revealed that bortezomib (**16**, Figure 10.2), a proteasome inhibitor developed for the chemotherapy of myeloma, displayed high anti-metacestodal activity with an EC_{50} of 0.6 μM, leading to an accumulation of ubiquinated proteins and unequivocally parasite death. In-gel zymography assays using *E. multilocularis* extracts demonstrated bortezomib-mediated inhibition of protease activity in a band of approximately 23 kDa, the same size at which the proteasome subunit beta 5 of *E. multilocularis* could be detected by Western blot. Treatment of BalB/c mice experimentally infected with *E. multilocularis* metacestodes with bortezomib led to reduced parasite weight, but to a degree that was not statistically significant, and it induced adverse effects such as diarrhea and neurological symptoms. Thus, this study identified the proteasome as a drug target in *E. multilocularis* metacestodes that can be efficiently inhibited and further investigations employing treatment adjustment and/or other proteasome inhibitors are necessary.

10.6.2 Drug Repurposing for the Discovery of Novel Compounds to Treat CE

Early studies carried out in experimentally infected mice and jirds assessed the *in vivo* effects of iodinated oil of thymol, ethyl-*N*-dimethyl ether of thymol fumarate, chloroguanide, rifampin, pentamidine isethionate, amphotericin B, suramin, and methotrexate on secondary CE. None of these compounds exhibited any meaningful reduction of cyst weight under the conditions used [91]. Other compounds were assessed for activity against *E. granulosus* protoscoleces such as cetrimide [131] and the ionophore monensin [132], but they were not effective against metacestodes. In contrast, levamisole and ivermectin, which are classically used against nematode infections, exhibited *in vitro* activities against protoscoleces as did benzimidazoles [133]. A novel prophylactic therapy approach that would avoid the formation of metastases due to spilling of *E. granulosus* protoscoleces was developed by exposing protoscoleces to praziquantel [30, 31], or a combination of praziquantel and ABZ [30] prior to injection into mice. Since then, combined ABZ/praziquantel therapy in the treatment of human CE has been controversially discussed [32, 134–136].

Benzimidazoles show variable efficacy. Albendazole and its metabolite ABZ-sulfoxide have been reported to be active against *E. granulosus* protoscoleces *in vitro* [137–139], but they act slowly over a period of several days to weeks. In contrast, MBZ and oxfendazole (fenbendazole-sulfoxide) seem to act more rapidly [138, 140]. A combination of fenbendazole and netobimin, which is a prodrug of ABZ used as a veterinary anthelmintic, was shown to act synergistically against *E. granulosus* infection in rodents [141]. Experiments carried out in *E. granulosus*-infected sheep and goats suggested that oxfendazole may be as efficacious as ABZ, but does not require daily uptake of the drug because of its prolonged bioavailability [142]. Other benzimidazoles exhibiting interesting protoscolicidal and metacestodicidal activity are flubendazole [143] and nocodazole [144]. Flubendazole combined with nitazoxanide did also exhibit profound efficacy in *E. granulosus* protoscoleces and metacestodes *in vitro,* and in mice experimentally infected by intraperitoneal injection of protoscoleces [145]. *In vitro* treatments with another broad-spectrum anti-infective compound, thiazolide nitazoxanide [85], resulted in severe damage to protoscoleces and the germinal layer of the respective metacestodes within a few days [52], but nitazoxanide treatment in experimentally *E. granulosus*-infected sheep was not effective [146]. However, oxfendazole treatment, and a combination of oxfendazole and nitazoxanide, significantly decreased the number of fertile cysts and increased the number of degenerated cysts in sheep [146]. In addition, nitazoxanide did also not affect hydatid cyst development in mice [145]. Surprisingly, two case reports suggested that nitazoxanide may be an effective treatment option in CE, particularly in patients with progressive disease who receive conventional therapy, but surely further studies need to be carried out to verify these findings in other patients [51, 53].

The immunosuppressant drug cyclosporin A, which is employed mainly during the management of organ transplants, has reported activity against CE in the murine model. Cyclosporin has a profound effect when administered early (from

two days) after infection, in five consecutive doses daily, resulting in reduced cyst numbers and mass after 20 weeks. When cyclosporin was administered 18 weeks postinfection, the wet weight was decreased by 42% compared to untreated controls. Transmission electron microscopy of the germinal membrane and laminated layer of late-treated *E. granulosus* revealed abnormalities in all cysts studied, whereas control and early-treated hydatid cysts were normal, indicating that cyclosporin exerts parasitostatic rather than parasiticidal effects [147].

Tamoxifen is a nonsteroidal selective estrogen receptor modulator binding to estrogen receptor-alpha, which is widely used against compounds for the treatment of primary breast cancer in premenopausal women and gynecomastia in men receiving hormonal therapy for prostatic carcinoma. At 10–50 μM, this compound impacted on *E. granulosus* protoscoleces and metacestode survival *in vitro*, and at a dose rate of 20 mg/kg of body weight, tamoxifen induced protection against the infection in mice. In the clinical efficacy studies, a reduction in cyst weight was observed after the administration of 20 mg/kg in mice with cysts developed during three or six months, compared to that of those collected from control mice [114]. Tamoxifen was also shown to be active against the cestodes *Taenia crassiceps* and *T. solium* [148, 149]. The activity of another anticancer drug, the proteasome inhibitor bortezomib, was demonstrated recently. Bortezomib was shown to exhibit considerable *in vitro* activity by eliciting endoplasmic reticulum stress and autophagy in *E. granulosus* protoscoleces and metacestodes, but no *in vivo* studies have been reported so far [150]. In addition, bortezomib was evaluated in combination with the kinase inhibitor rapamycin, and a synergistic effect on protoscolece viability could be observed with the combination. Rapamycin is a polyketide macrolide that binds to FK506-binding proteins, which are involved in protein folding, and known to be targets for antiproliferative drugs. The rapalogs rapamycin, FK506 (tacrolimus), and everolimus were reported to exhibit protoscolicdal effects *in vitro*, and synergistic scolicidal actions were observed during combined therapy with rapalogs plus cyclosporine [150]. *In vivo* studies on these rapalogs have not been reported to date.

Recent studies investigated the protoscolicidal effects of chenodeoxycholic acid (CDCA) and sodium arsenite *in vitro* using eosin staining and caspase detection assays to monitor apoptosis. CDCA is a bile acid that has been therapeutically tested against hepatitis C (in combination with bezafribate), and is used against constipation and against cerebral cholesterosis. Dose-dependent mortality and induction of apoptosis in protoscoleces was noted at concentrations of 500–3000 μM after a few days of treatment [151]. Sodium arsenite has strong teratogenic and carcinogenic effects, and is not surprisingly toxic for protoscoleces at lower concentrations (16 μM) [152]. However, the reported toxicity and the strong adverse reactions expected through treatment with sodium arsenite render its application rather unlikely.

The activities of two antidiabetic drugs against secondary CE in mice were demonstrated. Glibenclamide is a second-generation sulfonylurea receptor inhibitor, which has been shown to be active against protozoan parasites [153]. Metformin is a plant-derived antihyperglycemic and potential anticancer agent which may exert its antiproliferative effects via the induction of energetic stress

and activation of AMP-activated protein kinase [154]. No *in vivo* data is available for glibenclamide to date, but oral administration of metformin (50 mg/kg/d) in *E. granulosus*-infected mice was highly effective in reducing the weight and number of parasite cysts, yet its combination with the lowest recommended dose of ABZ (5 mg/kg/d) was even more effective [153].

Natural products obtained from essential oils from a variety of medicinal plants have been frequently studied as protoscolicidal agents, either to replace 20% NaCl and 95% ethanol during PAIR, to reduce metastasis formation by protoscoleces spillage during surgery, or to reduce cyst load by *E. granulosus* metacestodes. Of all the natural herb products tested, thymol and carvacrol (**17**, Figure 10.2), two isomers which differ only by the positioning of a hydroxyl group appear to represent the most promising option. Thymol and carvacrol are the main components of essential oils of *Thymus vulgaris* and *Origanum vulgare*, and they have reported antibacterial and antifungal properties. Prior to the development of other anthelminthics, thymol was used to treat hookworm infections in the United States [155]. Elissondo et al. demonstrated the efficacy of thymol against *E. granulosus* protoscoleces [156]. Due to its apparent lack of toxicity combined with good efficacy, it was proposed to be used as a scolicidal agent during hydatid cysts surgery and/or PAIR [157]. Further investigations on thymol-based therapy against murine CE demonstrated promising chemoprophylactic and therapeutic efficacy that was comparable to ABZ treatment, and the authors suggested that thymol could be applicable as an alternative treatment in human CE [158]. Similarly, Fabbri et al. demonstrated profound *in vitro* and *in vivo* efficacy of carvacrol against *E. granulosus* metacestodes in mice, comparable with ABZ, suggesting that this compound could be applied as a potential alternative treatment option [159].

Mentha piperita and *Mentha pulegium* essential oils were studied for their efficacy against protoscoleces and metacestodes *in vitro* [160]. *M. pulegium* essential oil induced dose- and time-dependent protoscolicidal effects, as well as loss of turgor in metacestodes maintained *in vitro*. These findings were substantiated by scanning and transmission electron microscopy studies of protoscoleces and germinal layer. The main compound in *M. pulegium* essential oil is piperitone oxide, and it was suggested, but unfortunately not conclusively demonstrated, that piperitone oxide could be responsible for protoscolicidal and metacestodicidal effects. Other natural products that were described to have significant anti-protoscolex activity are derived from endophytic *Pestalotiopsis* sp. [161], from the circassian walnut *Juglans regia* [162], *Myrtus communis L.* essential oil [163], and *Nectaroscordum tripedale L.* leaf extract [164]; but in all these studies, the corresponding active substances have not been determined.

10.7 Where to Go from Here?

For many of the compounds presented here, no *in vivo* studies were published after promising *in vitro* activities had been observed. This could be a result of (i) lack of project financing, (ii) lack of specificity and toxicity of the compound, or

(iii) the authors wanted to refrain from publishing "negative" results, which is, unfortunately, a commonly observed fact. Several substances were not pursued further, even though they looked promising in *in vivo* mouse trials, mainly due to side effects and toxicity. Others were not followed up most likely because of financial constraints.

A few drugs were, however, applied in human patients. Amphotericin B was used as a salvage treatment in humans, but is not recommended due to its intravenous route of administration and resulting nephrotoxicity [49]. Nitazoxanide was also applied against human AE, but it did not prove to be effective [49, 50]. Selected compounds received further attention regarding their formulation and mode of application in the *in vivo* mouse model: DB1127 was active when injected intraperitoneally, but not *per os* [92]. DB1127 was not further followed up because its prime indication project on protozoan parasites was discontinued, and data on bioavailability and pharmacokinetics were lacking [33]. Mefloquine was active in the mouse model when applied orally at a dosage of 100 mg/kg [101], but did not prove to be acting in a parasiticidal manner. Till date, there is reluctance to the long-term treatment of patients with AE using mefloquine, due to expected neurological side effects [165]. Thymol, an essential oil extracted from the plants *T. vulgaris* and *O. vulgare*, was successfully applied as an oral formulation in mice and exhibited a synergistic effect together with albendazole in both AE and CE mouse models. However, for AE, these findings on thymol could not be reproduced (own unpublished results). It is not known whether thymol acts parasiticidal and no studies have yet been performed on the potential toxicity of long-term application of thymol or its effects in the human patient [87].

Thus, none of the approaches carried out to date have identified alternatives with improved properties compared to the benzimidazoles used to date. However, some promising compounds (MMV665807, mefloquine, and thymol) are still being further investigated. Novel treatment options that act parasiticidal are still lacking. Thus, further screening efforts should focus on the screening of additional drug libraries to identify better compounds with increased efficacy, selective toxicity, and improved safety; and more biochemical and molecular studies are needed to identify relevant drug targets [33]. Combining drugs with different mechanisms of action, as has been done in a few instances, could be a possibility to improve treatment efficacy.

E. multilocularis and *E. granulosus* are highly adapted to a parasitic life style. Crucial genes and entire pathways for the *de novo* synthesis of pyrimidines, purines, and amino acids are absent in the genome, and genes for fatty acid and cholesterol *de novo* synthesis are largely missing [55]. Thus, these parasites need to acquire amino acids, lipids, purines, and other metabolites from their host, and transcripts coding for respective proteins involved in uptake and transport of these essential components are upregulated in the metacestode stage. These auxotrophies should be exploited for the development of novel therapeutic options. In addition, cestode parasites, similar to other helminths, express the malate dismutation pathway, with the anaerobic NADH-fumarate reductase system as a predominant component in the respiratory chain of *E. multilocularis*, and thus providing a unique opportunity to target the energy metabolism [45]. However, while much should/could be done, finances for building up a research

program on novel drugs for echinococcosis are difficult to acquire, and funding on this topic is not being regarded as a priority, neither by private nor public authorities. Hopefully, this will change in the near future.

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References

1 Alvarez Rojas, C.A., Romig, T., and Lightowlers, M.W. (2014). Echinococcus granulosus sensu lato genotypes infecting humans--review of current knowledge. *Int. J. Parasitol.* 44: 9–18.
2 Lymbery, A.J. (2017). Phylogenetic pattern, evolutionary processes and species delimitation in the genus echinococcus. *Adv. Parasitol.* 95: 111–145.
3 Lymbery, A.J., Jenkins, E.J., Schurer, J.M., and Thompson, R.C.A. (2015). Echinococcus canadensis, E. Borealis, and E. Intermedius. What's in a name? *Trends Parasitol.* 31: 23–29.
4 Thompson, R.C.A. (2017). Biology and systematics of echinococcus. *Adv. Parasitol.* 95: 65–109.
5 Xiao, N., Qiu, J., Nakao, M. et al. (2005). Echinococcus shiquicus N. Sp., a taeniid cestode from tibetan fox and plateau pika in China. *Int. J. Parasitol.* 35: 693–701.
6 Deplazes, P., Rinaldi, L., Alvarez Rojas, C.A. et al. (2017). Global distribution of alveolar and cystic echinococcosis. *Adv. Parasitol.* 95: 315–493.
7 Kern, P., Menezes da Silva, A., Akhan, O. et al. (2017). The echinococcoses: diagnosis, clinical management and burden of disease. *Adv. Parasitol.* 96: 259–369.
8 Gottstein, B., Stojkovic, M., Vuitton, D.A. et al. (2015). Threat of alveolar echinococcosis to public health – a challenge for Europe. *Trends Parasitol.* 31 (9): 407–12.
9 Deplazes, P. and Eckert, J. (2001). Veterinary aspects of alveolar echinococcosis--a zoonosis of public health significance. *Vet. Parasitol.* 98: 65–87.
10 Eckert, J. and Deplazes, P. (2004). Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clin. Microbiol. Rev.* 17: 107–135.
11 Jorgensen, P. and an der Heiden, M., Kern, P., Schöneberg, I., Krause, G., Alpers, K. (2008). Underreporting of human alveolar echinococcosis, Germany. *Emerg. Infect. Dis.* 14: 935–937.
12 Torgerson, P.R. and Macpherson, C.N.L. (2011). The socioeconomic burden of parasitic zoonoses: global trends. *Vet. Parasitol.* 182: 79–95.

13 Jenkins, D.J., Romig, T., and Thompson, R.C.A. (2005). Emergence/re-emergence of echinococcus spp.--a global update. *Int. J. Parasitol.* 35: 1205–1219.

14 Agudelo Higuita, N.I., Brunetti, E., and McCloskey, C. (2016). Cystic echinococcosis. *J. Clin. Microbiol.* 54: 518–523.

15 Brehm, K. (2010). Echinococcus multilocularis as an experimental model in stem cell research and molecular host–parasite interaction. *Parasitology* 137: 537–555.

16 Koziol, U., Rauschendorfer, T., Zanon Rodríguez, L. et al. (2014). The unique stem cell system of the immortal larva of the human parasite echinococcus multilocularis. *EvoDevo* 5: 10.

17 Koziol, U., Krohne, G., and Brehm, K. (2013). Anatomy and development of the larval nervous system in echinococcus multilocularis. *Front. Zool.* 10: 24.

18 Ali-Khan, Z., Siboo, R., Gomersall, M., and Faucher, M. (1983). Cystolytic events and the possible role of germinal cells in metastasis in chronic alveolar hydatidosis. *Ann. Trop. Med. Parasitol.* 77: 497–512.

19 Koziol, U. and Brehm, K. (2015). Recent advances in echinococcus genomics and stem cell research. *Vet. Parasitol.* .

20 Mehlhorn, H., Eckert, J., and Thompson, R.C.A. (1983). Proliferation and metastases formation of larval echinococcus multilocularis. *Z. Für. Parasitenkd.* 69: 749–763.

21 Hizem, A., M'rad, S., Oudni-M'rad, M. et al. (2016). Molecular genotyping of echinococcus granulosus using formalin-fixed paraffin-embedded preparations from human isolates in unusual tissue sites. *J. Helminthol.* 90: 417–421.

22 Bresson-Hadni, S., Delabrousse, E., Blagosklonov, O. et al. (2006). Imaging aspects and non-surgical interventional treatment in human alveolar echinococcosis. *Parasitol. Int.* 55 (Suppl.): S267–S272.

23 Siles-Lucas, M., Casulli, A., Conraths, F.J., and Müller, N. (2017). Laboratory diagnosis of echinococcus spp. in human patients and infected animals. *Adv. Parasitol.* 96: 159–257.

24 Gottstein, B., Wang, J., Blagosklonov, O. et al. (2014). Echinococcus metacestode: in search of viability markers. *Parasite Paris Fr.* 21: 63.

25 Kaltenbach, T.E.M., Graeter, T., Mason, R.A. et al. (2015). Determination of vitality of liver lesions by alveolar echinococcosis. Comparison of parametric contrast enhanced ultrasound (SonoVue®) with quantified 18F-FDG-PET-CT. *Nukl. Nucl. Med.* 54: 43–49.

26 Stumpe, K.D.M., Renner-Schneiter, E.C., Kuenzle, A.K. et al. (2007). F-18-fluorodeoxyglucose (FDG) positron-emission tomography of echinococcus multilocularis liver lesions: prospective evaluation of its value for diagnosis and follow-up during benzimidazole therapy. *Infection* 35: 11–18.

27 Kern, P. (2006). Medical treatment of echinococcosis under the guidance of good clinical practice (GCP/ICH). *Parasitol. Int.* 55 (Suppl.): S273–S282.

28 Brunetti, E., Kern, P., and Vuitton, D.A. (2010). Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Trop.* 114: 1–16.

29 Brunetti, E., Troia, G., Garlaschelli, A.L. et al. (2004). Twenty years of percutaneous treatments for cystic echinococcosis: a preliminary assessment of their use and safety. *Parassitologia* 46: 367–370.

30 Urrea-París, M.A., Casado, N., Moreno, M.J., and Rodriguez-Caabeiro, F. (2001). Chemoprophylactic praziquantel treatment in experimental hydatidosis. *Parasitol. Res.* 87: 510–512.

31 Urrea-París, M.A., Moreno, M.J., Casado, N., and Rodriguez-Caabeiro, F. (1999). Echinococcus granulosus: praziquantel treatment against the metacestode stage. *Parasitol. Res.* 85: 999–1006.

32 Cobo, F., Yarnoz, C., Sesma, B. et al. (1998). Albendazole plus praziquantel versus albendazole alone as a pre-operative treatment in intra-abdominal hydatisosis caused by echinococcus granulosus. *Trop. Med. Int. Health TM IH* 3: 462–466.

33 Hemphill, A., Stadelmann, B., Rufener, R. et al. (2014). Treatment of echinococcosis: albendazole and mebendazole – what Else? *Parasite Paris Fr.* 21: 70.

34 Kern, P. (2003). Echinococcus granulosus infection: clinical presentation, medical treatment and outcome. *Langenbecks Arch. Surg.* 388: 413–420.

35 Horton, R.J. (1989). Chemotherapy of echinococcus infection in man with albendazole. *Trans. R. Soc. Trop. Med. Hyg.* 83: 97–102.

36 Pawlowski, Z.S., Eckert, J., Vuitton, D.A. et al. (2001, 2001). Echinococcosis in humans: clinical aspects, diagnosis and treatment. In: *WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern*, 20–32. Paris: World Organization for Animal Health and World Health Organization.

37 Grüner, B., Kern, P., Mayer, B. et al. (2017). Comprehensive diagnosis and treatment of alveolar echinococcosis: a single-center, long-term observational study of 312 patients in Germany. *GMS Infect. Dis.* 1–12.

38 Reuter, S., Buck, A., Manfras, B. et al. (2004). Structured treatment interruption in patients with alveolar echinococcosis. *Hepatol. Baltim. Md.* 39: 509–517.

39 Ammann, R.W., Ilitsch, N., Marincek, B., and Freiburghaus, A.U. (1994). Effect of chemotherapy on the larval Mass. And the long-term course of alveolar echinococcosis swiss echinococcosis study group. *Hepatol. Baltim. Md.* 19: 735–742.

40 Torgerson, P.R., Schweiger, A., Deplazes, P. et al. (2008). Alveolar echinococcosis: from a deadly disease to a well-controlled infection. Relative survival and economic analysis in Switzerland over the last 35 years. *J. Hepatol.* 49: 72–77.

41 Lacey, E. (1990). Mode of action of benzimidazoles. *Parasitol. Today Pers. Ed.* 6: 112–115.

42 Brehm, K. and Koziol, U. (2014). On the importance of targeting parasite stem cells in anti-echinococcosis drug development. *Parasite Paris Fr.* 21: 72.

43 Xiao, S., Feng, J., and Yao, M. (1995). Effect of antihydatid drugs on carbohydrate metabolism of metacestode of echinococcus granulosus. *Chin. Med. J. (Engl.)* 108: 682–688.

44 Barrowman, M.M., Marriner, S.E., and Bogan, J.A. (1984). The fumarate reductase system as a site of anthelmintic attack in ascaris suum. *Biosci. Rep.* 4: 879–883.

45 Matsumoto, J., Sakamoto, K., Shinjyo, N. et al. (2008). Anaerobic NADH-fumarate reductase system is predominant in the respiratory chain of echinococcus multilocularis, providing a novel target for the chemotherapy of alveolar echinococcosis. *Antimicrob. Agents Chemother.* 52: 164–170.

46 Ingold, K., Bigler, P., Thormann, W. et al. (1999). Efficacies of albendazole sulfoxide and albendazole sulfone against *in vitro*-cultivated echinococcus multilocularis metacestodes. *Antimicrob. Agents Chemother.* 43: 1052–1061.

47 Küster, T., Stadelmann, B., Aeschbacher, D., and Hemphill, A. (2014). Activities of fenbendazole in comparison with albendazole against echinococcus multilocularis metacestodes *in vitro* and in a murine infection model. *Int. J. Antimicrob. Agents* 43: 335–342.

48 Ricken, F.J., Nell, J., Grüner, B. et al. (2017). Albendazole increases the inflammatory response and the amount of Em2-positive small particles of echinococcus multilocularis (spems) in human hepatic alveolar echinococcosis lesions. *PLoS Negl.Trop. Dis.* 11: e0005636.

49 Tappe, D., Müller, A., Frosch, M., and Stich, A. (2009). Limitations of amphotericin B and nitazoxanide in the treatment of alveolar echinococcosis. *Ann. Trop. Med. Parasitol.* 103: 177–181.

50 Kern, P.A., Abboud, P., Kern, W. et al. (2008). Critical appraisal of nitazoxanide for the treatment of alveolar echinococcosis. *Am. J. Trop. Med. Hyg.* 79: 119.

51 Pérez-Molina, J.A., Díaz-Menéndez, M., Gallego, J.I. et al. (2011). Evaluation of nitazoxanide for the treatment of disseminated cystic echinococcosis: report of five cases and literature review. *Am. J. Trop. Med. Hyg.* 84: 351–356.

52 Walker, M., Rossignol, J.F., Torgerson, P., and Hemphill, A. (2004). *In vitro* effects of nitazoxanide on echinococcus granulosus protoscoleces and metacestodes. *J. Antimicrob. Chemother.* 54: 609–616.

53 Winning, A., Braslins, P., and McCarthy, J.S. (2009). Case report: nitazoxanide for treatment of refractory bony hydatid disease. *Am. J. Trop. Med. Hyg.* 80: 176–178.

54 Spiliotis, M., Tappe, D., Sesterhenn, L., and Brehm, K. (2004). Long-term *in vitro* cultivation of echinococcus multilocularis metacestodes under axenic conditions. *Parasitol. Res.* 92: 430–432.

55 Tsai, I.J., Zarowiecki, M., Holroyd, N. et al. (2013). The genomes of four tapeworm species reveal adaptations to parasitism. *Nature* 496: 57–63.

56 Spiliotis, M., Lechner, S., Tappe, D. et al. (2008). Transient transfection of echinococcus multilocularis primary cells and complete *in vitro* regeneration of metacestode vesicles. *Int. J. Parasitol.* 38: 1025–1039.

57 Fernández, C., Gregory, W.F., Loke, P., and Maizels, R.M. (2002). Full-length-enriched cDNA libraries from echinococcus granulosus contain separate populations of oligo-capped and trans-spliced transcripts and a high level of predicted signal peptide sequences. *Mol. Biochem. Parasitol.* 122: 171–180.

58 Parkinson, J., Wasmuth, J.D., Salinas, G. et al. (2012). A transcriptomic analysis of echinococcus granulosus larval stages: implications for parasite biology and host adaptation. *PLoS Negl. Trop. Dis.* 6: e1897.

59 Zheng, H., Zhang, W., Zhang, L. et al. (2013). The Genome of the hydatid tapeworm echinococcus granulosus. *Nat. Genet.* 45: 1168–1175.

60 Rausch, R. and Jentoft, V.L. (1957). Studies on the helminth Fauna of Alaska. XXXI. Observations on the propagation of the larval echinococcus multilocularis leuckart, 1863, *in vitro*. *J. Parasitol.* 43: 1–8.

61 Hemphill, A. and Gottstein, B. (1995). Immunology and morphology studies on the proliferation of *in vitro* cultivated echinococcus multilocularis metacestodes. *Parasitol. Res.* 81: 605–614.

62 Jura, H., Bader, A., Hartmann, M. et al. (1996). Hepatic tissue culture model for study of host–parasite interactions in alveolar echinococcosis. *Infect. Immun.* 64: 3484–3490.

63 Hemphill, A., Stadelmann, B., Scholl, S. et al. (2010). Echinococcus metacestodes as laboratory models for the screening of drugs against cestodes and trematodes. *Parasitology* 137: 569–587.

64 Spiliotis, M. and Brehm, K. (2009). Axenic *in vitro* cultivation of echinococcus multilocularis metacestode vesicles and the generation of primary cell cultures. *Methods Mol. Biol. Clifton, NJ* 470: 245–262.

65 Stadelmann, B., Rufener, R., Aeschbacher, D. et al. (2016). Screening of the open source malaria box reveals an early Lead compound for the treatment of alveolar echinococcosis. *PLoS Negl. Trop. Dis.* 10: e0004535.

66 Stadelmann, B., Scholl, S., Müller, J., and Hemphill, A. (2010). Application of an *in vitro* drug screening assay based on the release of phosphoglucose isomerase to determine the structure–activity relationship of thiazolides against echinococcus multilocularis metacestodes. *J. Antimicrob. Chemother.* 65: 512–519.

67 Cumino, A.C., Nicolao, M.C., Loos, J.A. et al. (2012). Echinococcus granulosus tegumental enzymes as *in vitro* markers of pharmacological damage: a biochemical and molecular approach. *Parasitol. Int.* 61: 579–585.

68 Mahanty, S., Paredes, A., Marzal, M. et al. (2011). Sensitive *in vitro* system to assess morphological and biochemical effects of praziquantel and albendazole on taenia solium cysts. *Antimicrob. Agents Chemother.* 55: 211–217.

69 Müller, J., Sidler, D., Nachbur, U. et al. (2008). Thiazolides inhibit growth and induce glutathione-*S*-transferase pi (GSTP1)-dependent cell death in human colon cancer cells. *Int. J. Cancer* 123: 1797–1806.

70 Mizukami, C., Spiliotis, M., Gottstein, B. et al. (2010). Gene silencing in echinococcus multilocularis protoscoleces using RNA interference. *Parasitol. Int.* 59: 647–652.

71 Spiliotis, M., Mizukami, C., Oku, Y. et al. (2010). Echinococcus multilocularis primary cells: improved isolation, small-scale cultivation and RNA interference. *Mol. Biochem. Parasitol.* 174: 83–87.

72 Siles-Lucas, M. and Hemphill, A. (2002). Cestode parasites: application of *in vivo* and *in vitro* models for studies on the host–parasite relationship. *Adv. Parasitol.* 51: 133–230.

73 Küster, T., Hermann, C., Hemphill, A. et al. (2013). Subcutaneous infection model facilitates treatment assessment of secondary alveolar echinococcosis in mice. *PLoS Negl. Trop. Dis.* 7: e2235.

74 Stettler, M., Rossignol, J.F., Fink, R. et al. (2004). Secondary and primary murine alveolar echinococcosis: combined albendazole/nitazoxanide chemotherapy exhibits profound anti-parasitic activity. *Int. J. Parasitol.* 34: 615–624.

75 Küster, T., Zumkehr, B., Hermann, C. et al. (2012). Voluntary ingestion of antiparasitic drugs emulsified in honey represents an alternative to gavage in mice. *J. Am. Assoc. Lab. Anim. Sci. JAALAS* 51: 219–223.

76 Gorgas, D., Marreros, N., Rufener, R. et al. (2017). To see or not to see: non-invasive imaging for improved readout of drug treatment trials in the murine model of secondary alveolar echinococcosis. *Parasitology* 144: 937–944.

77 Hübner, C., Wiehr, S., Kocherscheidt, L. et al. (2010). Effects of *in vitro* exposure of echinococcus multilocularis metacestodes to cytostatic drugs on *in vivo* growth and proliferation of the parasite. *Parasitol. Res.* 107: 459–463.

78 Ritler, D., Rufener, R., Sager, H. et al. (2017). Development of a movement-based *in vitro* screening assay for the identification of new anti-cestodal compounds. *PLoS Negl. Trop. Dis.* 11: e0005618.

79 Klinkert, M.-Q. and Heussler, V. (2006). The use of anticancer drugs in antiparasitic chemotherapy. *Mini Rev. Med. Chem.* 6: 131–143.

80 Marchiondo, A.A., Ming, R., Andersen, F.L. et al. (1994). Enhanced larval cyst growth of echinococcus multilocularis in praziquantel-treated jirds (meriones unguiculatus). *Am. J. Trop. Med. Hyg.* 50: 120–127.

81 Miyaji, S., Katakura, K., Matsufuji, S. et al. (1993). Failure of treatment with alpha-difluoromethylornithine against secondary multilocular echinococcosis in mice. *Parasitol. Res.* 79: 75–76.

82 Stettler, M., Fink, R., Walker, M. et al. (2003). *In vitro* parasiticidal effect of nitazoxanide against echinococcus multilocularis metacestodes. *Antimicrob. Agents Chemother.* 47: 467–474.

83 Reuter, S., Manfras, B., Merkle, M. et al. (2006). *In Vitro* activities of itraconazole, methiazole, and nitazoxanide versus echinococcus multilocularis larvae. *Antimicrob. Agents Chemother.* 50: 2966–2970.

84 Hemphill, A. and Müller, J. (2009). Alveolar and cystic echinococcosis: toward novel chemotherapeutical treatment options. *J. Helminthol.* 83: 99–111.

85 Hemphill, A., Mueller, J., and Esposito, M. (2006). Nitazoxanide, a broad-Spectrum thiazolide anti-infective agent for the treatment of gastrointestinal infections. *Expert Opin. Pharmacother.* 7: 953–964.

86 Albani Clara, C.M. and Elissondo María, C. (2014). Efficacy of albendazole in combination with thymol against echinococcus multilocularis protoscoleces and metacestodes. *Acta Trop.* 140: 61–67.

87 Albani, C.M., Pensel, P.E., Elissondo, N. et al. (2015). *In vivo* activity of albendazole in combination with thymol against echinococcus multilocularis. *Vet. Parasitol.* 212: 193–199.

88 Reuter, S., Merkle, M., Brehm, K. et al. (2003). Effect of amphotericin B on larval growth of echinococcus multilocularis. *Antimicrob. Agents Chemother.* 47: 620–625.

89 Reuter, S., Buck, A., Grebe, O. et al. (2003). Salvage treatment with amphotericin B in progressive human alveolar echinococcosis. *Antimicrob. Agents Chemother.* 47: 3586–3591.

90 Soeiro, M.N.C., Werbovetz, K., Boykin, D.W. et al. (2013). Novel amidines and analogues as promising agents against intracellular parasites: a systematic review. *Parasitology* 140: 929–951.

91 Kammerer, W.S. and Perez-Esandi, M.V. (1975). Chemotherapy of experimental echinococcus granulosus infection. Trials in CF1 mice and jirds (meriones unguiculatus). *Am. J. Trop. Med. Hyg.* 24: 90–95.

92 Küster, T., Kriegel, N., Boykin, D.W. et al. (2013). *In vitro* and *in vivo* activities of dicationic diguanidino compounds against echinococcus multilocularis metacestodes. *Antimicrob. Agents Chemother.* 57: 3829–3835.

93 Stadelmann, B., Küster, T., Scholl, S. et al. (2011). *In vitro* efficacy of dicationic compounds and mefloquine enantiomers against echinococcus multilocularis metacestodes. *Antimicrob. Agents Chemother.* 55: 4866–4872.

94 Küster, T., Kriegel, N., Stadelmann, B. et al. (2014). Amino ozonides exhibit *in vitro* activity against echinococcus multilocularis metacestodes. *Int. J. Antimicrob. Agents* 43: 40–46.

95 Spicher, M., Roethlisberger, C., Lany, C. et al. (2008). *In vitro* and *in vivo* treatments of echinococcus protoscoleces and metacestodes with artemisinin and artemisinin derivatives. *Antimicrob. Agents Chemother.* 52: 3447–3450.

96 Keiser, J., Chollet, J., Xiao, S.-H. et al. (2009). Mefloquine--an aminoalcohol with promising antischistosomal properties in mice. *PLoS Negl. Trop. Dis.* 3: e350.

97 Manneck, T., Haggenmüller, Y., and Keiser, J. (2010). Morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult flukes of schistosoma mansoni. *Parasitology* 137: 85–98.

98 Keiser, J., Odermatt, P., and Tesana, S. (2009). Dose–response relationships and tegumental surface alterations in opisthorchis viverrini following treatment with mefloquine *in vivo* and *in vitro*. *Parasitol. Res.* 105: 261–266.

99 Walter, R.D., Wittich, R.M., and Kuhlow, F. (1987). Filaricidal effect of mefloquine on adults and microfilariae of brugia patei and brugia malayi. *Trop. Med. Parasitol. Off. Organ Dtsch. Tropenmedizinische Ges. Dtsch. Ges. Tech. Zusammenarbeit GTZ* 38: 55–56.

100 Küster, T., Stadelmann, B., Hermann, C. et al. (2011). *In vitro* and *in vivo* efficacies of mefloquine-based treatment against alveolar echinococcosis. *Antimicrob. Agents Chemother.* 55: 713–721.

101 Küster, T., Stadelmann, B., Rufener, R. et al. (2015). Oral treatments of echinococcus multilocularis-infected mice with the antimalarial drug mefloquine that potentially interacts with parasite ferritin and cystatin. *Int. J. Antimicrob. Agents* 46: 546–551.

102 Tanowitz, H.B., Weiss, L.M., and Wittner, M. (1993). Diagnosis and treatment of intestinal helminths. I. Common intestinal cestodes. *Gastroenterologist* 1: 265–273.

103 Liu, J., Chen, X., Ward, T. et al. (2016). Niclosamide inhibits epithelial-mesenchymal transition and tumor growth in lapatinib-resistant human epidermal growth factor receptor 2-positive breast cancer. *Int. J. Biochem. Cell Biol.* 71: 12–23.

104 Mathis, A., Wild, P., Boettger, E.C. et al. (2005). Mitochondrial ribosome as the target for the macrolide antibiotic clarithromycin in the helminth echinococcus multilocularis. *Antimicrob. Agents Chemother.* 49: 3251–3255.

105 Rodriguez-Fonseca, C., Amils, R., and Garrett, R.A. (1995). Fine structure of the peptidyl transferase Centre on 23 S-like rRNAs deduced from chemical probing of antibiotic-ribosome complexes. *J. Mol. Biol.* 247: 224–235.

106 Sander, P., Prammananan, T., Meier, A. et al. (1997). The role of ribosomal RNAs in macrolide resistance. *Mol. Microbiol.* 26: 469–480.

107 Liance, M., Nemati, F., Bories, C., and Couvreur, P. (1993). Experience with doxorubicin-bound polyisohexylcyanoacrylate nanoparticles on murine alveolar echinococcosis of the liver. *Int. J. Parasitol.* 23: 427–429.

108 Sarciron, M.E., Audin, P., Delabre, I. et al. (1993). Synthesis of propargylic alcohols and biological effects on echinococcus multilocularis metacestodes. *J. Pharm. Sci.* 82: 605–609.

109 Messina, M.J. (1999). Legumes and soybeans: overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr.* 70: 439S–450S.

110 Pike, A.C., Brzozowski, A.M., Hubbard, R.E. et al. (1999). Structure of the ligand-binding domain of estrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* 18: 4608–4618.

111 Naguleswaran, A., Spicher, M., Vonlaufen, N. et al. (2006). *In vitro* metacestodicidal activities of genistein and other isoflavones against echinococcus multilocularis and echinococcus granulosus. *Antimicrob. Agents Chemother.* 50: 3770–3778.

112 Spiliotis, M., Konrad, C., Gelmedin, V. et al. (2006). Characterization of EmMPK1, an ERK-like MAP kinase from echinococcus multilocularis which is activated in response to human epidermal growth factor. *Int. J. Parasitol.* 36: 1097–1112.

113 Stadelmann, B., Aeschbacher, D., Huber, C. et al. (2014). Profound activity of the anti-cancer drug bortezomib against echinococcus multilocularis metacestodes identifies the proteasome as a novel drug target for cestodes. *PLoS Negl. Trop. Dis.* 8: e3352.

114 Nicolao, M.C., Elissondo, M.C., Denegri, G.M. et al. (2014). *In vitro* and *in vivo* effects of tamoxifen against echinococcus granulosus larval stage. *Antimicrob Agents Chemother.* 58 (9): 5146–54.

115 Spicher, M., Naguleswaran, A., Ortega-Mora, L.M. et al. (2008). *In Vitro* and *in Vivo* effects of 2-methoxyestradiol, either alone or combined with albendazole, against echinococcus metacestodes. *Exp. Parasitol.* 119: 475–482.

116 Brehm, K. (2010). The role of evolutionarily conserved signaling systems in *Echinococcus multilocularis* development and host–parasite interaction. *Med. Microbiol. Immunol. (Berl.)* 199: 247–259.

117 Brehm, K. and Koziol, U. (2017). Echinococcus-host interactions at cellular and molecular levels. *Adv. Parasitol.* 95: 147–212.

118 Brehm, K. and Spiliotis, M. (2008). The influence of host hormones and cytokines on echinococcus multilocularis signaling and development. *Parasite Paris Fr.* 15: 286–290.

119 Bellei, B., Pitisci, A., Izzo, E., and Picardo, M. (2012). Inhibition of melanogenesis by the pyridinyl imidazole class of compounds: possible involvement of the wnt/β-catenin signaling pathway. *PLoS One* 7: e33021.

120 Gelmedin, V., Caballero-Gamiz, R., and Brehm, K. (2008). Characterization and inhibition of a p38-like mitogen-activated protein kinase (MAPK) from echinococcus multilocularis: antiparasitic activities of p38 MAPK inhibitors. *Biochem. Pharmacol.* 76: 1068–1081.

121 Gelmedin, V., Spiliotis, M., and Brehm, K. (2010). Molecular characterization of MEK1/2- and MKK3/6-like mitogen-activated protein kinase kinases (MAPKK) from the fox tapeworm echinococcus multilocularis. *Int. J. Parasitol.* 40: 555–567.

122 Hemer, S. and Brehm, K. (2012). *In vitro* efficacy of the anticancer drug imatinib on echinococcus multilocularis larvae. *Int. J. Antimicrob. Agents* 40: 458–462.

123 Schubert, A., Koziol, U., Cailliau, K. et al. (2014). Targeting echinococcus multilocularis stem cells by inhibition of the polo-like kinase EmPlk1. *PLoS Negl. Trop. Dis.* 8: e2870.

124 Fernández, V., Chalar, C., Martínez, C. et al. (2000). Echinococcus granulosus: molecular cloning and phylogenetic analysis of an inducible glutathione S-transferase. *Exp. Parasitol.* 96: 190–194.

125 Liebau, E., Müller, V., Lucius, R. et al. (1996). Molecular cloning, expression and characterization of a recombinant glutathione *S*-transferase from echinococcus multilocularis. *Mol. Biochem. Parasitol.* 77: 49–56.

126 Basto, A.P., Müller, J., Rubbiani, R. et al. (2017). Characterization of the activities of dinuclear thiolato-bridged arene ruthenium complexes against toxoplasma gondii. *Antimicrob. Agents Chemother.* 61.

127 Corrêa, R.S., da Silva, M.M., Graminha, A.E. et al. (2016). Ruthenium(II) complexes of 1,3-thiazolidine-2-thione: cytotoxicity against tumor cells and anti-trypanosoma cruzi activity enhanced upon combination with benznidazole. *J. Inorg. Biochem.* 156: 153–163.

128 Macedo, T.S., Colina-Vegas, L., Paixão, M.D.A. et al. (2016). Chloroquine-containing organoruthenium complexes are fast-acting multistage antimalarial agents. *Parasitology* 143: 1543–1556.

129 Southam, H.M., Butler, J.A., Chapman, J.A., and Poole, R.K. (2017). The microbiology of ruthenium complexes. *Adv. Microb. Physiol.* 71: 1–96.

130 Küster, T., Lense, N., Barna, F. et al. (2012). A new promising application for highly cytotoxic metal compounds: η6-areneruthenium(II) phosphite complexes for the treatment of alveolar echinococcosis. *J. Med. Chem.* 55: 4178–4188.

131 Frayha, G.J., Bikhazi, K.J., and Kachachi, T.A. (1981). Treatment of hydatid cysts (echinococcus granulosus) by cetrimide (R). *Trans. R. Soc. Trop. Med. Hyg.* 75: 447–450.

132 Rogan, M.T. and Richards, K.S. (1986). Echinococcus granulosus: *in vitro* effect of monensin on the tegument of the protoscolex. *Parasitology* 93 (Pt 2): 347–355.

133 Martinez, J., Perez-Serrano, J., Bernadina, W.E., and Rodriguez-Caabeiro, F. (1999). Echinococcus granulosus: *in vitro* effects of ivermectin and praziquantel on hsp60 and hsp70 levels. *Exp. Parasitol.* 93: 171–180.

134 Bygott, J.M. and Chiodini, P.L. (2009). Praziquantel: neglected drug? Ineffective treatment? Or therapeutic choice in cystic hydatid disease? *Acta Trop.* 111: 95–101.

135 Nazligul, Y., Kucukazman, M., and Akbulut, S. (2015). Role of chemotherapeutic agents in the management of cystic echinococcosis. *Int. Surg.* 100: 112–114.

136 Yasawy, M.I., Alkarawi, M.A., and Mohammed, A.R. (2001). Prospects in medical management of Echinococcus granulosus. *Hepatogastroenterology* 48: 1467–1470.

137 Chinnery, J.B. and Morris, D.L. (1986). Effect of albendazole sulphoxide on viability of hydatid protoscoleces *in vitro*. *Trans. R. Soc. Trop. Med. Hyg.* 80: 815–817.

138 Morris, D.L., Chinnery, J.B., and Ubhi, C. (1987). A comparison of the effects of albendazole, its sulphone metabolite, and mebendazole on the viability of protoscoleces of echinococcus granulosus in an *in vitro* culture system. *Trans. R. Soc. Trop. Med. Hyg.* 81: 804–806.

139 Pérez-Serrano, J., Casado, N., and Guillermo, null, Denegri, null, Rodriguez-Caabeiro, F. (1994). The effects of albendazole and albendazole sulphoxide combination-therapy on echinococcus granulosus *in vitro*. *Int. J. Parasitol.* 24: 219–224.

140 Rodriguez-Caabeiro, F., Casado, N., and Juarez-Pelaez, E. (1989). Efecto in vitro de praziquantel, mebendazole Y oxfendazole sobre protoscolex de Echinococcus granulosus. *Rev. Iber. Parasitol.* 49: 77–83.

141 García-Llamazares, J.L., Alvarez-de-Felipe, A.I., Redondo-Cardeña, P. et al. (1997). *In vivo* inhibition of the regenerative capacity of hydatid material after treatment with netobimin. *Parasitol. Res.* 83: 105–108.

142 Dueger, E.L., Moro, P.L., and Gilman, R.H. (1999). Oxfendazole treatment of sheep with naturally acquired hydatid disease. *Antimicrob. Agents Chemother.* 43: 2263–2267.

143 Elissondo, M., Ceballos, L., Dopchiz, M. et al. (2007). *In vitro* and *in vivo* effects of flubendazole on Echinococcus granulosus metacestodes. *Parasitol. Res.* 100: 1003–1009.

144 Shkoliar, N.A., Kukhaleva, I.V., Legon'kov, I.A., and Kovalenko, F.P. (2014). Efficacy of nocodazole et experimental invasion echinococcus granulosus of white mice. *Med. Parazitol. (Mosk.)* 2: 42–46.

145 Laura, C., Celina, E., Sergio, S.B. et al. (2015). Combined flubendazole-nitazoxanide treatment of cystic echinococcosis: pharmacokinetic and efficacy assessment in mice. *Acta Trop.* 148: 89–96.

146 Gavidia, C.M., Gonzalez, A.E., Lopera, L. et al. (2009). Evaluation of nitazoxanide and oxfendazole efficacy against cystic echinococcosis in naturally infected sheep. *Am. J. Trop. Med. Hyg.* 80: 367–372.

147 Hurd, H., Mackenzie, K.S., and Chappell, L.H. (1993). Anthelmintic effects of cyclosporin a on protoscoleces and secondary hydatid cysts of echinococcus granulosus in the mouse. *Int. J. Parasitol.* 23: 315–320.
148 Escobedo, G., Palacios-Arreola, M.I., Olivos, A. et al. (2013). Tamoxifen treatment in hamsters induces protection during taeniosis by Taenia solium. *BioMed. Res. Int.* 2013: 280496.
149 Vargas-Villavicencio, J.A., Larralde, C., De León-Nava, M.A. et al. (2007). Tamoxifen treatment induces protection in murine cysticercosis. *J. Parasitol.* 93: 1512–1517.
150 Nicolao, M.C., Loos, J.A., Rodriguez Rodrigues, C. et al. (2017). Bortezomib initiates endoplasmic reticulum stress, elicits autophagy and death in Echinococcus granulosus larval stage. *PLoS One* 12: e0181528.
151 Shi, H., Lei, Y., Wang, B. et al. (2016). Protoscolicidal effects of chenodeoxycholic acid on protoscoleces of echinococcus granulosus. *Exp. Parasitol.* 167: 76–82.
152 Xing, G., Wang, B., Lei, Y. et al. (2016). *In vitro* effect of sodium arsenite on echinococcus granulosus protoscoleces. *Mol. Biochem. Parasitol.* 207: 49–55.
153 Loos, J.A., Dávila, V.A., Rodrígues, C.R. et al. (2017). Metformin exhibits preventive and therapeutic efficacy against experimental cystic echinococcosis. *PLoS Negl. Trop. Dis.* 11: e0005370.
154 Loos, J.A. and Cumino, A.C. (2015). *In vitro* anti-echinococcal and metabolic effects of metformin involve activation of AMP-activated protein kinase in larval stages of echinococcus granulosus. *PLoS One* 10: e0126009.
155 Ferrell, J.A. (1914). The rural school and hookworm disease. In: *US Bureau of Education Bulletin*, vol. 20. Washington, DC: US: Government Printing Office.
156 Elissondo, M.C., Albani, C.M., Gende, L. et al. (2008). Efficacy of thymol against echinococcus granulosus protoscoleces. *Parasitol. Int.* 57: 185–190.
157 Elissondo, M.C., Pensel, P.E., and Denegri, G.M. (2013). Could thymol have effectiveness on scolices and germinal layer of hydatid cysts? *Acta Trop.* 125: 251–257.
158 Maggiore, M., Pensel, P.E., Denegri, G., and Elissondo, M.C. (2015). Chemoprophylactic and therapeutic efficacy of thymol in murine cystic echinococcosis. *Parasitol. Int.* 64: 435–440.
159 Fabbri, J., Maggiore, M.A., Pensel, P.E. et al. (2016). *In vitro* and *in vivo* efficacy of carvacrol against echinococcus granulosus. *Acta Trop.* 164: 272–279.
160 Maggiore, M.A., Albanese, A.A., Gende, L.B. et al. (2012). Anthelmintic effect of mentha spp. Essential oils on Echinococcus granulosus protoscoleces and metacestodes. *Parasitol. Res.* 110: 1103–1112.
161 Verma, V.C., Gangwar, M., Yashpal, M., and Nath, G. (2013). Anticestodal activity of endophytic pestalotiopsis Sp. on protoscoleces of hydatid cyst Echinococcus granulosus. *BioMed. Res. Int.* 2013: 308515.
162 Streliaeva, A.V., Polzikov, V.V., Prokina, E.S. et al. (2011). Development of a new hydrocarbon extract from the medicinal raw material of circassian walnut (Juglans regia) and study of its antiparasitic activity. *Med. Parazitol. (Mosk.)* 3: 28–31.

163 Mahmoudvand, H., Fallahi, S., Mahmoudvand, H. et al. (2016). Efficacy of myrtus communis L. to inactivate the hydatid cyst protoscoleces. *J. Investig. Surg. Off. J. Acad. Surg. Res.* 29: 137–143.

164 Mahmoudvand, H., Ezatpour, B., Rashidipour, M. et al. (2016). Report: evaluation of the scolicidal effects of nectaroscordum tripedale extract and its acute toxicity in mice model. *Pak. J. Pharm. Sci.* 29: 2125–2128.

165 Tickell-Painter, M., Maayan, N., Saunders, R. et al. (2017). Mefloquine for preventing malaria during travel to endemic areas. *Cochrane Database Syst. Rev.* 10: CD006491.

166 Lundström-Stadelmann, B., Rufener, R., Ritler, D., Zurbriggen, R., and Hemphill A. (2019). The importance of being parasiticidal... an update on drugdevelopment for the treatment of alveolar echinococcosis. Food and Waterborne Parasitology 12: e00040.

11

New Insights into the Treatment of Foodborne Trematode Infections

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11.1 Introduction

Foodborne trematode (FBT) infections are among the most neglected parasitic diseases, both in terms of research funding and public media. The class Trematoda is a major group of parasites and almost 100 species of FBTs are known to infect humans, including liver flukes, lung flukes, and intestinal flukes [1, 2]. It is estimated that about 80 million people are currently infected with FBT and about 1 billion are at risk of infection with a global burden of 3.62 million disability-adjusted life-years (DALYs) [1, 3]. FBT infections generally cause chronic diseases with a variety of signs, symptoms, and pathological consequences depending on the fluke species. FBT infections are traditionally considered as tropical diseases, but currently constitute an emerging public health problem that may occur beyond the tropics [1, 2]. Regarding the geographical distribution of FBT infections, it should be noted that the traditional picture is changing in recent years. FBT infections cannot be considered as parasitic diseases limited to low- and middle-income countries in tropical areas, since factors such as migration flows, increased international tourism, improved transportation systems, or the growing international markets are expanding geographical limits and the population at risk worldwide.

Despite all these facts, the impact of FBT has been commonly underestimated, a number of control campaigns have been stopped, and funding for improving prevention is scarce. Furthermore, research on this topic is impeded by several reasons such as the difficulties entailed in the diagnosis, the complexities of human cultural behaviors, and poor knowledge of the potential economic consequences of FBT infections.

In this chapter, we summarize the key characteristics of FBT infections, depicting the life cycle of the parasite and the epidemiological and clinical features of the disease. Special attention is paid to current therapeutic approaches to and drug discovery strategies for these important parasitic diseases.

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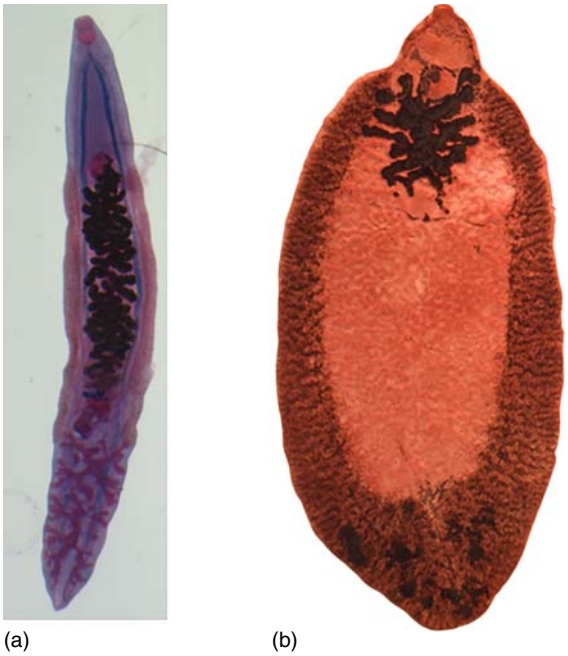
11.2 Morphology and Biology of Foodborne Trematodes

Digenean trematodes causing FBT infections are dorsoventrally flattened, with a bilateral symmetry. They are leaf shaped and ranging from a few millimeters to 8–10 cm. An oral sucker surrounds the mouth at the anterior end and a blind muscular ventral sucker is present on the ventral surface. The tegument is a syncytial epithelium that is involved in several processes including nutrient adsorption, secretion, osmoregulation, or sensory functions [4]. Most of the trematodes are hermaphroditic, containing both the female and male reproductive systems in the same individual. Trematodes lack body cavity, and internal organs are embedded in connective tissue or parenchyma (Figure 11.1).

Diagnosis of FBT infection is usually achieved by detection of eggs in feces or sputum. They are typically oval and operculated and may range from 18 to above 150 μm in length and between 12–14 μm and above 90 μm in width [5] (Figure 11.2).

The life cycle of FBT is complex, involving a number of diverse larval stages (egg, miracidium, sporocyst, redia, cercariae, and metacercaria) apart from the adult stage (Figure 11.3). Within the definitive host, FBTs can be found in practically every organ, but the lung, liver, and intestine are the most common. Typically, the life cycles of major FBTs include two or three hosts: a vertebrate definitive host, an invertebrate intermediate host, and, frequently, a second intermediate host that carries the encysted metacercarial stage. Eggs produced by adult worms following sexual reproduction are released via feces or sputum



(a) (b)

Figure 11.1 Adult worms of some foodborne trematode infections: (a) *Clonorchis sinensis* and (b) *Fasciola hepatica*.

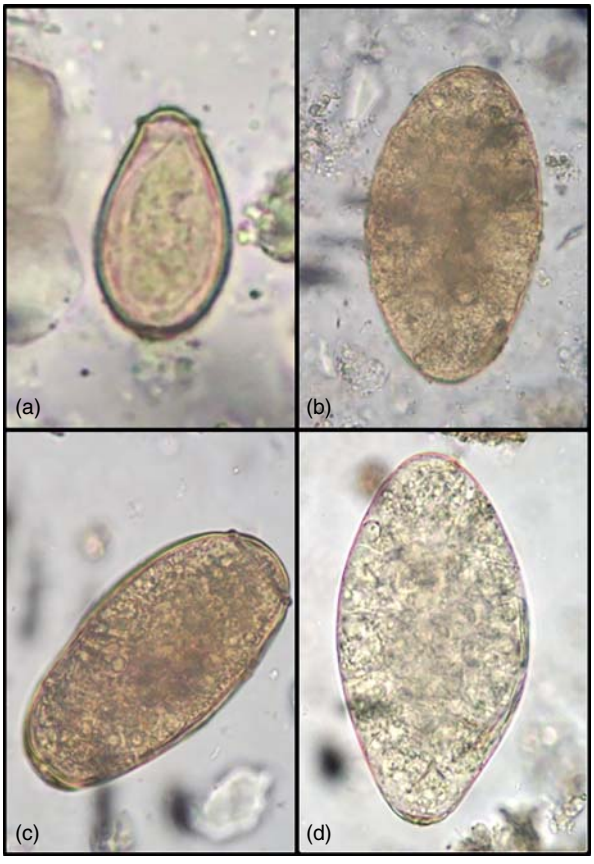


Figure 11.2 Photomicrographs of various eggs of foodborne trematode infections detected in formalin-preserved human fecal material: (a) *Clonorchis sinensis* (30.0 × 17.5 μm); (b) *Fasciola hepatica* (127.5 × 72.5 μm); (c) *Paragonimus westermani* (95.0 × 45.0 μm); and (d) *Echinostoma* sp. (127.5 × 72.5 μm).

(lung FBT). The eggs of some FBTs are fully developed when laid, but others require some time in the environment for embryonation. A "sac-like" ciliated larva or miracidium emerges from the egg and actively penetrates a snail, the first intermediate host, or is ingested by the snail. In the case of *Clonorchis sinensis, Opisthorchis* spp., and Heterophyidae, eggs are directly ingested by the snails and the miracidia hatch in the gastrointestinal tract of the snail. Various species of snails can act as the first intermediate host and commonly are specific to the trematode species.

Within the snail host, miracidia develop into sporocysts or, most rarely, directly give rise to redia. The germinal cells within the sporocysts multiply and produce new germinal masses, which produce daughter sporocysts or rediae. Development of sporocysts and rediae follow different patterns depending on the FBT species. Finally, these larval stages produce cercariae. Cercariae leave the snail by active escape or passive extrusion. The free-swimming cercariae meet a compatible second intermediate host in which they penetrate and encyst (i.e. Echnostomatidae, *C. sinensis*, or *Opithorchis* spp.). As second intermediate hosts, several species of fishes, crustaceans, snails, or tadpoles have been reported. Alternatively, the cercariae of some species (i.e. *Fasciola* spp. or *Fasciolopsis*

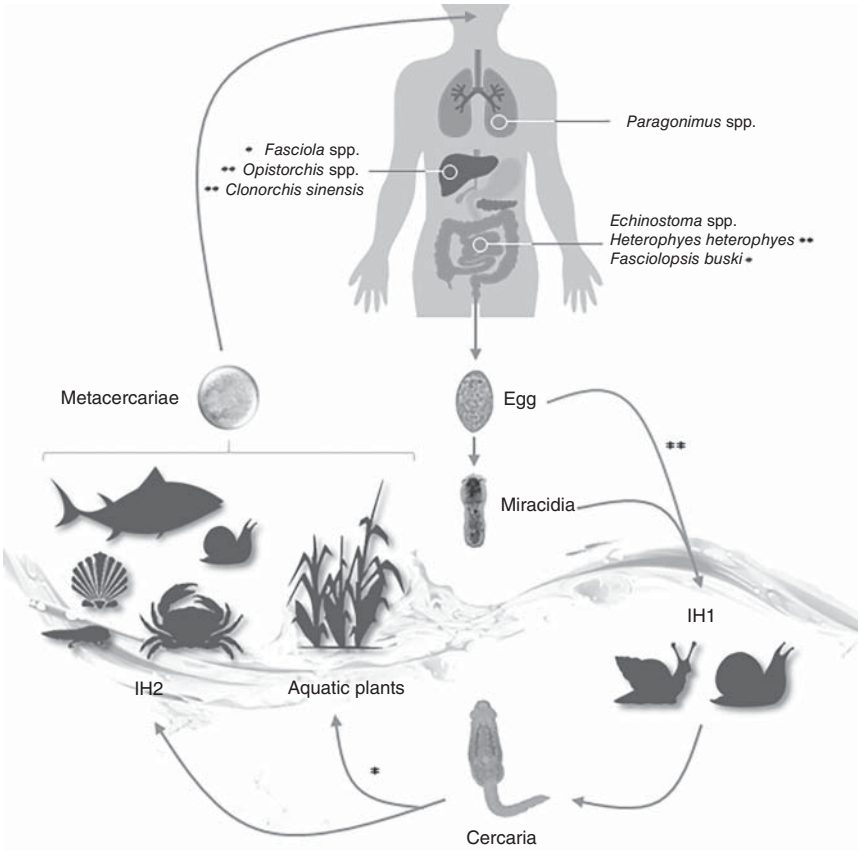


Figure 11.3 Schematic life cycle of the major foodborne trematodes.

buski) encyst on aquatic vegetation such as watercress, water lotus, water caltrop, water chestnut, or water lily. Human or animal definitive hosts become infected when eating raw or insufficiently cooked second intermediate host, vegetation or, even, by drinking contaminated water [6]. Metacercariae excyst in the gastrointestinal system, releasing juvenile worms that migrate to the target organ where they mature to adults. Infection with *Paragonimus* spp. might also occur by consumption of wild boar or venison acting as a paratenic host [7, 8].

11.3 Epidemiology and Global Impact

Although FBT infections are commonly considered as tropical diseases, they are not limited to the tropics. FBT infections occur in areas where the first and second intermediate host coexist and humans eat raw or undercooked fish or other aquatic products, which explain their focal distribution. However, increased international travels, human migration, or food trade explain the increased occurrence of allochthonous cases worldwide [1, 9–11].

The global burden of FBT infections is difficult to determine, although several approaches have been done in recent years. Fürst and colleagues [1] estimated that almost 60 million people were infected in 2005, with 7.9 million developing severe sequelae and 7158 died. The estimated global burden was 665 352 DALYs. More recently, it was estimated that the prevalence of FBT infections in 2013 was about 80 million people, which constituted an increase of about 50% from 1990 [3]. However, these estimations may constitute only a small part of the problem.

The number of infected people varies depending on the trematode species. The global estimate for clonorchiasis is greater than 15 million people. For opisthorchiasis, it is estimated that 8.4 million people are infected, causing more than 1000 deaths. Estimates for fascioliaisis ranges from 2.4 to 17 million people. More than 23 million people suffer from paragonimiasis and about 7 million people are infected with species of intestinal trematodes [1].

11.4 Major Foodborne Trematodes

Herein, we review the main features of the major FBTs causing human infections. We divide them into liver, lung, and intestinal FBT infections.

11.4.1 Liver Foodborne Trematode Infections

The most relevant liver FBTs are *C. sinensis, Opisthorchis* spp., and *Fasciola* spp.

11.4.1.1 Clonorchiasis and Opisthorchiasis

C. sinensis, Opisthorchis viverrini, and *Opisthorchis felineus* are liver flukes that have close morphological and biological characteristics. These species follow a three-host life cycle. Embryonated eggs are released via feces and in an aquatics environment are ingested by a snail (commonly *Bithynia* spp.). After hatching, miracidia give rise to sporocyst, redia, and, finally, cercariae that leave the host. Ceracariae may penetrate in several species of fishes, where they encyst. Humans become infected after ingestion of raw or undercooked fish. Adult worms inhabit the intrahepatic bile duct, but also can be found in the common bile duct, cystic duct, and gallbladder. They can survive for up to 26 years.

C. sinensis is endemic in East Asia including PR China, Korea, Taiwan, and Thailand, and, moreover, several cases have been reported in Far East Russia. *O. viverrini* is found in Thailand, Laos PDR, Cambodia, and Vietnam; and *O. felineus h*as been detected in several continental European countries such as Belarus, Germany, Greece, Italy, Romania, Russia, Spain, the Ukraine, the Baltic countries, Moldova, and Kazakhstan [2, 12, 13].

Usually, chronic opisthorchiasis and clonorchiasis show a few specific symptoms, and a palpable liver is the most common sign [14]. High worm burdens (up to 25 000 flukes) are associated with acute pain in the right upper quadrant, obstructive jaundice, cirrhosis, cholangitis, acalculous cholecystitis, fibrosis in the bile duct, or bile peritonitis [15]. Periductal fibrosis is the cause of hepatobiliary disease, leading to increased risk of cholangiocarcinoma (CCA) [13]. In fact,

O. viverrini and *C. sinensis* are classified by the International Agency of Research on Cancer (IARC) as carcinogenics class I and II, respectively [16, 17]. Moreover, about 400 cases of CCA occurred in patients heavily infected with *O. felineus* [18]. CCA is a cancer of the epithelial cells in the bile ducts arising along either the intra- or extrahepatic biliary tree with extremely poor prognosis [17]. CCA is responsible for about 15% of liver cancers worldwide [19].

11.4.1.2 Fascioliasis

Fascioliasis is caused by *Fasciola hepatica* or *Fasciola gigantica*. Despite *F. hepatica* being found worldwide, *F. gigantica* appears to be restricted to areas of Africa and Asia [20]. Adult worms, inhabiting the bile ducts, release eggs that are passed to the feces. Embryonation and hatching occur in freshwater and the free-swimming miracidia infect the first intermediate host. *F. hepatica* mainly uses snail species belonging to the so-called *Galba*/*Fossaria* group, including *G. truncatula*, as the first intermediate host and *F. gigantica* is transmitted by *Radix* spp., mainly *Radix natalensis*, in Africa [20]. Within the snail, the parasite undergoes stages: sporocyst, redia, and cercariae. Released cercariae swim to adhere to vegetation where they encyst. Humans become infected after ingestion of contaminated wild and cultivated aquatic and terrestrial plants, and even beverages made from plants or untreated water. The parasite excysts in the small intestine and migrates to the bile duct to develop into adult worms, which occurs about 12 weeks after infection [20].

Hyperendemic human fascioliasis has been reported in the Altiplano region of Bolivia, the Nile Delta, and Northern Iran [20]. More sporadically, human cases occur in Cuba, France, Portugal, Spain, or the United States, among other countries. Acute fascioliasis, corresponding to the migration of juvenile worms, is characterized by abdominal pain, fever, hepatomegaly, and gastrointestinal symptoms. Chronic fascioliasis, corresponding to the presence of adult worms in the bile ducts, may be asymptomatic or show hepatic signs such as cholangitis, cholecystitis, and cholelithiasis [21].

11.4.2 Lung Foodborne Trematode Infections (Paragonimiasis)

Paragonimisasis is the zoonotic disease caused by lung flukes belonging to the genus *Paragonimus*. There are about 15 species of *Paragonimus* known to infect humans, although *Paragonimus westermani* is the most common worldwide. *Paragonimus* spp. have a three-host life cycle. Adult worms inhabit the lung and ley eggs that are coughed up and ejected with the sputum by spitting or swallowed and released by the feces. In a freshwater environment, hatched miracidia invade snails, mainly of the genus *Semisulcospira*, and the sporocyst, redia, and cercariae stages occur. Crustacea, the second intermediate host, acquire the infection by consuming cercariae or the snails harboring the mature cercariae. Humans become infected by consuming raw or undercooked freshwater crustaceans such as crabs, shrimps, or crayfishes. The metacercariae excyst in the small intestine and penetrate the abdominal cavity and migrate to the lungs, where they become adults.

It is estimated that more that 23 million people in 48 countries are currently infected with *Paragonimus* spp. and almost 300 million people live at risk of

infection [7]. Among FBT infections, paragonimiasis is the most relevant in terms of DALYs [22]. Paragonimiasis occurs in focal areas in Asia (PR China, Japan, Korea, Laos PDR, the Philippines, Taiwan, Vietnam, and Thailand), South and Central America (Ecuador, Costa Rica, Peru, and Columbia), and Africa (Cameroon, Gambia, and Nigeria).

The main pathological features are related to the mechanical damage induced by the migration of the parasite and the toxins released by the migratory worms. Moreover, ectopic paragonimiasis is quite common. Cerebral, cutaneous, abdominal, and hepatic ectopic infections are the most common [7]. Adult flukes in the lung cause hemorrhage, inflammation, parenchymal necrosis, and fibrosis encapsulation. Moreover, hemoptysis and chronic cough occur, with brown and blood-streaked pneumonia-like sputum [12].

11.4.3 Intestinal Foodborne Trematode Infections

Intestinal FBT infections can be caused by a large number of species. Herein, we review the most relevant of those species.

11.4.3.1 Diplostomiasis

Diplostomiasis is caused by a member of the family Diplostomidae, mainly *Neodiplostomum seoulensis*. Human infections have been reported in the Republic of Korea, and the total number of cases in this country was estimated as 1000 [23]. Human infections occur by eating raw frogs, the second intermediate host harboring the metacercaria, or snakes acting as the paratenic host. Adult worms, inhabiting the intestinal tract, release eggs with feces that, in freshwater, hatch the miracidium that infect the first intermediate host. Fork-tailed cercariae, produced in sporocysts, penetrate the second intermediate host.

11.4.3.2 Echinostomiasis

Echinostomes constitute a heterogeneous group of cosmopolitan digeneans that parasitize several vertebrates, including humans. Currently, echinostomiasis is attributed to at least 24 species belonging to the family Echinostomatidae and is endemic in Southeast Asia and the Far West. Endemic foci are located in China, India, Indonesia, Korea, Malaysia, the Philippines, or Thailand, although occasional cases are detected in other countries [24].

Humans become infected after eating raw or insufficiently cooked snails, clams, frogs, tadpoles, or fishes that act as the second intermediate host. Adult worms inhabit the small intestine and release eggs with feces into ponds, streams, or lakes. Miracidia hatch from eggs to actively locate and penetrate the first intermediate host (snails belonging to Planorbidae, Lymnaeidae, or Bulinidae). After a single generation of sporocysts and two of rediae, cercariae emerge and invade the second intermediate host where they encyst. Interestingly, it has been suggested that drinking untreated water may be a source of human infection [25].

The clinical symptoms of echinostomiasis depend on the parasite load and may be severe in cases of elevated worm burden. The most common symptoms are epigastric and abdominal pain, fatigue, diarrhea, weight loss, acid belching, nausea, anorexia, and vomiting [26].

11.4.3.3 Fasciolopsiasis

Fasciolopsiasis is caused by the intestinal fluke *F. buski*, belonging to the family Fasciolidae. Fasciolopsiasis is restricted to PR China, India, Bangladesh, Thailand, Malaysia, Borneo, Sumatra, and Myanmar [27]. Humans become infected through the ingestion of raw freshwater vegetables containing metacercariae. Metacercariae excyst and juvenile worms attach to the wall of the duodenum and jejunum, where they mature. In freshwater sources, miracidia hatch and infect the first intermediate host (snails of the family Planorbidae). After transformation to sporocysts and rediae, cercariae emerge and swim to reach freshwater plants to encyst. Commonly, fasciolopsiasis only causes mild symptoms including diarrhea, abdominal colic, flatulence, vomiting, fever, and duodenal ulcer. Some deaths have been reported in chronic heavy infections [12, 27].

11.4.3.4 Gymnophalloidiasis

This infection is caused by *Gymnophalloides seoi* that has been exclusively reported in Korea, where it is highly prevalent among villagers in the southwestern coastal islands [23, 28]. The source of human infection are oysters (*Crassotrea gigas*) harboring metacercariae that are commonly eaten raw in this area. The first intermediate host is not known. Symptomatology may vary between individuals, although gastrointestinal discomfort is common.

11.4.3.5 Heterophyasis

At least 26 species belonging to 12 genera of the family Heterophyidae have been proved to infect humans. However, *Heterophyes heterophyes* and *Metagonimus yokogawai* are the most prevalent. *H. heterophyes* has been reported in Egypt, Iran, Korea, and Sudan; and there have been several sporadic cases in Europe. Human infections with *M. yokogawai* mainly occur in the Far East, India, the Balkan states, Israel, and Siberia [27]. The main source of infection is raw, pickled, or poorly cooked fish harboring metacercariae. Adult worms live between the villi of the anterior region of the small intestine, releasing embryonated eggs. Littorine snails, which act as the first intermediate host, ingest the eggs. Intramolluscan stages include sporocysts and rediae; and the released cercariae infect shrimps or shorefish, where they encyst on the surface or muscles [29]. Low-grade infections have no clinical consequences. In heavy infections, diarrhea, abdominal pain, dyspepsia, nausea, and vomiting may occur. Moreover, anaphylactic reactions can occur [30].

11.5 Current Drugs Used Against Foodborne Intestinal Trematodes

11.5.1 Praziquantel

Praziquantel (PZQ) is currently the drug of choice for most FBT infections. PZQ was developed by Merck as a potential tranquilizer and passed to Bayer to be screened for anthelminthic activity [31, 32]. Initially, the emphasis was on

its activity against cestodes and the drug was marketed for veterinary use as Droncit® [33, 34]. Activity against trematodes, with emphasis on *Schistosoma* spp., was first tested in animals [35]. Thereafter, it was successfully assayed in humans in collaboration with the WHO [36–38]. This success, together with the absence of side effects, made PZQ the drug of choice for schistosomiasis as it still remains today. Due to its broad spectrum of activity against other trematodes, PZQ also became the drug of choice against most of the FBTs [15, 39].

PZQ was marketed for human use as Biltricide®, available in tablets of 600 mg. In the early 1980s, the Korean company Shin Poong Pharmaceutical Co Ltd developed an alternative method for synthesis of PZQ based on local R&D, resulting in the disruption of the monopoly hold by Bayer until then and in a conspicuous price reduction. Subsequently, several producers started to commercialize PZQ under license of Shin Poong [32]. Currently, it is available under various names such as Distocide® (Shin Poong, EIPICO), Bilharzid® (Egyptian International Pharmaceutical Co Ltd) or Prazitel® (Cosmos), and even as generic presentations. In general, almost all the PZQ presentations have been shown to be of sufficient quality in terms of drug content, purity, disintegration, and dissolution. However, some of the generic presentations did not contain PZQ at all [40–42].

PZQ is the generic name for 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino[2,1-*a*]isoquinolin-4-one (Figure 11.4). It is a colorless or nearly white crystalline powder, insoluble in water, scarcely soluble in methanol, and soluble in organic solvents such as chloroform or dimethylsulfoxide. It is stable under normal conditions and melts at 136–140 °C. PZQ possesses an asymmetric center in position 11b (Figure 11.4). Commercial PZQ is a racemate composed of "levo" and "dextro" isomers. Only the (−) enantiomer is endowed with anti-trematode activity [43, 44].

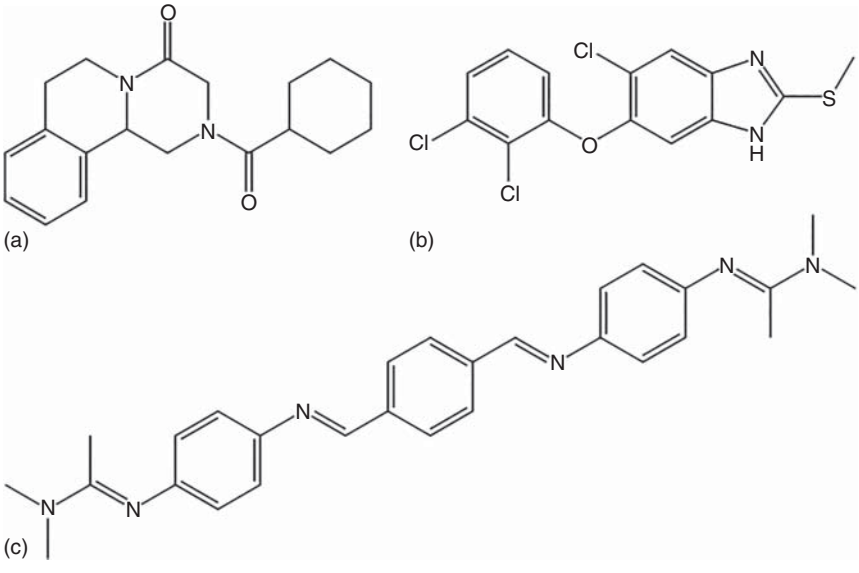


Figure 11.4 Chemical structure of (a) praziquantel, (b) triclabendazole, and (c) tribendimidine.

Orally administered PZQ is rapidly adsorbed, reaching maximal serum concentration (C_{max}) at one to two hours [36]. PZQ undergoes a marked liver first-pass metabolism and rapidly disappears from circulation. The plasma half-life is about one hour, and it is almost completely eliminated in 24 hours through the urine and the feces [45]. Metabolites have a longer half-life (about four hours) and they are mainly represented by monohydrixulated PZQ. The bioavailability of PZQ is increased by simultaneous administration of inhibitors of cytochrome P450 such as cimetidine, 17-α-ethynylestradiol, or diphenylhydramine. In contrast, simultaneous administration of some antiepileptics such as carbamazepine, phenytoin, or dexamethasone decreases the bioavailability [46].

The mechanism of action of PZQ is not yet well defined [47, 48]. PZQ induces an influx of Ca^{2+}, which results in muscular contraction and spastic paralysis of the musculature and morphological alterations such as vacuolization at the base of the syncytium and surface blebbing [49–51]. Although it has been hypothesized that calcium influx only represents one of the components of the complex mechanism of action, most of the studies are exclusively addressed toward identifying the molecular targets that become affected by calcium circulation. It was suggested that PZQ acts through theβ-subunit variant to the Ca^{2+} channel, which results in massive binding of Ca^{2+}. Moreover, expression of the ATP-binding cassette (ABC) superfamily, including multidrug resistance-associated protein 2, becomes affected after treatment with PZQ [52]. You and coworkers [51], using the genome silencing procedure RNA interference, showed that a member of the calcium signaling pathway (calcium/calmodulin-dependent protein kinase type II-camK II) augmented the effect of PZQ, suggesting that camK II has a key role in the activity of PZQ.

Toxicity of PZQ is low in animals and no genotoxic risks have been demonstrated [53]. A clastogenic or pre-clastogenic role has been suggested [54, 55]. Side effects have been observed in up to 50% of patients but are commonly short lived and spontaneously subside [56]. Most common side effects are related to gastrointestinal tract (abdominal pain and discomfort, nausea, vomiting, anorexia, and/or diarrhea), central nervous system (headache or dizziness), skin (pruritus or eruptions), or are nonspecific (fever or fatigue).

As mentioned, PZQ shows a wide spectrum of activity against trematodes and it has become the drug of choice for clonorchiasis, opisthorchiasis, paragonimiasis, and intestinal fluke infections. In the case of clonorchiasis, efficacy of PZQ depends on the treatment schedule [57, 58]. According to WHO recommendations, treatment with 25 mg/kg twice daily for two consecutive days reports cure rates of 94–100% [59]. A total dose of 150 mg/kg reports a cure rate higher than 90% and a nearly complete reduction of egg elimination [60]. High reduction of egg elimination was also achieved with a one-day treatment schedule of three doses of 25 mg/kg, although the cure rates were low [61]. Regarding the chemoprophylaxis of clonorchiasis, it was shown that repeated mass treatment or selective treatment every 6–12 months was effective, yielding low prevalence in heavily endemic areas. In moderately endemic areas, only one or two selective treatments were required in combination with health education [62, 63]. However, these protocols did not allow complete control of the disease. The treatment schedule of opisthorchiasis is similar to that of clonorchiasis.

Treatment with 25 mg/kg yielded a cure rate in patients infected with *O. viverrini* [64]. Although chemoprophylaxis can be applied, recent studies have suggested that repeated infection with *O. viverrini* and treatment with PZQ increases the risk of CCA [65, 66].

PZQ has a long history of use against paragonimiasis. First studies were performed in Korea in the early 1980s, with high cure rates being reported [67]. The recommended treatment schedule (25 mg/kg for two to three days) [7, 68] reports complete cure rates of about 90%. A single dose of 25 mg/kg only cured 77% of patients [68]. Repeated rounds of treatment may be required, especially when pleural effusion is present [69]. PZQ can be also used for treatment of cerebral paragonimiasis. Coadministration of anti-inflammatories can be recommended to avoid reactions due to substances released by dead worms [69]. An important risk inherent to treatment of paragonimiasis is the fact that PZQ can eliminate a pulmonary infection, but this might be the source of a subsequent cerebral invasion [70].

At present, PZQ is the drug of choice against intestinal trematodes. A single dose of 25 mg/kg is recommended [11]. However, other schedules can be used [2]. For example, single doses of 10 and 40 mg/kg also reported cure rates of 100% in infected patients [1, 2] in Thailand [71, 72]. A treatment schedule of 75 mg/kg in three divided doses for one day has also been used [1]. In gymnophalloidiasis, a single dose of 10 mg/kg is recommended [73].

Currently, PZQ is recommended for individuals aged four years and older [59]. However, tablets of PZQ are large (600 mg) and bitter in taste, and therefore difficult to use in infants. A PZQ syrup formulation is available, although its bioequivalence and which excipients are required for dose uniformity or stability are not known [40, 74].

Finally, it should be mentioned that that there is emerging evidence that tolerance or resistance to PZQ is developing in animals [51, 74]. Although drug resistance has not yet been definitively proved, resistance could be related to the mechanisms of action described earlier. Analysis of gene expression revealed that camk II is upregulated in response to PZQ, which can stabilize Ca^{2+} fluxes within the parasite [51]. Furthermore, it was suggested that upregulation of parasite defense, including ATPases, gives rise to resistance [51]. Multidrug resistance transporters may be also important for drug susceptibility of trematodes [52].

11.5.2 Triclabendazole

Treatment of fasciolosis has been problematic since PZQ has no efficacy. Historically, several drugs have been used for the treatment of fasciolosis, although most of them have been abandoned in relation to their toxicity and the low cure rates (see Section 11.5.1). Currently, triclabendazole (TCBZ) is the drug of choice and, moreover, it constitutes a useful alternative for paragonimiasis [7, 20].

TCBZ is the generic name of 6-chloro-5-(2,3-dichlorophenoxy)-2-(methylthio) benzimidazole) (Figure 11.4). The halogenated benzimidazole was synthesized by Ciba-Geigy in 1978. TCBZ is a white crystalline powder, poorly water soluble but soluble in methanol [75]. The drug is available as Fantinex® for veterinary use since 1985. In 1990, the WHO and the pharmaceutical Ciba-Geigy

developed clinical trials for treatment of fasciolosis and paragonimiasis. Different doses were studied and it was concluded that 10 mg/kg and 20 mg/kg in two equal doses were the most efficient doses for fasciolosis and paragonimiasis, respectively. Unlike what happens with PZQ, TCBZ has weak or inconsistent activity against *Schistosoma* spp. [76]. Working in conjunction with WHO and Novartis Pharma Inc. (the successor company of Ciba-Geigy), the government of Egypt registered TCBZ for human use as Egaten® [77]. TCBZ was included in the WHO model list as the drug of choice for fasciolosis [78]. TCBZ is available for human use in a few countries such as Egypt or Peru. In other countries, it can be obtained directly from Novartis [72].

Mechanisms of TCBZ metabolism are complex, but this complexity serves to maintain active concentrations of TCBZ and its metabolites for a considerable period of time, enhancing drug efficacy [79]. The pharmacokinetics of TCBZ was first studied by Henessy and coworkers [80]. TCBZ is rapidly removed from portal blood by the liver. It is rapidly oxidized to the sulfoxide (TCBA.SO) and sulfone (TCBZ·SO_2) metabolites, which are the main metabolites detectable in the plasma. In fact, only low concentrations of the parent drug are detected in human plasma after oral administration of TCBZ [81]. Furthermore, after hydroxylation of TCBZ and its metabolites, the hidroxymetabolites are secreted in the bile. The flavin monoxygenase pathway is the main pathway involved in the conversion of TCBZ to TCBZ·SO and collaborates with cytochrome P450 enzyme system to the sulfonation to TCBZ·SO_2.

The pharmacokinetics of the metabolites of TCBZ was revised in detail by Keiser and colleagues [82]. Postprandial administration enhances the absorption of TCBZ in relation to acid gastric secretion, food-induced drug solubility, and altered gastrointestinal motility. Significant differences in the area under the plasma concentration time curve and the C_{max} of TCBZ and its metabolites were found between fasting and postprandial conditions. Maximal plasma concentrations of both of the metabolites were reached at four hours after postprandial oral administration and the elimination half-life were 11.2 and 11.8 hours for TCBZ·SO and TCBZ·SO_2, respectively. There are not available data on protein binding and drug distribution in humans.

The extensive metabolism of TCBZ implies that adult flukes are exposed to different forms of TCBZ that may be active against worms. However, TCBZ·SO has been suggested to be the active form [79, 83–85]. The mechanism of action of TCBZ, TCBZ·SO, and TCBZ·SO_2 is not well known. All the evidence indicates that TCBZ binds to α- and β-tubulin within the cell of the parasite causing ultrastructural disruption in several tissues, which is consistent with the inhibition of microtubule-based processes [86, 87]. Furthermore, TCBZ and its metabolites appear to inhibit the RNA and protein synthesis in *F. hepatica* [88]. Recently, it has been shown that TCBZ inhibits adenylate cyclase or its association with GTP-Ras, which is crucial in *F. hepatica* [87]. The recommended treatment for fasciolosis is two doses of 10 mg/kg 24 hours apart [20]. However, different regimes ranging from 5 to 30 mg/kg using one, two, or three dosing have been investigated [82]. Best results are obtained after food intake, probably in relation to an improved availability of the drug [81].

TCBZ is also effective against paragonimiasis and can be used as an alternative to PZQ. The recommended regime is 10 mg/kg in a single oral dose, although it may be repeated after 12–24 hours in heavy infections or given 20 mg/kg in two separate doses of 10 mg/kg in the same day [7, 89]. TCBZ can be a better option than PZQ for mass treatment since the one or two doses required can be administered under supervision, avoiding treatment at home if several doses are required [7].

TCBZ also has been investigated as potential treatment for other FBTs in animal models. TCBZ shows activity against echinostomes infecting mice using single doses from 40 to 1600 mg/kg. In contrast, a treatment regime of 30 mg/kg in pigs infected with *F. buski* yielded a worm reduction of 79% [82]. In the case of opisthorchiasis, no effect was observed in dogs [90]. Regarding clonorchiasis, the existing data are confused since no effect was observed in dogs using two doses of 100 mg/kg 24 hours apart, but, in contrast, a single dose of 10 mg/kg stopped egg secretion in dogs [91].

TCBZ is generally well tolerated and only mild adverse effects occur. The most common side effects include weakness, mild and transient abdominal and epigastric pain, pruritus, and, less commonly, vomiting, nausea, headache, dizziness, cough, liver enlargement, or altered liver function. After a triple dose, reversible increase of liver enzymes in serum and obstructive jaundice were reported [73, 82]. Neither genotoxicity nor embryocidal or teratogenic effects have been reported [82]. TCBZ is not registered for pediatric use and there is no experience in children younger than six years [92].

Evidences of resistance against TCBZ have been observed in livestock infected with *F. hepatica* [93, 94]. Resistance has been associated with enhanced tubulin organization after treatment and reduced surface disruption [95, 96]. It has been shown that *F. hepatica*-resistant isolates develop higher ability than susceptible isolates to transform TCBZ·SO, the most active metabolite of TCBZ, to TCBZ·SO_2, that is relatively inert [97, 98]. Moreover, mutations in the β-tubulin molecule appear to lead to resistance, as previously described in nematodes. These mutations would affect the drug-binding site and consist in substitutions at position 200 (phenylalanine-tyrosine) or 198 (glutamic acid-alanine) [79]. Overexpression of stress-related proteins induced in resistant isolates could be also involved in resistance [87]. In humans, several cases of resistance to TCBZ have been proved. A 46-year-old Korean woman displayed an allergic reaction to PZQ. Alternative treatment with TCBZ did not resolve the infection. Cure was finally achieved with PZQ followed by rapid desensitization [99]. Similar cases of resistance to TCBZ were reported in a Netherlander sheep farmer, a Turkish child, and also in a group of seven patients who became infected in Peru by ingesting watercress [100, 101]. However, it is suspected that more cases may exist, especially in endemic areas [102].

11.5.3 Tribendimidine

TBDD is currently emerging as a promising broad-spectrum anthelminthic and may constitute an alternative to PZQ, especially in the treatment of clonorchiasis and opisthorchiasis. Efficacy of TBDD against these infections is similar to

that of PZQ but shows less adverse effects. TBDD was first synthesized at the National Institute of Parasitic Diseases in Shanghai (PR China). In 1983, Chinese scientists synthesized amidantel and its derivatives, including TBDD, to further structure–activity relationships. Initially, TBDD was investigated against nematodes, but later it was also found to be effective against trematodes. Subsequently, it was marketed in PR China as an oral anthelminthic of broad spectrum for human use by Shaanxi Hanwang Chinese Medicine Co. Ltd.

TBDD is the generic name for *N*,*N*-Di-[4-(1-dimethylaminoethylimine) phenyl]-1,4-xylene imine (Figure 11.4). It forms a yellow column or crystalline powder and is tasteless. TBDD is readily soluble in chloroform, although less so in dimethylformamide and only marginally in anhydrous methanol, methanol, and acetone. It does not dissolve in water and is not easy deliquesced at air temperature. Pharmacokinetics after oral administration was initially studied in animal models, although several studies have also been performed in humans. TBDD is unstable and is quickly broken down to its primary metabolite *p*-(1-dimethylamino ethylimino) aniline (dADT) that is further metabolized to its acetylated form (adADT) and terephtaldehyde (TPAL), which is completely oxidized into terephtalic acid (TPAC). dADT and adADT are present in human plasma after oral administration, but TPAC is present in the urine [103, 104]. Only 40% of the original dose is recovered in urine, suggesting that other metabolites are produced [103]. Xiao and coworkers [104] analyzed the pharmacokinetics of TBDD using single doses of 200, 400, and 600 mg. Independently of the doses, time to maximum concentration (T_{max}) and elimination half-life were similar. In contrast, the C_{max} of dADT and area under the concentration time curve (AUC_{0-24}) were enhanced proportionally to dose.

From all the known metabolites, TPAL and TPAC are pharmacologically inactive, whereas dADT is highly active and adADT has marginal or no anthelminthic activity [105]. In fact, curation of opithorchiasis has been associated with higher dADT values of C_{max} and AUC_{0-24} in cured than in uncured patients [106]. Recently, Vanobberghen and colleagues [107] studied the population pharmacokinetics of TBDD in patients infected with *O. viverrini* and compared the 200 mg with 50 mg enteric coating formulation. In general, values (i.e. C_{max} and half-life) were comparable with previous studies, but T_{max} and AUC_{0-24} for dADT were higher than previously described. The 200 mg formulation had a 40% higher mean transit adsorption time, a 113% higher dADT volume of distribution, and a 364% higher adADT volume of distribution than the 50 mg formulation.

The mechanism of action of TBDD has been studied using the *Caenorhabditis elegans* model. However, this mechanism is scarcely known and seems to vary depending on the host species [108]. TBDD produces depolarization antagonized by the nicotinic antagonist of mecamylamine and is an agonist of muscle nAChRs [109]. In fact, TBDD causes reduction in egg laying, lost motility, body damage, and developmental arrest as with other cholinesterase inhibitors such as levamisole. Several *C. elegans* have been found to be simultaneously resistant to both TBDD and levamisole [110]. Despite these facts, there are several data suggesting that TBDD does not behave as do other cholinesterase inhibitors. TBDD paralyzes worms more rapidly and, moreover, several *C. elegans* mutants sensitive

to other cholinesterase inhibitors are resistant to TBDD [110]. However, this may be related to the nAChR subtype selectivity of TBDD. TBDD can activate a different population of parasite nAChRs than do other cholinesterase inhibitors. TBDD is more selective for the B-subtype than for the L-subtype of nAChR as levamisole [109].

In animal models, TBDD has an efficacy similar to that of PZQ against trematodes. In *C. sinensis*-infected rats and hamsters, single doses of 150 and 100 mg/kg, respectively, induced worm burden reductions of 98% and 100% [105]. In humans, the standard dose is 200 mg for children aged 5–14 years and 400 mg for adults older than 15 years [111]. In patients infected with *C. sinensis*, a cure rate of 44% was achieved using a single dose of 400 mg. The values increased to 58% using the same dose for three consecutive days, which is similar to results obtained with PZQ using the same schedule [61]. A recent study showed that effectiveness of TBDD against human infections with *O. viverrini* might depend on the dose, e.g. doses of 100, 200, and 400 mg [112].

Adverse effects are mild and transient and include vertigo, headache, nausea, and fatigue [112]. Toxicity of TBDD has been studied in animals. The acute median lethal dose (LD_{50}) of oral TBDD in mice and rats were found to be 950 ± 207 and 2001 ± 79 mg/kg, respectively. At long term, no death of rats and no side effects were observed using doses lower than 200 mg/kg after 14 days of daily treatment [113]. Using daily doses of higher than 500 mg/kg, about 26% of rats died and liver damage was observed [113]. In beagle dogs, salivation and vomiting were observed from the fifth day of treatment using daily doses of 60 and 120 mg/kg. Histopathological studies have shown that TBDD targets the digestive system and the major changes are intestinal congestion, desquamation of epithelial cells, and swelling of hepatic cells. However, these anomalies become resolved at one month posttreatment [114].

11.5.4 Other Drugs

A historical review of the literature shows that a large number of drugs against FBT have been investigated and/or used. Most of them have been abandoned mainly in relation to their side effects and/or low efficacy. Herein, we briefly review the characteristics of the most relevant of these drugs.

Albendazole or mebendazole, benzimidazole derivatives, has been used for treatment of different FBT infections. In the case of opisthorchiasis, doses of 400 mg/kg of albendazole twice a day for three days yielded cure rates of 12–33% and egg release reduction of about 95% [115]. Daily doses of 10 mg/kg for three and seven days reported cure rates of 40% and 100%, respectively, in patients infected with *C. sinensis* [116]. Mebendazole has been used against fascioliasis, opisthorciasis, and echinostomiasis. A single patient of fascioliasis was treated with 4 g/d for three weeks, achieving complete cure [117]. A cure rate of 94% of opisthorchiasis was yielded using 30 mg/d for three to four weeks [118]. Regarding echnostomiasis, 50 patients infected with *E. fujianensis* were given tablets of either 40 or 800 mg of mebendazole with cure rates of 71% and 85%, respectively [119]. Mebendazole has no effect against *Paragonimus* or *Fasciolopsis* [72].

Bithionol, a halogenated phenol derivative, was the drug of choice against fascioliasis for almost three decades in the last century. The recommended doses were 30–50 mg/kg daily, divided in three doses for 20–30 days, although a second round could be required. However, bithionol is toxic and numerous side effects may appear [20]. Before bithionol, the drug of choice against *F. hepatica* was dehydroemetine or the related compound emetine, which showed a 93% cure rate [120]. The main drawback of this drug was its toxicity, including cardiac side effects such as hypertension [121]. Chloroquine, an aminoquinoline derivative, was also used against fascioliasis. This drug significantly decreased clinical symptoms when used in the acute phase of the disease. In the dissolved Soviet Union and PR China, a xylol derivative, hexachloro-*para*-xylol, also was used [20]. Nitanoxazide, the prototype member of the thiazolides, has shown to have effects against *F. hepatica* [122, 123].

11.6 Natural Products and Drug Discovery against Foodborne Trematodes

The small number of available effective drugs against foodborne trematodiasis, together with the recent appearance of drug resistance and the unavailability of vaccines, urges the discovery and development of new trematocidal drugs to fight these infections [124, 125]. Motivated by the World Health Organization to ensure safety, efficacy, and quality of herb-derived traditional medicines, a number of studies have arisen exploring the trematocidal potential of plant-derived products, mainly in the last decade (Table 11.1). Despite the increasing number of papers in this field, results are still poorly conclusive due to the vast heterogeneity across studies, regarding parasite species, plant extracts, and the way in which wormicidal effect is evaluated. In this section, current studies on trematocidal activity of natural products are reviewed, and used to illustrate the screening strategies and animal models that are being used to discover and develop new drugs with suitable properties for the control of foodborne trematodiasis.

In vitro, whole parasite screens are still the basis of trematocidal drug discovery. Main laboratory models for testing anti-FBT drugs involve the liver flukes *F. hepatica* and *F. gigantica*, and the intestinal species *F. buski* and *E. caproni* [159]. However, the scarcity, even lack, of studies with other important groups such as liver trematodes belonging to the family Opistorchiidae or lung flukes of genus *Paragonimus* is noticeable. *In vitro* studies with parasites in culture are predominant, and are usually performed either on adult worms or newly excysted juveniles (NEJs) (Table 11.1). Panic and coworkers [160] stated that NEJs are suitable stages for in *vivo* drug testing, as they mirror the results that several drugs have against adult specimens. The use of NEJs allows reducing the use of laboratory animals and improving the extent and affordability of *in vitro* screening studies. However, species-specific and/or drug-specific differences in stage sensibility cannot be denied. An ideal trematocidal drug should be active against all the developmental stages that grow inside the vertebrate host [124]. Hence, it is highly recommendable to confirm the effectiveness against adult specimens before proceeding with further studies [159].

Table 11.1 *In vitro* and *in vivo* natural product screening: laboratory models, natural products, and methods for assay evaluation.

***In vivo* assays**

Trematode sp. (stage) [Ref]	Plant/natural product	Product	Active compound(s)	Evaluation of assay
Liver foodborne trematodes				
Fasciola hepatica (newly excysted juveniles [NEJs]) [126]	*Cartaegus mexicana, Eriobotrya japonica, Ternstroemia pringlei, Tilia mexicana Coffea arabica*	Chloroform, methanol, or water extracts of dried plant material (nonspecified)	Non-defined	Worm motility and viability over time
		Infusion of ground toasted grains		
F. hepatica (adults) [127]	*Cocos nucifera*	Chloroform extract	Non-defined	Worm viability; light microscopy
F. hepatica (adults) [128, 129]	*Artemisia annua, Artemisia abisithium, Asiminia triloba*	Ethanolic extracts of leaves	Artemisinin; acetogenins (asimicin and bullatacin)[PChA]	Worm viability over time
F. hepatica (adults) [129]	*Achillea millefolium, A. absinthium, Artemisia mexicana, Castela tortuosa, Chemopodium graveolens, Gymnosperma glutinosum, Justicia spicigera, Limpia critridora, Mentha piperita, Populus alba, Thymus vulgaris*	Hexane extracts of leaves, flowers, and stems	Non-defined	Worm viability

(Continued)

Table 11.1 (Continued)

Trematode sp. (stage) [Ref]	Plant/natural product	Product	Active compound(s)	Evaluation of assay
F. hepatica (adults) [130]	*Garcinia mangostana*	Mangostin (commercial product)	Mangostin (xanthone derivative)	Worm viability
F. hepatica (adults) [131]	*Hagenia abyssinica*	Sequential extracts of crude female flowers with *n*-haptane, methanol, and water	Nonpolar kosins[PChA]	Worm viability over time
F. hepatica (NEJs) [94]	*A. mexicana, Bocconia frutescens, Cajannus cajan, Lantana camara, Piper auritum*	Sequential extracts of dried leaves with hexane, ethyl acetate, and methanol	Non-defined	Worm motility and viability over time; light microscopy
F. hepatica (NEJs and adults) [132]	*Lycium chinense*	7-keto-sempervirol isolated from the root	7-keto-sempervirol	Motility and morphology of NEJs; worm viability addressed (helminth fluorescent bioassay); scanning electron microscopy (SEM)
Fasciola gigantica (adults) [133]	*Buchholzia coriaceae, Gynandropsis gynandra*	Sequential extract of leaves and stem, separately, in hexane and methanol	Non-defined	Worm motility and viability
F. gigantica (adults) [134]	*Propolis*	Propolis (exudates from plants mixed with beeswax)	Non-defined	SEM
F. gigantica (NEJs and adults) [135]	*Atrocarpus lakoocha*	Crude extract	2,4,3′,5′-Tethrahydroxy stilbene[PChA]	Relative motility; light microscopy (tegument) and SEM
F. gigantica (adults) [136]	*Allium sativum, Piper longum*	Essential oils	Non-defined	Worm motility (spontaneous muscular activity)

F. gigantica (adults) [137, 138]	*Commiphora molmol*	Myrrh (oleo-gum resin from the stem) Mirazid® (pharmaceutical preparation from myrrh)	Non-defined	Light microscopy (tegument) and SEM
F. gigantica (adults) [139]	*Dregea volubilis*	Methanolic extract of dried leaves	Non-defined	Worm motility and viability; SEM
F. gigantica (NEJs and adults) [140]	*Plumbago* spp.	Plumbagin (extractable from the root of *Plumbago* spp.)	Plumbagin (5-hydroxy-2-methyl-1, 4-naphtoquinone)	Worm motility and viability; SEM
F. gigantica (adults) [141]	*Conyza canadensis, Cymbopogon jwarancusa*	Crude methanolic extract	Non-defined	Worm viability over time
Clonorchis sinensis (adults) [131]	*H. abyssinica*	Sequential extracts of crude female flowers with *n*-haptane, methanol and water	Nonpolar kosins[PChA]	Worm viability over time
Intestinal foodborne trematodes				
Fasciolopsis buski (adults) [142–145]	*Flemingia vestita*	Alcoholic extract of fresh root-tuber peel; Genistein (4′,5,7-hydroxyisoflavone)	Genistein (4′,5,7-hydroxyisoflavone)	Worm motility and viability; SEM; biochemical assays; others
F. buski (adults) [146]	*Cannabis sativa*	Crude extract of leaves	Non-defined	Worm motility and viability; SEM
F. buski (adults) [147–150]	*Alpinia nigra*	Ethanolic shoot extract; astragalin	Astragalin (flavone glycoside); Others? (i.e. kaempfarol-3-*o*-glucoronide, β-pinene, α-pinene)[PChA]	Worm motility and viability; SEM Transmission electron microscopy (TEM) (tegument and gastrodermis); biochemical assays; others

(Continued)

Table 11.1 (Continued)

Trematode sp. (stage) [Ref]	Plant/natural product	Product	Active compound(s)	Evaluation of assay
F. buski (adults) [151]	*Lysimachia ramosa*	Ethanolic extract of dried leaves	Non-defined	Worm motility and viability; SEM
F. buski (adults) [149]	*Potentilla fulgens* *Carex baccanus*	Ethanolic extracts of dried root peel Ethanolic extracts of dried root-tuber	Non-defined	Worm motility and viability; biochemical assays; others
Echinostoma caproni (adults) [127]	*C. nucifera*	Chloroform extract	Non-defined	Worm viability; light microscopy
E. caproni (adults) [128]	*A. annua, A. abisithium, A. triloba*	Ethanolic extracts of leaves	Artemisinin, acetogenins (asimicin and bullatacin)^{PChA}	Worm viability over time
E. caproni (adults) [130]	*G. mangostana*	Mangostin	Mangostin (xanthone derivative)	Worm viability
E. caproni (adults) [152]	A total of 27 medical plants used in Côte d'Ivoire against parasitic diseases	A total of 36 ethanolic extracts from roots, leaves, stem bark, or whole plant	Non-defined	Worm viability
E. caproni (adults) [131]	*H. abyssinica*	Sequential extracts of crude female flowers with *n*-haptane, methanol, and water	Nonpolar kosins^{PChA}; non-defined polar compounds	Worm viability over time

***In vivo* assays**

Trematode sp. (host)	Plant	Product	Active compound(s)	Evaluation of assay
Liver foodborne trematodes				
F. gigantica (goat) [153]	*Albizia anthelmintica* *Balanites aegyptiaca*	Stem bark, water extract; Fruit mesocarp, water extract	Non-defined	Worm burden reduction; eggs per gram of feces (EPG), pathology, hematology
F. gigantica (rabbit) [154]	*C. molmol*	Mirazid®	Non-defined	Worm burden reduction; EPG; antibody response
F. hepatica (rat) [155]	*C. molmol*	Mirazid®	Non-defined	SEM; TEM (tegument and gastrodermis)
Opistorchis viverrini (hamster) [156]	*G. mangostana*	Methanolic extract of pericarp	Mangostin	Worm burden^{nsd}; adult morphometry; worm fecundity
Intestinal foodborne trematodes				
E. caproni (mouse) [127]	*Allium cepa*	Polyethylene glycol/propylene carbonate extracts	Non-defined	Worm burden reduction
E. caproni (mouse) [130]	*G. mangostana*	Mangostin; Mangostin diacetate	Mangostin (xanthone derivative)	Worm burden reduction
E. caproni (mouse) [157]	*A. sativum*	Paste of dried cloves	Non-defined	Worm burden^{nsd}; EPG^{nsd}; SEM; TEM (tegument); protein secretion in culture; differences in secreted proteins
Pygidiopsis genata (–) [158]	*C. molmol*	Mirazid®	Non-defined	Worm burden reduction; SEM

PCh: based on phytochemical analysis; nsd: nonstatistical differences.

In the absence of suitable high-throughput molecular-based screens, one of the foremost advantages of *in vitro* studies is that they allow for rapid screening of potentially active compounds. Most of these studies evaluate drug efficacy based on worm viability, although there is an increasing tendency to assay evaluation by scanning electron microscopy and transmission electron microscopy of the tegument and gastrodermis, the two main routes for drug entry (Table 11.1). These phenotypic screens are useful methods to test the direct trematocidal effect of potential drugs, although they do not provide any information regarding the mechanism of action nor the molecular targets of drugs. Only a few studies have gone further to preliminarily investigate potential molecular targets, following two different strategies: (i) biochemical assays of metabolic enzymes [148, 149] and (ii) comparative proteomics [157]. In any case, molecular-based approaches are still very scarce and the publication of draft genomes of model FBTs (e.g. *F. hepatica* and *E. caproni*) has not prompted them in the magnitude it was expected [159]. Not only the cellular targets but also the speed of action should be considered when evaluating the activity of trematocidal compounds [161]. Vera-Montenegro and coworkers [126] found high efficacy (over 90%) of different plant extracts against *F. hepatica*, although it took three days of culture *in vitro*. The rapid action of a trematocidal drug is desirable, because it facilitates the development of easily administrable, single-dose formulations, and an easy dosage is crucial for implementing cost-effective control programs [124].

Because of the features discussed, presently, *in vitro* studies appear as the prevailing strategy for preliminary, large-scale screening of potentially active molecules and plant extracts [126, 129, 152]. However, *in vivo* studies in animal models are required to evaluate drug bioavailability, pharmacokinetics, and toxicity to determine if a compound is worth being considered for in-depth investment in medicinal chemistry and pharmacology [162]. A number of plant-derived products that have demonstrated trematocidal effect *in vitro* have failed to function *in vivo* [127, 129, 130, 152]. For instance, 36 plant extracts with lethal activity were identified on *E. caproni in vitro*, although none of them showed effect *in vivo* [152]. Likewise, active principle mangostin, a xanthone derivative from the pericarp and fruit of *Gracinia mangostana*, was effective against *F. hepatica in vitro*, but not *in vivo* [130]. *In vivo* trials with plant-derived products are scarce and display huge variability in terms of dosage. These are based on animal models and, similar to *in vitro* studies, the majority of them are performed using complex plant extracts; and assay evaluation consists mostly in worm burden reduction and electron microscopy of recovered worms (Table 11.1).

Regarding the nature and composition of the plant-based products employed, a wide heterogeneity is also noticeable across studies. Most of the studies employ plant extracts, consisting of complex mixtures of compounds, which makes it difficult to identify the active principle(s). Moreover, a number of variables, e.g. seasonal and environmental conditions, protocols of collection and storage of plant material, extraction procedures, or solvent properties may affect the composition of the final product and its content in active principle(s) [163]. Thomsen and coworkers [131] found differences in anthelmintic activity of *Hagenia abyssinica* female flower extracts depending on the polarity of the

solvent. Similarly, different extracts of *Cocos nucifera* showed different efficacies against *E. caproni* and *F. hepatica* [127]. The other common shortcoming is that the bioavailability of the active compound in the host is not always directly related to the concentration in the plant [163], which may explain the eventual inconsistency between *in vitro* and *in vivo* studies.

Some studies achieve to infer the active compound(s) in plant extracts based on phytochemical analysis, although only a few have tested the isolated active principles (Table 11.1) [94, 128, 131, 135]. Activity against FBTs have been attributed to some definite, plant-isolated molecules of different chemical nature (Table 11.1). The naphtoquinone plumbagin, from *Plumbago* spp., and 7-keto-sempervirol, a diterpenoid from *Lycium chinense*, have been shown to be active against *F. hepatica* NEJs and adults *in vitro* [132, 140]. The flavone derivatives genistein and astragalin, isolated from *Flemingia vestita* and *Alpinia nigra*, respectively, showed *in vitro* activity against *F. buski* [145, 150]. Leaving out studies with synthetic and semisynthetic artemisinins [164], the xanthone derivative mangostin is the unique plant-derived active compound that, to our knowledge, has been tested *in vivo* against FBTs till date. Mangostin and mangostin diacetate caused up to 54% worm burden reduction in *E. caproni*-infected mice [130]. In contrast, nonsignificant worm burden reductions have been observed against liver trematodes *F. hepatica* and *O. viverrini*, although impairment of worm development and fecundity has been reported in *O. viverrini* [130, 156]. A desirable characteristic of trematocidal drugs is displaying a wide spectrum, i.e. being active against the major human trematode species [124]. Therefore, studies in this field should test potentially active compounds against different groups of trematodes with different niches inside the host.

Although elucidating the active compound(s) in complex plant preparations appears crucial to rational design of plant-derived drugs, in some cases, the anthelminthic effect of an extract may be due to the synergistic action of several compounds in the mixture. Myrrh, an aromatic oleo gum resin obtained from the stem of the *Commiphora molmol* tree, is the base of Mirazid®, a trematocidal drug marketed in Egypt since 2001 [165]. Myrrh is composed of polysaccharides, proteins, and volatile oil-containing steroids, sterols, and terpenes. Although its mechanism of action is poorly understood, a number of studies support the activity of Mirazid, both experimentally and clinically [166]. Effectiveness of Mirazid have been reported against liver (*F. hepatica* and *D. dendriticum*) and intestinal (*H. heterophyes*) FBTs in humans [167–177]. Nevertheless, other studies are questioning the effectiveness of this drug [165, 178].

In summary, research on potential anti-FBT drugs is remarkably sparse and underscores the neglected situation of these infections, which affect over 40 million people worldwide, with around 750 million individuals at risk [68]. Advances in chemotherapy and chemotherapy-based control of foodborne trematodiasis are normally behind the progression done with schistosomiasis. Studies exploring new potential active compounds are still very scarce, although a tendency to evaluate traditional, plant-based products is gaining in popularity. Nevertheless, those studies are still preliminary and, in the best-case scenario, far off from leading to a new drug in the near future. Furthermore, current studies consist in low-scale phenotypic screens rather than in mechanism-based

models that enable high-throughput screening with simple readouts, and huge heterogeneity exists across studies. In this scenario, drug repurposing has supplied the emptiness of the drug discovery pipeline against FBTs [125]. Preclinical studies with artemisinins, synthetic peroxides, and tribendimidine are in progress and appear as the nearest alternative to fight resistance development against currently in-use drugs.

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References

1 Fürst, T., Keiser, J., and Utzinger, J. (2012). Global burden of human foodborne trematodiasis: a systematic review and meta-analysis. *Lancet Infect. Dis.* 12: 210–221.
2 Toledo, R., Esteban, J.G., and Fried, B. (2012). Current status of foodborne trematode infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 31: 1705–1718.
3 Herricks, J.R., Hotez, P.J., Wanga, V. et al. (2017). The global burden of disease study 2013: What does it mean for the NTDs? *PLoS Negl. Trop. Dis.* 11: e0005424.
4 Peoples, R.C. and Fried, B. (2014). Form and function in the digenea. *Adv. Exp. Med. Biol.* 766: 3–20.
5 Esteban, J.G., Muñoz-Antoli, C., Toledo, R., and Ash, L.R. (2014). Diagnosis of human trematode infections. *Adv. Exp. Med. Biol.* 766: 293–327.
6 Lun, Z.R., Gasser, R.B., Lai, D.H. et al. (2005). Clonorchiasis: a key foodborne zoonosis in China. *Lancet Infect. Dis.* 5: 31–41.
7 Blair, D. (2014). Paragonimiasis. *Adv. Exp. Med. Biol.* 766: 115–152.
8 Yoshida, A., Matsuo, K., Moribe, J. et al. (2016). Venison, another source of *Paragonimus westermani* infection. *Parasitol. Int.* 65: 607–612.
9 Fürst, T., Duthaler, U., Sripa, B. et al. (2012). Trematode infections: liver and lung flukes. *Infect. Dis. Clin. North. Am.* 26: 399–419.
10 Fürst, T., Sayasone, S., Odermatt, P. et al. (2012). Manifestation, diagnosis, and management of foodborne trematodiasis. *BMJ* 344: e4093.
11 WHO (1995). Control of Foodborne Trematode Infections. *Report of a WHO Study Group*. World Health Organization, Geneva.
12 Sripa, B., Kaewkes, S., Intapan, P.M. et al. (2010). Foodborne trematodiases in Southeast Asia: epidemiology, pathology, clinical manifestation and control. *Adv. Parasitol.* 72: 305–350.

13 Saijuntha, W., Sithithaworn, P., Kaitsopit, N. et al. (2014). Liver flukes: *Clonorchis* and *Opisthorchis*. *Adv. Exp. Med. Biol.* 766: 153–199.
14 Rim, H.J. (2005). Clonorchiasis: an update. *J. Helminthol.* 79: 269–281.
15 Keiser, J. and Utzinger, J. (2005). Emerging foodborne trematodiasis. *Emerg. Infect. Dis.* 11: 1507–1514.
16 Mayer, D.A. and Fried, B. (2007). The role of helminth infections in carcinogenesis. *Adv. Parasitol.* 65: 239–296.
17 Sripa, B. and Pairojkul, C. (2008). Cholangiocarcinoma: lessons from Thailand. *Curr. Opin. Gastroenterol.* 24: 349–356.
18 Hotez, P.J. and Alibek, K. (2011). Central Asia's hidden burden of neglected tropical diseases. *PLoS Negl. Trop. Dis.* 5: e1224.
19 Parkin, D.M. (2006). The global health burden of infection-associated cancers in the year 2002. *Int. J. Cancer* 118: 3030–3044.
20 Mas-Coma, S., Valero, M.A., and Bargues, M.D. (2014). Fascioliasis. *Adv. Exp. Med. Biol.* 766: 77–114.
21 Robinson, M.W. and Dalton, J.P. (2009). Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364: 2763–2776.
22 Murray, C.J.L. (1994). Quantifying the burden of disease: the technical basis for disability adjusted life years. *Bull. World Health Organ.* 72: 428–445.
23 Chai, J.Y. and Lee, S.H. (2002). Foodborne intestinal trematode infections in the Republic of Korea. *Parasitol. Int.* 51: 129–154.
24 Toledo, R. and Esteban, J.G. (2016). An update on human echinostomiasis. *Trans. R. Soc. Trop. Med. Hyg.* 110: 37–45.
25 Xiao, X., Lu, D., Wang, T. et al. (1995). (1995) Studies on mode of human infection with *Echinochasmus liliputanus*. *Chinese J. Parasitol. Parasit. Dis.* 13: 197–199.
26 Toledo, R., Esteban, J.G., and Fried, B. (2006). Immunology and pathology of intestinal trematodes in their definitive hosts. *Adv. Parasitol.* 63: 285–365.
27 Toledo, R., Muñoz-Antoli, C., and Esteban, J.G. (2014). Intestinal trematode infections. *Adv. Exp. Med. Biol.* 766: 201–240.
28 Chai, J.Y., Lee, G.C., Park, Y.K. et al. (2000). Persistent endemicity of *Gymnophalloides seoi* infection in a southwestern coastal village of Korea with special reference to its egg laying capacity in the human host. *Korean J. Parasitol.* 38: 51–57.
29 Sohn, W.M. (2009). Fish-borne zoonotic trematode metacercariae in the Republic of Korea. *Korean J. Parasitol.* 47: S103–S113.
30 Marty, A.M. and Andersen, E.M. (2000). Fasciolopsiasis and other intestinal trematodiasis. In: *Pathology of Infectious Diseases, Vol I Helminthiases* (ed. W.M. Meyers, R.C. Neafie, A.M. Marty and D.J. Wear). Washington DC: Armed Forces Institute of Pathology (AFIP) and American Registry of Pathology.
31 Cioli, D., Pica-Mattoccia, L., and Archer, S. (1995). Antischistosomal drugs: past, present ...and future? *Pharmacol. Ther.* 68: 35–85.
32 Cioli, D. and Pica-Mattoccia, L. (2003). Praziquantel. *Parasitol. Res.* 90: S3–S9.

33 Thomas, H. and Gönnert, R. (1977). The efficacy of praziquantel against cestodes in animals. *Z. Parasitenkd.* 52: 117–127.

34 Thomas, H. and Gönnert, R. (1978). The efficacy of praziquantel against cestodes in cats, dogs and sheep. *Res. Vet. Sci.* 24: 20–25.

35 Gönnert, R, Andrews, P (1977). Praziquantel, a new board-spectrum antischistosomal agent. *Z. Parasitenkd.* 52: 129–150.

36 Leopold, G., Ungethüm, W., Groll, E. et al. (1978). Clinical pharmacology in normal volunteers of praziquantel, a new drug against schistosomes and cestodes. An example of a complex study covering both tolerance and pharmacokinetics. *Eur. J. Clin. Pharmacol.* 14: 281–291.

37 Davis, A. (1978). *Schistosomicides in the Developmental Stage*, 6–8. WHO Expert Committee.

38 Katz, N., Rocha, R., and Chaves, A. (1979). Preliminary trials with praziquantel in human infections due to *Schistosoma mansoni*. *Bull. World Health Organ.* 57: 781–785.

39 Keiser, J., Duthaler, U., and Utzinger, J. (2010). Update on the diagnosis and treatment of food-borne trematode infections. *Curr. Opin. Infect. Dis.* 23: 513–520.

40 Doenhoff, M., Kimani, G., and Cioli, D. (2000). Praziquantel and the control of schistosomiasis. *Parasitol. Today* 16: 364–366.

41 Appleton, C.C. and Mbaye, A. (2001). Praziquantel--quality, dosages and markers of resistance. *Trends Parasitol.* 17: 356–357.

42 Sulaiman, S.M., Traoré, M., Engels, D. et al. (2001). Counterfeit praziquantel. *Lancet* 358: 666–667.

43 Andrews, P., Thomas, H., Pohlke, R., and Seubert, J. (1983). Praziquantel. *Med. Res. Rev.* 3: 147–200.

44 Liu, Y.H., Qian, M.X., Wang, X.G. et al. (1986). Comparative efficacy of praziquantel and its optic isomers in experimental therapy of schistosomiasis japonica in rabbits. *Chin. Med. J.* 99: 935–9340.

45 Steiner, K., Diekmann, H.W., Garbe, A., and Nowak, H. (1976). Pharmacokinetics of pramiverine in rats, dogs, and monkeys. *Arzneim.-Forsch.* 26: 717–721. (German).

46 Metwally, A., Bennett, J.L., Botros, S., and Ebeid, F. (1995). Effect of cimetidine, bicarbonate and glucose on the bioavailability of different formulations of praziquantel. *Arzneim.-Forsch.* 45: 516–518.

47 Day, T.A., Bennett, J.L., and Pax, R.A. (1002). Praziquantel: the enigmatic antiparasitic. *Parasitol. Today* 8: 342–344.

48 Cioli, D. (2000). Praziquantel: is there real resistance and are there alternatives? *Curr. Opin. Infect. Dis.* 13: 659–663.

49 Becker, B., Mehlhorn, H., Andrews, P. et al. (1980). Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni, Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) in vitro. *Z. Parasitenkd.* 63: 113–128.

50 Mehlhorn, H., Becker, B., Andrews, P. et al. (1981). In vivo and in vitro experiments on the effects of praziquantel on *Schistosoma mansoni*. A light and electron microscopic study. *Arzneim.-Forsch.* 31: 544–554.

51 You, H., McManus, D.P., Hu, W. et al. (2013). Transcriptional responses of in vivo praziquantel exposure in schistosomes identifies a functional role for calcium signalling pathway member CamKII. *PLoS Pathog.* 9: e1003254.

52 Kasinathan, R.S. and Greenberg, R.M. (2012). Pharmacology and potential physiological significance of schistosome multidrug resistance transporters. *Exp. Parasitol.* 132: 2–6.

53 Frohberg, H. (1984). Results of toxicological studies on praziquantel. *Arzneim.-Forsch.* 34: 1137–1144.

54 Anwar, W.A., Au, W.W., Ramanujam, V.M., and Legator, M.S. (1989). Enhancement of benzene clastogenicity by praziquantel in mice. *Mutat. Res.* 222: 283–289.

55 Herrera, L.A., Ostrosky-Wegman, P., Montero, R. et al. (1994). Evaluation of the carcinogenic and genotoxic potential of praziquantel in the Syrian hamster embryo cell transformation assay. *Mutat. Res.* 305: 175–180.

56 Davis, A. (1993). Antischistosomal drugs and clinical practice. In: *Human Schistosomiasis* (ed. P. Jordon, G. Webbe and R.F. Sturrock). Wallingford: CAB International.

57 Qian, M.B., Utzinger, J., Keiser, J., and Zhou, X.N. (2016). Clonorchiasis. *Lancet* 387: 800–810.

58 Tang, S., Chen, L., Qian, M. et al. (2016). Pharmacokinetics of a new ivermectin/praziquantel suspension after intramuscular administration in sheep. *Vet. Parasitol.* 221: 54–58.

59 WHO (2002). *Report of the WHO Informal Consultation on the Use of Praziquantel During Pregnancy/Lactation and Albendazole/Mebendazole in Children under 24 Months*. Geneva: World Health Organization.

60 Rim, H.J. and Yoo, K.S. (1979). Chemotherapeutic effect of praziquantel (Embay 8440) in the treatment of Clonorchiasis sinensis. *Korea Univ. Med. J.* 16: 459–470.

61 Qian, M.B., Yap, P., Yang, Y.C. et al. (2013). Efficacy and safety of tribendimidine against *Clonorchis sinensis*. *Clin. Infect. Dis.* 56: e76–e82.

62 Hong, S.T., Yoon, K., Lee, M. et al. (1998). Control of clonorchiasis by repeated praziquantel treatment and low diagnostic efficacy of sonography. *Korean J. Parasitol.* 36: 249–254.

63 Choi, M.H., Park, S.K., Li, Z. et al. (2010). Effect of control strategies on prevalence, incidence and re-infection of clonorchiasis in endemic areas of China. *PLoS Negl. Trop. Dis.* 4: e601.

64 Wegner, D.H. (1984). The profile of the trematodicidal compound praziquantel. *Arzneim.-Forsch.* 34: 1132–1136.

65 Kamsa-Ard, S., Luvira, V., Pugkhem, A. et al. (2015). Association between praziquantel treatment and cholangiocarcinoma: a hospital-based matched case-control study. *BMC Cancer* 15: 776.

66 Saengsawang, P., Promthet, S., and Bradshaw, P. (2016). Reinfection by *Opisthorchis viverrini* after treatment with praziquantel. *Asian Pac. J. Cancer Prev.* 17: 857–862.

67 Rim, H.J., Chang, Y.S., Lee, J.S. et al. (1981). Clinical evaluation of praziquantel (Embay 8440; Biltricide) in the treatment of *Paragonimus westermani*. *Kisaengchunghak Chapchi* 19: 27–37.

68 Keiser, J. and Utzinger, J. (2009). Foodborne trematodiases. *Clin. Microbiol. Rev.* 22: 466–483.

69 Oh, I.J., Kim, Y.I., Chi, S.Y. et al. (2011). Can pleuropulmonary paragonimiasis be cured by only the 1st set of chemotherapy? Treatment outcome and clinical features of recently developed pleuropulmonary paragonimiasis. *Intern. Med.* 50: 1365–1370.

70 Lv, S., Zhang, Y., Steinmann, P. et al. (2010). Helminth infections of the central nervous system occurring in Southeast Asia and the Far East. *Adv. Parasitol.* 72: 351–408.

71 Bunnag, D., Radomyos, P., and Harinasuta, T. (1983). Field trial on the treatment of fasciolopsiasis with praziquantel. *Southeast Asian J. Trop. Med. Public. Health.* 14: 216–219.

72 Keiser, J. and Utzinger, J. (2004). Chemotherapy for major foodborne trematodes: a review. *Expert Opin. Pharmacother.* 5: 1711–1726.

73 Lee, S.H. and Chai, J.Y. (2001). A review of *Gymnophalloides seoi* (Digenea: Gymnophallidae) and human infections in the Republic of Korea. *Korean J. Parasitol.* 39: 85–118.

74 Albonico, M., Levecke, B., LoVerde, P.T. et al. (2015). Monitoring the efficacy of drugs for neglected tropical diseases controlled by preventive chemotherapy. *J. Glob. Antimicrob. Resist.* 3: 229–236.

75 Robinson, C.P. (1985). Triclabendazole. *Drugs Today* 21: 227–233.

76 Keiser, J., El Ela, N.A., El Komy, E. et al. (2006). Triclabendazole and its two main metabolites lack activity against *Schistosoma mansoni* in the mouse model. *Am. J. Trop. Med. Hyg.* 75: 287–291.

77 WHO (ed.) (1998). *Triclabendazole and Fascioliasis: Reports on Individual Drugs*. Geneva: World Health Organization.

78 WHO (ed.) (2015). *WHO Model List of Essential Medicines*. Geneva: World Health Organization.

79 Fairweather, I. (2009). Triclabendazole progress report, 2005-2009: an advancement of learning? *J. Helminthol.* 83: 139–150.

80 Hennessy, D.R., Lacey, E., Steel, J.W., and Prichard, R.K. (1987). The kinetics of triclabendazole disposition in sheep. *J. Vet. Pharmacol. Ther.* 10: 64–72.

81 Lecaillon, J.B., Godbillon, J., Campestrini, J. et al. (1998). Effect of food on the bioavailability of triclabendazole in patients with fascioliasis. *Br. J. Clin. Pharmacol.* 45: 601–604.

82 Keiser, J., Engels, D., Büscher, G., and Utzinger, J. (2005). Triclabendazole for the treatment of fascioliasis and paragonimiasis. *Expert Opin. Investig. Drugs* 14: 1513–1526.

83 Mottier, L., Moreno, L., Alvarez, L. et al. (2004). Measurement of triclabendazole and its metabolites in liver flukes: method development and full validation. *J. Pharm. Biomed. Anal.* 35: 991–999.

84 Mottier, L., Alvarez, L., Ceballos, L., and Lanusse, C. (2006). Drug transport mechanisms in helminth parasites: passive diffusion of benzimidazole anthelmintics. *Exp. Parasitol.* 113: 49–57.

85 Mottier, L., Virkel, G., Solana, H. et al. (2004). Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidized metabolites into *Fasciola hepatica*. *Xenobiotica* 34: 1043–1057.

86 Brennan, G.P., Fairweather, I., Trudgett, A. et al. (2007). Understanding triclabendazole resistance. *Exp. Mol. Pathol.* 82: 104–109.

87 Kelley, J.M., Elliott, T.P., Beddoe, T. et al. (2016). Current threat of triclabendazole resistance in *Fasciola hepatica*. *Trends Parasitol.* 32: 458–469.

88 Fairweather, I. and Boray, J.C. (1999). Fasciolicides: efficacy, actions, resistance and its management. *Vet. J.* 158: 81–112.

89 WHO (2011). *Report of the WHO Expert Consultation on Foodborne Trematode Infections and Taeniasis/Cysticercosis*. Geneva: World Health Organization.

90 Moskvina, T.V. and Ermolenko, A.V. (2016). Helminth infections in domestic dogs from Russia. *Vet. World* 9: 1248–1258.

91 Shiramian, K., Abu, M., Hara, A., and Haida, Y. (1989). The effect of triclabendazole to canine Clonorchiasis. *Yamaguchi J. Vet. Med.* 16: 113–116.

92 Keiser, J., Ingram, K., and Utzinger, J. (2011). Antiparasitic drugs for paediatrics: systematic review, formulations, pharmacokinetics, safety, efficacy and implications for control. *Parasitology* 138: 1620–1632.

93 Overend, D.J. and Bowen, F.L. (1995). Resistance of *Fasciola hepatica* to triclabendazole. *Aust. Vet. J.* 72: 275–276.

94 Alvarez-Mercado, J.M., Ibarra-Velarde, F., Alonso-Diaz, M.A. et al. (2015). In vitro antihelmintic effect of fifteen tropical plant extracts on excysted flukes of *Fasciola hepatica*. *BMC Vet. Res.* 11: 45.

95 Robinson, M.W., Trudgett, A., Hoey, E.M., and Fairweather, I. (2002). Triclabendazole-resistant *Fasciola hepatica*: beta-tubulin and response to in vitro treatment with triclabendazole. *Parasitology* 124: 325–338.

96 Walker, S.M., McKinstry, B., Boray, J.C. et al. (2004). Response of two isolates of *Fasciola hepatica* to treatment with triclabendazole in vivo and in vitro. *Parasitol. Res.* 94: 427–438.

97 Robinson, M.W., Lawson, J., Trudgett, A. et al. (2004). The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. *Parasitol. Res.* 92: 205–210.

98 Alvarez-Sánchez, M.A., Mainar-Jaime, R.C., Pérez-García, J., and Rojo-Vázquez, F.A. (2006). Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. *Vet. Rec.* 159: 424–425.

99 Kyung, S.Y., Cho, Y.K., Kim, Y.J. et al. (2011). A paragonimiasis patient with allergic reaction to praziquantel and resistance to triclabendazole: successful treatment after desensitization to praziquantel. *Korean J. Parasitol.* 49: 73–77.

100 Winkelhagen, A.J., Mank, T., de Vries, P.J., and Soetekouw, R. (2012). Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. *Emerg. Infect. Dis.* 18: 1028–1089.

101 Cabada, M.M., Lopez, M., Cruz, M. et al. (2016). Treatment failure after multiple courses of triclabendazole among patients with fascioliasis in Cusco, Peru: a case series. *PLoS Negl. Trop. Dis.* 10: e0004361.

102 Cwiklinski, K., O'Neill, S.M., Donnelly, S., and Dalton, J.P. (2016). A prospective view of animal and human Fasciolosis. *Parasit. Immunol.* 38: 558–568.

103 Yuan, G., Xu, J., Qu, T. et al. (2010). Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. *Drugs R. D.* 10: 83–90.

104 Xiao, S.H., Utzinger, J., Tanner, M. et al. (2013). Advances with the Chinese anthelminthic drug tribendimidine in clinical trials and laboratory investigations. *Acta Trop.* 126: 115–126.

105 Xiao, S.H., Xue, J., Xu, L.L. et al. (2009). The in vitro and in vivo effect of tribendimidine and its metabolites against *Clonorchis sinensis*. *Parasitol. Res.* 105: 1497–1507.

106 Duthaler, U., Sayasone, S., Vanobbergen, F. et al. (2016). Single-ascending-dose pharmacokinetic study of tribendimidine in *Opisthorchis viverrini*-infected patients. *Antimicrob. Agents Chemother.* 60: 5705–5715.

107 Vanobberghen, F., Penny, M.A., Duthaler, U. et al. (2016). Population pharmacokinetic modeling of tribendimidine metabolites in *Opisthorchis viverrini*-infected adults. *Antimicrob. Agents Chemother.* 60: 5695–5704.

108 Kulke, D., Krücken, J., Welz, C. et al. (2012). In vivo efficacy of the anthelmintic tribendimidine against the cestode *Hymenolepis microstoma* in a controlled laboratory trial. *Acta Trop.* 123: 78–84.

109 Robertson, A.P., Puttachary, S., Buxton, S.K., and Martin, R.J. (2015). Tribendimidine: mode of action and nAChR subtype selectivity in *Ascaris* and *Oesophagostomum*. *PLoS Negl. Trop. Dis.* 9: e0003495.

110 Hu, Y., Xiao, S.H., and Aroian, R.V. (2009). The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. *PLoS Negl. Trop. Dis.* 3: e499.

111 Steinmann, P., Zhou, X.N., Du, Z.W. et al. (2008). Tribendimidine and albendazole for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp.: open-label randomized trial. *PLoS Negl. Trop. Dis.* 2: e322.

112 Sayasone, S., Odermatt, P., Vonghachack, Y. et al. (2016). Efficacy and safety of tribendimidine against *Opisthorchis viverrini*: two randomised, parallel-group, single-blind, dose-ranging, phase 2 trials. *Lancet Infect. Dis.* 16: 1145–1153.

113 Ren, H.N., Yang, Y.Q., Cheng, B.Z. et al. (1988). Toxicity of tribendimidin, a new anti-hookworm drug. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 6: 199–201. (Chinese).

114 Xiao, S.H., Hui-Ming, W., Tanner, M. et al. (2005). Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. *Acta Trop.* 94: 1–14.

115 Pungpark, S., Bunnag, D., and Harinasuta, T. Albendazole in the treatment of opisthorchiasis and concomitant intestinal helminthic infections. *Southeast Asian J. Trop. Med. Public Health* 15: 44–50.

116 Liu, Y.H., Wang, X.G., and Gao, P. (1991). Qian, M.X. Experimental and clinical trial of albendazole in the treatment of Clonorchiasis sinensis. *Chin. Med. J.* 104: 27–31.

117 Dugernier, T., Geubel, A., Bigaignon, G. et al. (1986). Human fascioliasis: cure by mebendazole? A case report. *Gastroenterol. Clin. Biol.* 10: 513–516.

118 Jaroonvesama, N., Charoenlarp, K., and Cross, J.H. (1981). Treatment of *Opisthorchis viverrini* with mebendazole. *Southeast Asian J. Trop. Med. Public Health.* 12: 595–597.

119 Chen, Y., Xu, G., Feng, Z. et al. (1997). Studies on efficacy of praziquantel and mebendazole-medicated salt in treatment of *Echinochasmus fujianensis* infection. *Southeast Asian J. Trop. Med. Public Health* 28: 344–346.

120 Chen, M.G. and Mott, K.E. (1990). Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. *Trop. Dis. Bull.* 87: R1–R38.

121 Hawn, T.R. and Jong, E.C. (1999). Update on hepatobiliary and pulmonary flukes. *Curr. Infect. Dis. Rep.* 1: 427–433.

122 Favennec, L., Jave Ortiz, J., Gargala, G. et al. (2003). Double-blind, randomized, placebo-controlled study of nitazoxanide in the treatment of fascioliasis in adults and children from northern Peru. *Aliment Pharmacol. Ther.* 17: 265–270.

123 Zumaquero-Ríos, J.L., Sarracent-Pérez, J., Rojas-García, R. et al. (2013). Fascioliasis and intestinal parasitoses affecting schoolchildren in Atlixco, Puebla State, Mexico: epidemiology and treatment with nitazoxanide. *PLoS Negl. Trop. Dis.* 7: e2553.

124 Keiser, J. and Utzinger, J. (2007). Advances in the discovery and development of trematocidal drugs. *Expert Opin. Drug Discovery* 2: S9–S23.

125 Panic, G., Duthaler, U., Speich, B., and Keiser, J. (2014). Repurposing drugs for the treatment and control of helminth infections. *Int. J. Parasitol. Drugs Drug. Resist.* 4: 185–200.

126 Vera-Montenegro, Y., Ibarra-Velarde, F., Ramirez-Avila, G., and Munguia-Xochihua, J. (2008). In vitro fasciolicide activity of some plant extracts against newly excysted flukes. *Ann. N.Y. Acad. Sci.* 1149: 180–182.

127 Abdel-Ghaffar, F., Semmler, M., Al-Rasheid, K.A. et al. (2011). The effects of different plant extracts on intestinal cestodes and on trematodes. *Parasitol. Res.* 10: 979–984.

128 Ferreira, J.F., Peaden, P., and Keiser, J. (2011). In vitro trematocidal effects of crude alcoholic extracts of *Artemisia annua, A. absinthium, Asimina triloba,* and *Fumaria officinalis*: trematocidal plant alcoholic extracts. *Parasitol. Res.* 109: 1585–1592.

129 Ibarra-Moreno, S., Ibarra-Velarde, F., and Ávila-Acevedo, J.G. (2010). Obtaining the minimum lethal dose against *Fasciola hepatica* in vitro using plant extract hexanes with fasciolicide activity and toxicity evaluation in CD1 male mice. *Am. J. Plant. Sci.* 3: 899–903.

130 Keiser, J., Vargas, M., and Winter, R. (2012). Anthelminthic properties of mangostin and mangostin diacetate. *Parasitol. Int.* 61: 369–371.

131 Thomsen, H., Reider, K., Franke, K. et al. (2012). Characterization of constituents and anthelmintic properties of *Hagenia abyssinica*. *Sci. Pharm.* 80: 433–446.

132 Edwards, J., Brown, M., Peak, E. et al. (2015). The diterpenoid 7-keto-sempervirol, derived from *Lycium chinense*, displays anthelmintic

activity against both *Schistosoma mansoni* and *Fasciola hepatica*. *PLoS Negl. Trop. Dis.* 9: e0003604.

133 Ajaiyeoba, E.O., Onocha, P.A., and Olarenwaju, O.T. (2001). In vitro anthelmintic properties of *Buchholzia coriaceae* and *Gynandropsis gynandra* extracts. *Pharm. Biol.* 39: 217–220.

134 Hegazi, A.G., Abd El Hady, F.K., and Shalaby, H.A. (2007). An in vitro effect of propolis on adult worms of *Fasciola gigantica*. *Vet. Parasitol.* 144: 279–286.

135 Saowakon, N., Tansatit, T., Wanichanon, C. et al. (2009). *Fasciola gigantica*: anthelmintic effect of the aqueous extract of *Artocarpus lakoocha*. *Exp. Parasitol.* 122: 289–298.

136 Singh, T.U., Kumar, D., Tandan, S.K., and Mishra, S.K. (2009). Inhibitory effect of essential oils of *Allium sativum* and *Piper longum* on spontaneous muscular activity of liver fluke, *Fasciola gigantica*. *Exp. Parasitol.* 123: 302–308.

137 Massoud, A.M., Shalaby, H.A., El Khateeb, R.M. et al. (2012). Effects of Mirazid and myrrh volatile oil on adult *Fasciola gigantica* under laboratory conditions. *Asian Pac. J. Trop. Biomed.* 2: 875–884.

138 Massoud, A.M., Shalaby, H.A., El Khateeb, R.M. et al. (2013). Tegumental histological effects of Mirazid and myrrh volatile oil on adult *Fasciola gigantica*. *Asian Pac. J. Trop. Biomed.* 3: 501–504.

139 Hossain, E., Chandra, G., Nandy, A.P. et al. (2013). Possible fasciocidal activity of methanol extract of *Dregea volubilis* leaves. *Exp. Parasitol.* 135: 183–187.

140 Lorsuwannarat, N., Piedrafita, D., Chantree, P. et al. (2014). The in vitro anthelmintic effects of plumbagin on newly excysted and 4-weeks-old juvenile parasites of *Fasciola gigantica*. *Exp. Parasitol.* 136: 5–13.

141 Shafiq, A., Kanwal, R., Ullah Qureshi, R., and Riaz Chaudhry, F. (2015). In vitro screening of *Cymbopogon jwarancusa* and *Conyza canadensis* against liver flukes. *Trop. Biomed.* 32: 407–412.

142 Roy, B. and Tandon, V. (1996). Effect of root-tuber extract of *Flemingia vestita*, a leguminous plant, on *Artyfechinostomum sufrartyfex* and *Fasciolopsis buski*: a scanning electron microscopy study. *Parasitol. Res.* 82: 248–252.

143 Kar, P.K. and Tandon, V. Anthelmintic efficacy of genistein, the active principle of *Flemingia vestita* (Fabaceae): alterations in the activity of the enzymes associated with the tegumental and gastrodermal interfaces of the trematode, *Fasciolopsis buski*. *J. Parasit. Dis.* 28: 45–56.

144 Kar, P.K., Tandon, V., and Saha, N. (2002). Anthelmintic efficacy of *Flemingia vestita*: genistein-induced effect on the activity of nitric oxide synthase and nitric oxide in the trematode parasite, *Fasciolopsis buski*. *Parasitol. Int.* 51: 249–257.

145 Kar, P.K., Tandon, V., and Saha, N. (2004). Anthelmintic efficacy of genistein, the active principle of *Flemingia vestita* (Fabaceae): alterations in the free amino acid pool and ammonia levels in the fluke, *Fasciolopsis buski*. *Parasitol. Int.* 53: 287–291.

146 Roy, B. and Tandon, V. (1997). In vitro flukicidal effect of leaf extract of *Cannabis sativa* Linn. on the trematode *Fasciolopsis buski*. *Ind. J. Exp. Biol.* 35: 80–82.

147 Roy, B., Dasgupta, S., and Tandon, V. (2009). Ultrastructural observations on *Fasciolopsis buski* and its alterations caused by shoot extract of *Alpinia nigra*. *Microsc. Res. Tech.* 72: 61–66.

148 Roy, B. and Swargiary, A. (2009). Anthelmintic efficacy of ethanolic shoot extract of *Alpinia nigra* on tegumental enzymes of *Fasciolopsis buski*, a giant intestinal parasite. *J. Parasit. Dis.* 33: 48–53.

149 Swargiary, A., Roy, B., Giri, B.R., and Ronghang, B. (2013). A comparative study on the anthelmintic efficacy of some medical plants of North-East India: alteration in th glycolytic enzymes of *Fasciolopsis buski*, a giant intestinal fluke. *Asian Pac. J. Trop. Med.* 6: 412–420.

150 Swargiary, A. and Roy, B. (2015). In vitro anthelmintic efficacy of *Alpinia nigra* and its bioactive compound, astragalin against *Fasciolopsis buski*. *Int. J. Pharm. Pharm. Sci.* 7: 30–35.

151 Challam, M., Roy, B., and Tandon, V. (2010). Effect of *Lysimachia ramosa* (Primulaceae) on helminth parasites: motility, mortality and scanning electron microscopic observations on surface topography. *Vet. Parasitol.* 169: 214–218.

152 Kone, W.M., Vargas, M., and Keiser, J. (2012). Anthelmintic activity of medicinal plants used in Cote d'Ivoire for treating parasitic diseases. *Parasitol. Res.* 110: 2351–2362.

153 Koko, W.S., Galal, M., and Khalid, H.S. (2000). Fasciolicidal efficacy of *Albizia anthelmintica* and *Balanites aegyptiaca* compared with albendazole. *J. Ethnopharmacol.* 71: 247–252.

154 Mahmoud, M.S., Abou-El Dobal, S., and Soliman, K. (2008). Immune response in *Fasciola gigantica* experimentally infected rabbits treated with either carnosine or Mirazid®. *Res. J. Parasitol.* 2008, 3: 40–49.

155 Abdelaal, M.M., Brennan, G.P., Abdel-Aziz, A., and Fairweather, I. (2016). Ultrastructural changes to the tegumental system and gastrodermal cells of adult *Fasciola hepatica* following treatment in vivo with a commercial preparation of myrrh (Mirazid). *J. Helminthol.* 20: 1–14.

156 Aukkanimart, R., Boonmars, T., Sriraj, P. et al. (2015). Anthelmintic, anti-inflammatory and antioxidant effects of *Garcinia mangostana* extract in hamster opisthorchiasis. *Exp. Parasitol.* 154: 5–13.

157 Cortés, A., García-Ferrús, M., Sotillo, J. et al. (2017). Effects of dietary intake of garlic on intestinal trematodes. *Parasitol. Res.* 116: 2119–2129.

158 Fathy, F.M., Salama, O., and Massoud, A.M. (2005). Effect of Mirazid (*Commiphora molmol*) on experimental heterophyidiasis. *J. Egypt. Soc. Parasitol.* 35: 1037–1050.

159 Keiser, J. (2010). In vitro and in vivo trematode models for chemotherapeutic studies. *Parasitology* 137: 589–603.

160 Panic, G., Ingram, K., and Keiser, J. (2013). Development of an in vitro drug sensitivity assay based on newly excysted larvae of *Echinostoma caproni*. *Parasit. Vectors* 6: 237.

161 O'Neill, J.F., Johnston, R.C., Halferty, L. et al. (2015). Ultrastructural changes in the tegument and gut of adult *Fasciola hepatica* following in vivo treatment with artesunate. *Exp. Parasitol.* 154: 143–154.

162 Geary, T.G., Sakanari, J.A., and Caffrey, C.R. (2015). Anthelmintic drug discovery: into the future. *J. Parasitol.* 101: 125–133.

163 Athanasiadou, S., Githiori, J., and Kyriazakis, I. (2007). Medicinal plants for helminth parasite control: facts and fiction. *Animal* 1: 1392–1400.

164 Keiser, J. and Utzinger, J. (2007b). Artemisinins ans synthetic trioxolanes in the treatment of helminth infections. *Curr. Opin. Infect. Dis.* 20: 605–612.

165 Barakat, R., Elmorshedy, H., and Fenwick, A. (2005). Efficacy of myrrh in the treatment of human *Schistosomiasis mansoni*. *Am. J. Trop. Med. Hyg.* 73: 365–367.

166 Abdul-Ghani, R.A., Loutfy, N., and Hassan, A. (2009). Myrrh and trematodoses in Egypt: an overview of safety, efficacy and effectiveness profiles. *Parasitol. Int.* 58: 210–214.

167 Hegab, M.H. and Hassan, R.M. (2003). Role of circulating *Fasciola* antigens and IgG4 isotype in assessment of cure from fascioliasis. *J. Egypt. Soc. Parasitol.* 33: 561–570.

168 Massoud, A., Morsy, T.A., and Haridy, F.M. (2003). Treatment of Egyptian dicrocoeliasis in man and animals with Mirazid. *J. Egypt. Soc. Parasitol.* 33: 337–442.

169 Massoud, A.M., El-Kholy, N.M., El-Shennawy, F.A., and Farag, R.E. (2004). Study of some immune aspects in patients with fascioliasis before and after *Chommiphora molmol* (Mirazid) treatment. *J. Egypt. Soc. Parasitol.* 34: 315–332.

170 Massoud, A.M., El-Shazly, A.M., and Morsy, T.A. (2007). Mirazid (*Commiphora molmol*) in treatment of human heterophyiasis. *J. Egypt. Soc. Parasitol.* 37: 395–410.

171 Massoud, A.M., El-Sherbini, E.T., Mos, N. et al. (2010). Mirazid in treatment of three zoonotic trematodes in Beni-Sweif and Dakhalia Governorates. *J. Egypt. Soc. Parasitol.* 40: 119–134.

172 Abo-Madyan, A.A., Morsy, T.A., Motawea, S.M., and Morsy, A.T. (2004). Clinical trial of Mirazid in treatment of human fascioliasis, Ezbet El-Bakly (Tamyia Center) Al-Fayoum Governorate. *J. Egypt. Soc. Parasitol.* 34: 807–818.

173 Al-Mathal, E.M. and Fouad, M.A. (2004). Myrrh (*Commiphora molmol*) in treatment of human and sheep dicrocoeliasis dendriticum in Saudi Arabia. *J. Egypt. Soc. Parasitol.* 34: 713–720.

174 Al-Mathal, E.M. and Fouad, M.A. (2005). Human fascioliasis among immigrant workers in Saudi Arabia. *J. Egypt. Soc. Parasitol.* 35: S1199–S1207.

175 Hassan, M.M., Abbaza, B.E., El-Karamany, I. et al. (2004). Detection of anti-*Fasciola* isotypes among patients with fascioliasis before and after treatment with Mirazid. *J. Egypt. Soc. Parasitol.* 34: 857–864.

176 Soliman, O.E., El-Arman, M., Abdul-Samie, E.R. et al. (2004). Evaluation of myrrh (Mirazid) therapy in fascioliasis and intestinal schistosomiasis in children: immunological and parasitological study. *J. Egypt. Soc. Parasitol.* 34: 941–966.

177 Ella, O.H. and Mohammad, A.E. (2015). *Dicrocoelium dendriticum* infection in a patient with chronic Schistosomiasis haematobium. *J. Egypt. Soc. Parasitol.* 45: 629–632.

178 Osman, M.M., El-Taweel, H.A., Shehab, A.Y., and Farag, H.F. (2010). Ineffectiveness of myrrh-derivative Mirazid against schistosomiasis and fascioliasis in humans. *East. Mediterr. Health J.* 16: 932–936.

Part IV

Bacteria

12

Buruli Ulcer

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12.1 Etiology and Epidemiology

Mycobacterium ulcerans is the causative agent of the neglected tropical skin disease Buruli ulcer (BU) [1]. BU occurs mainly in Central and West Africa, but the disease has also been reported from Australia/Oceania, Asia, and the Americas [2]. *M. ulcerans* has evolved from the ubiquitous waterborne organism *Mycobacterium marinum* by acquisition of a virulence plasmid [2, 3] encoding the enzymes required for the production of a polyketide-derived macrolide toxin, designated mycolactone [4]. *M. marinum* infects fish and amphibians and only occasionally causes granulomatous, self-limiting skin lesions in humans, indicating that the ability to produce mycolactone was a first step in the emergence of *M. ulcerans* as an infectious agent of increased virulence for humans. Genome reduction indicates that *M. ulcerans* is further adapting to a more stable ecological niche [5]. Two lineages have been distinguished among *M. ulcerans* strains isolated from human BU lesions [6]. The ancestral lineage found in patients from Asia, South America, and Mexico is associated with only sporadic disease cases in humans. The classical lineage is found in Africa and Australia, where local incidence rates can be >1/1000 per year. While the genomic diversity within disease isolates from different African countries is very limited, the Australian strains represent a distinct classical sub-lineage. The mode of transmission of *M. ulcerans* has not been fully elucidated so far [7, 8]. Direct human-to-human transmission seems to be rare. Occurrence of the disease is associated with slow-flowing and stagnant water bodies, and it is assumed that infection may derive from an environmental reservoir through trauma of the skin or insect bites. In Africa, the majority of patients with BU are children. The population age-adjusted prevalence shows a biphasic pattern, with the lowest prevalence in the <4-year-olds, and peak prevalences in the 12 to 14 age group and in the >50-year-olds [9]. In contrast, the median age of Australian patient cohorts from Queensland and Victoria is much higher (45 and 61 years, respectively) [10, 11]. Many individuals living in BU-endemic regions

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and exposed to *M. ulcerans* seem to seroconvert and do not develop clinical disease [12–14].

BU is characterized by indolent necrotizing skin lesions. *M. ulcerans* grows optimally at temperatures below the core body temperature, favoring infection of the skin. Mycolactone is the key factor in the pathogenesis of BU [4]. The toxin causes increased expression of the proapoptotic regulator Bim, driving the exposed mammalian cells into apoptosis [15]. At low concentrations, mycolactone affects many functions of tissue-resident macrophages and monocytes [16–18] and suppresses dendritic cell maturation [19–21]. Most BU lesions are located at the limbs; skin manifestations range from non-ulcerative (papules, nodules, plaques, edema) to ulcerative forms [22]. In established BU lesions, extracellular clusters of *M. ulcerans* are found in completely necrotic subcutaneous tissue [23]. However, in the early phase of the infection, there may be an intra-macrophage growth phase [24]. The necrotic core of early BU lesions is surrounded by a belt of infiltrating leukocytes, which seem to be unable to reach the extracellular bacteria surrounded by a protective cloud of mycolactone [25].

12.2 Current Treatments

While surgical excision has been the only treatment recommended for BU traditionally, the World Health Organization (WHO) published in 2004 a provisional guidance recommending a combination therapy of rifampicin (RIF) and streptomycin (STR) with daily applications for a period of eight weeks [26]. The current recommendation for treatment of BU in the WHO Guidance for Healthworkers is [27] as follows:

- *Standard antibiotic treatment:* Rifampicin at 10 mg/kg body weight by mouth daily for eight weeks and streptomycin at 15 mg/kg body weight by intramuscular injection daily for eight weeks (contraindicated in pregnancy).
- *Antibiotic treatment for pregnant women:* A pregnant patient in Benin was successfully treated with a combination of rifampicin and clarithromycin [28]. There were subsequently other reports of successful treatment with this combination. The recommendation, based on expert opinion, is therefore, rifampicin at 10 mg/kg body weight by mouth daily for eight weeks and clarithromycin at 7.5 mg/kg body weight by mouth twice daily for eight weeks. The extended-release formulation of clarithromycin may be used at 15 mg/kg body weight once daily, although it has yet to be tested.
- *Antibiotic treatment used in Australia* [29–31] *and French Guiana* [32]*:* The recommended treatment, based on vast clinical practice, is rifampicin at 10 mg/kg body weight by mouth daily for eight weeks and clarithromycin at 7.5 mg/kg body weight by mouth twice daily for eight weeks, or rifampicin at 10 mg/kg body weight by mouth once daily for eight weeks and moxifloxacin at 400 mg by mouth once daily for eight weeks (for adults only) [33].

Results of a randomized controlled trial with rifampicin and clarithromycin in Africa are expected to become available in 2019 and based on the results, it will most likely be recommended that STR be replaced by clarithromycin as standard therapy.

12.3 Unmet Needs

While the currently recommended standard combination therapy with RIF and STR for eight weeks [26, 27] is highly effective, there are several drawbacks associated with this relatively long and intense treatment. For the injection of STR, patients need to seek help in health care facilities; and injections are generally more prone to entail blood-borne infections. Furthermore, in particular, STR has strong side effects, including ototoxicity and nephrotoxicity [34, 35]. Therefore, STR is replaced by either clarithromycin or moxifloxacin in the treatment guidelines for Australia and French Guiana [27, 36]. Also, for African endemic countries, the WHO Technical Advisory Group for BU recently recommended that STR be replaced by clarithromycin and the switch be made to this fully oral treatment with fewer side effects.

RIF is the most effective bactericidal drug against *M. ulcerans* [37, 38], and there is currently no replacement in case RIF-resistant *M. ulcerans* strains emerge. Molecular assays for the detection of drug resistance mutations in the *rpoB* gene have been developed [39], but RIF-resistant clinical isolates with mutations in the *rpoB* gene have not been described so far. However, resistant strains have been isolated after RIF monotherapy in experimentally treated mice [40], and a study with 70 *M. ulcerans* isolates from Ghana revealed in 12 strains phenotypic resistance to RIF at a concentration of 40 μg/ml [41]. While the potential impact of RIF resistance on treatment success and the current level of RIF resistance among strains circulating in BU-endemic regions are unknown, it would in any case be desirable to have alternatives to RIF treatment in patients experiencing adverse effects such as hepatotoxicity or hypersensitivity reactions.

As with other mycobacterial diseases, there is a need for the development of novel, fast-acting, affordable antimycobacterial drugs with high potency, making shorter antibiotic treatment regimens possible. The new medication should be highly effective without severe side effects and should, in particular, be suitable for pediatric patients, since most patients with BU worldwide are children in the BU-endemic regions in Africa.

12.4 Diagnostics

Since no effective BU vaccine is available and transmission of *M. ulcerans* is not well understood, early case detection and rapid initiation of treatment are currently the key elements of BU control. BU can present in a variety of clinical manifestations, complicating the clinical diagnosis. The differential diagnosis of skin conditions with similar manifestations is broad [1, 42] and misclassification of clinically suspected cases is therefore not uncommon. Laboratory tests routinely used for diagnosis of BU include the microscopic detection of acid-fast bacilli (AFB) in stained smears from clinical specimens and detection of the *M. ulcerans*–specific insertion sequence (IS) element IS*2404* by PCR [43]. Also, histopathological analysis of sections from the affected tissue, revealing characteristic hallmarks of BU pathogenesis, can support diagnosis. Since *M. ulcerans* grows extremely slowly, primary cultivation of the bacteria

is only suitable for retrospective reconfirmation of clinical diagnosis. Of the established diagnostic methods, IS*2404* PCR is the most sensitive and specific, if performed under strict quality control [44, 45]. In comparison, the sensitivity of microscopic detection of acid-fast bacilli, the only diagnostic test that can be currently performed at the hospital level, is low. However, PCR requires sophisticated laboratory infrastructure and well-trained personnel and is, in resource-poor settings, usually only available at reference centers located far away from the rural BU-endemic areas of Africa. Therefore, the diagnosis of BU is often based on clinical judgment only and great efforts are made to develop a simple and rapid point-of-care diagnostic test for BU.

12.5 Discovery Models

12.5.1 *In Vitro* Test Formats

In general, laboratory work with *M. ulcerans* is challenging. First of all, with a generation time of two to four days, *M. ulcerans* is an extremely slow-growing mycobacterium, even when compared to *M. tuberculosis*, which has a doubling time of around 24 hours. Furthermore, *M. ulcerans* produces biofilms and its clumpy growth generates problems when it comes to the quantification of the bacteria, affecting the overall reproducibility of experiments. Methods to reduce the level of aggregation have been described [46, 47], but use of aggregated cultures may better reflect the situation in the host tissue, where bacteria are typically found in clusters [23].

Determination of minimum inhibitory concentrations (MICs) is considered the "gold standard" for the assessment of the susceptibility of microbes to antimicrobial agents and is often used to also evaluate the performance of other susceptibility testing methods. The MIC is defined as the lowest concentration of an antimicrobial that will inhibit the growth of an organism. Two different *in vitro* test formats are most commonly used for *M. ulcerans*: the agar dilution and the broth dilution method. In the agar dilution method, different concentrations of the antimicrobial substance are added to a nutrient agar medium followed by inoculation of the surface of the agar plate with a standardized number of cells. For the broth dilution method, bacteria are inoculated into a liquid growth medium in the presence of different concentrations of the antimicrobial agent. For this method, 96-well microtiter plates are commonly used. Bacterial growth is assessed after incubation for a defined period of time. For a first evaluation of the activity of new compounds, dilution steps down from 0.1 mg/ml are suitable. For MIC determinations, usually analyses with twofold dilution steps are accepted. The most common readouts are the determination of the number of colony-forming units (CFUs) on agar plates or the measurement of the metabolic activity of the bacterial suspension in growth medium.

CFU counting allows for the determination of the number of live bacterial cells (rather, of cell clusters in the case of *M. ulcerans*) that can give rise to bacterial colonies. Bacteria are either preincubated in liquid medium containing different concentrations of compound and plated out after different times of incubation or

plated directly onto both drug-free and drug-containing mycobacterial agar, such as 7H10 or 7H11. In the case of *M. ulcerans,* plates have to be subsequently incubated at permissive temperature (28–32 °C) for several months, which is raising issues with respect to compound stability. First *M. ulcerans* colonies can be found after about two months, but CFU counts may further increase after prolonged incubation. The required long incubation periods make CFU counting unsuitable for series of sequential tests, where new experiments are guided by the results of previous analyses.

The metabolic activity of *M. ulcerans* bacteria is most commonly analyzed in a 96-well plate format using the redox-sensitive reagent resazurin. After incubation of *M. ulcerans* with the serially diluted antimicrobial in growth medium for different times, resazurin is added, which is reduced by metabolically active cells into fluorescent resorufin. For *M. ulcerans*, determination of the metabolic activity with either wild-type bacteria or luminescent/GFP-tagged transfectants [48–50] is more practical than CFU counting, since various conditions can be tested within a shorter time period. However, since inhibition of the metabolic activity does not necessarily reflect bacterial death, this method cannot distinguish between bactericidal and bacteriostatic effects.

Also, agar disk-diffusion testing is used for antimicrobial susceptibility testing. Here, agar plates are inoculated with a standardized inoculum of the test microorganism and filter paper disks containing different concentrations of the test compound are placed on the agar surface. The antimicrobial agent diffuses into the agar and the formation of a clear inhibition zone around the filter paper suggests that the compound has bactericidal activity. The determination of the diameters of the inhibition growth zones yields qualitative results that are used to categorize microorganisms as susceptible, intermediate, or resistant. However, without the availability of stored algorithms, the method is not suitable to determine MIC values. For new compounds, the rate of diffusion into the agar is not known, and in the case of *M. ulcerans*, compound stability is also a major issue in view of the required long incubation periods.

12.5.2 *In Vivo* Testing

A range of host organisms, including reptiles (anole lizard [51, 52]) and mammals (armadillos [53], grass cutters [54], guinea pigs [55–57], mice, pigs [58, 59], and monkeys [60]) have been used for experimental infection studies with *M. ulcerans*. For *in vivo* drug testing, the mouse footpad infection model is most commonly used. Clinical testing of the efficacy of the RIF/STR combination treatment was encouraged by mouse footpad model studies [61–63], indicating that positive results in this model have predictive value for the human situation. Different experimental details, including different mouse and *M. ulcerans* strains, inoculation doses, and intervals between inoculation and start of treatment, have been used to test a broad range of compounds in the mouse model.

Progression of the infection and effectiveness of treatments are most commonly assessed by measuring footpad thickness. Also, recombinant bioluminescent *M. ulcerans* strains expressing luxAB from *Vibrio harveyi* that produce light by a luciferase-catalyzed reaction [64, 65] have been used for the

real-time evaluation of treatment efficacy *in vivo* [66]. A good correlation was found between the CFU counts and the measured luminescence in infected mouse footpads, suggesting that the produced bioluminescence represents a versatile marker for the detection of viable bacteria [66, 67]. Recently, optimized *M. ulcerans* strains expressing the entire luxCDABE operon were engineered. These recombinant strains are autoluminescent and do not require the addition of an exogenous substrate; they were successfully used to perform serial, noninvasive assessments of drug activities in live mice [68]. *Ex vivo*, the bacterial burden in footpads of the sacrificed animal has been analyzed by qPCR and by plating lysates to determine the number of CFUs. Also, histopathological analysis can give insights into treatment efficacy [15].

12.6 Testing of Compounds for Activity Against *M. ulcerans*

12.6.1 Preclinical Profiling of Currently Recommended Antibiotic Treatment Regimens for BU

That RIF has *in vitro* activity against *M. ulcerans* was shown in 1975 [69, 70]. Nevertheless, the combination treatment with RIF and STR was introduced only in 2004. Clinicians who anecdotally tried antibiotic treatment before were most likely discouraged by the apparent worsening of lesions during treatment, including progressive ulceration of primary lesions and the occasional development of new lesions before the onset of healing. In fact, >30% of patients enrolled in the BURULICO antimicrobial trial showed at week 8 (end of antibiotic treatment) an increase in lesion size as compared to week 6 [35]. Furthermore, 83% of non-ulcerative lesions ulcerated after start of treatment and 7% of the patients developed new lesions during or after treatment. While such paradoxical responses were historically misinterpreted as failure to respond to antibiotic treatment, mouse model studies (Table 12.1) strongly encouraged clinical evaluation and introduction of antibiotic therapies with RIF in combination with STR, clarithromycin, or moxifloxacin.

12.6.2 Repurposing of Tuberculosis Drug Candidates

For the identification of new scaffolds with activity against *M. ulcerans*, whole-cell-based assays rather than target-based drug discovery approaches have been used. In view of the major costs involved in drug discovery and development [84], development of new drugs for BU is not achievable under market economy conditions. However, drug repurposing [85], in particular that of new antibiotics under development for the treatment of tuberculosis, may be a viable strategy. Since repurposing is building on detailed information on pharmacology, formulation, and safety generated by previous research and development efforts, new candidate therapies could progress rapidly to efficacy testing for BU. As a slow-growing mycobacterium closely related to the *M. tuberculosis* complex [86], certain molecular drug targets may be conserved between *M. tuberculosis* and

Table 12.1 Preclinical profiling of antibiotics currently recommended for BU treatment (RIF, STR, clarithromycin, and moxifloxacin).

Publication	*In vitro* activity	*In vivo* activity in mouse models
Leach and Fenner 1954 [71]	Sensitive to 1 µg/ml STR	No effect of STR treatment of advanced lesions
Feldman and Karlson 1957 [72]		Limited efficacy of STR
Stanford and Philips 1972 [63]		Cure after 10 wk, but not after 4 wk of RIF treatment
Havel and Pattyn 1975 [69]	MIC of RIF 5 µg/ml	MED of RIF 10–15 mg/kg body weight/d
Krieg et al. 1979 [73]		RIF treatment was more effective in combination with heat treatment
Portaels et al. 1998 [74]	MIC of clarithromycin 0.125–2 µg/ml	
Thangaraj et al. 2000 [75]	MIC of RIF between 0.1 and 2 µg/ml	
Dega et al. 2000 [61]		Treatment with RIF, rifabutin, or amikacin 5 d a week was effective; treatment with clarithromycin, minocycline, and sparfloxacin was not effective
Bentoucha et al. 2001 [76]		With 4-wk treatment regimens, best results were obtained with a combination of RIF and amikacin
Dega et al. 2002 [62]		Twelve-week combination treatment with RIF and amikacin 5 d a week was curative and superior to a combination of RIF with clarithromycin and sparfloxacin
Marsollier et al. 2003 [40]		No relapses after treatment with a combination of RIF and amikacin. After RIF monotherapy, relapse with strains with higher MIC for RIF

(Continued)

Table 12.1 (Continued)

Publication	*In vitro* activity	*In vivo* activity in mouse models
Ji et al. 2006 [37]	MIC values: moxifloxacin < STR < RIF	After 8 wk of treatment, footpads of mice treated with RIF and combinations of RIF with STR, moxifloxacin, or amikacin were culture negative; taking into account the cost, potential toxicity, and availability, the combination of RIF with moxifloxacin was suggested for application in the field
Lefrancois et al. 2007 [77]		Eight weeks, but not four weeks, of combination treatment with RIF and STR or amikacin was curative
Ji et al. 2007 [38]		Eight-week treatment with RIF alone or RIF plus STR, clarithromycin, or moxifloxacin was sterilizing
Ji et al. 2008 [78]		Eight-week combination treatment with RIF and clarithromycin, STR, or moxifloxacin was sterilizing; the combination of moxifloxacin and clarithromycin was not sterilizing
Ji et al. 2009 [79]		No relapses after 8-wk daily treatment with a combination of RIF and STR, but not after treatment for 5 d a week
Almeida et al. 2011 [80]		Results suggest that an entirely oral daily treatment with rifapentin and clarithromycin may be as effective as treatment with RIF and STR
Almeida et al. 2013 [81]		Bactericidal activity of different combination treatment regimens with RIF or rifapentin and STR did not predict sterilizing activity (relapse-free cure)
Converse et al. 2015 [82]		No relapse after 6 wk of treatment with RIF and STR, 5% relapse with RIF and clofazimine, 50% relapse with RIF and clarithromycin
Chauffour et al. 2016 [83]		Sterilizing activity of twice per week combination treatment with rifapentin and clarithromycin, STR, or bedaquiline

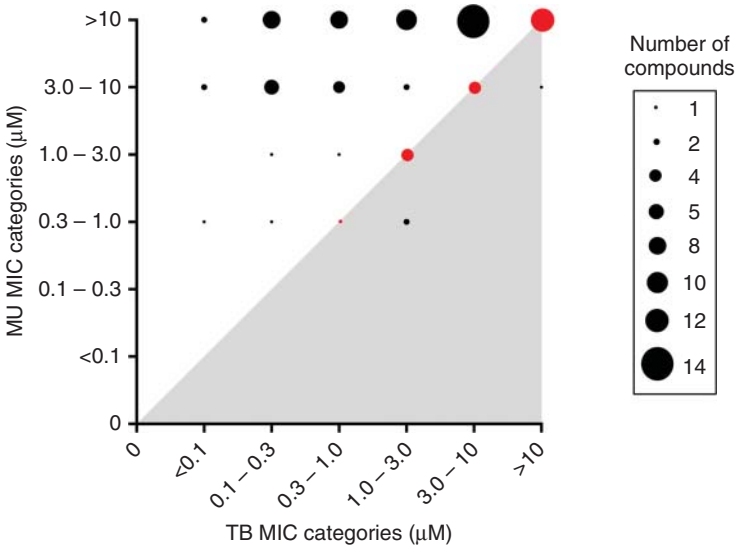

Figure 12.1 *M. tuberculosis* active compounds of different development stages were tested on *M. ulcerans* and MIC values for both pathogens were compared. Each compound is defined by a coordinate (MIC for *M. ulcerans* versus MIC for *M. tuberculosis*) and the size of the dot represents the number of compounds at a given coordinate. The gray triangle contains compounds that are more active against *M. ulcerans* than against *M. tuberculosis*, while the white triangle indicates the opposite. The four dots shown in red represent compounds which have the same activity against both mycobacterial species. Figure adapted from Ref. [88].

M. ulcerans [87]. In a recent study comparing activities of a chemically diverse set of 83 antimycobacterial agents against *M. tuberculosis* and *M. ulcerans*, it was found that most *M. tuberculosis* active compounds (in different stages of development) were inactive or only weakly active against *M. ulcerans* (Figure 12.1) [88].

The pronounced resistance of *M. ulcerans* to many compounds may in part be related to the highly hydrophobic extracellular matrix [89], but loss of drug target structures due to genome reduction [5] may also play a role. No correlation between different physicochemical properties (*C*log *P*, molecular weight, polar surface area, and ion class) of the compounds and the MIC values measured for *M. ulcerans* was found. While compounds with high activity against the *M. tuberculosis* complex are thus not necessarily suitable leads for *M. ulcerans* drug development, imidazopyridine amide (IPA) compounds, including the anti-tuberculosis drug candidate Q203 [90], targeting the respiratory cytochrome bc_1:aa_3 (cyt-bc_1:aa_3), showed outstanding activity against *M. ulcerans* [91]. Although the cyt-bc_1:aa_3 is the primary terminal oxidase in *M. tuberculosis*, the presence of an alternate *bd*-type terminal oxidase limits the sterilizing potency of IPA compounds. In contrast, *M. ulcerans* strains belonging to the classical lineage found in Africa and Australia have lost, in the course of reductive evolution, all alternate terminal electron acceptors. Since they, therefore, rely exclusively on the cyt-bc_1:aa_3 to respire, some IPA derivatives are

bactericidal at very low concentrations against *M. ulcerans*. Profiling of a series of IPA compounds for activity against *M. ulcerans* has revealed comparable structure–activity relationships for *M. ulcerans* and *M. tuberculosis* [91]. Q203 was the most active derivative with an outstanding MIC_{50} below 1 nM. While Q203 is only bacteriostatic for *M. tuberculosis,* it is bactericidal for *M. ulcerans*. The high *in vitro* activity of Q203 against *M. ulcerans* translated into high treatment efficacy in the experimental BU mouse footpad infection model, where an oral dose of 0.5 mg per kg body weight three times per week for four weeks was highly curative [91]. *M. ulcerans* mutant selection experiments showed that reversion to a functional cytochrome *bd* oxidase under Q203 pressure has a low probability to be selected.

12.6.3 Compound Screening

A broad range of other antimicrobials has been tested for activity against *M. ulcerans,* with a few showing promising properties (Table 12.2).

Recently, activity of avermectins against mycobacteria, including *M. tuberculosis* [102, 103], motivated testing against *M. ulcerans*. Avermectins are 16-membered macrocyclic lactones produced by *Streptomyces avermitilis* that show potent anthelmintic and insecticidal activity and are inexpensive, well-tolerated after oral/topical administration, and widely available. Some avermectins, indeed, showed activities in the low micromolar range against *M. ulcerans* [100, 101].

Patients in remote BU-endemic areas of Africa often first seek help from traditional healers and herbalists [104], who apply herbal remedies prepared from bark, roots, or leaves. The activity of extracts prepared from diverse medicinal plants has been tested against *M. ulcerans* [105–111], but only very few bioactive compounds with moderate activity have been identified so far [112, 113]. Nevertheless, herbal household remedies may contribute to healing of BU lesions [114].

12.7 Clinical Studies

Prior to the publication of the "Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer)" in 2004 [26], various treatment regimens have been evaluated anecdotally for BU treatment without encouraging results. Controlled clinical trials in humans have indicated that cotrimoxazole [115] and clofazimine [116] are both ineffective for treating ulcerative lesions. Furthermore, only limited efficacy has been reported for a RIF/dapsone combination treatment [117]. Encouraged by the efficacy of treatments with RIF in combination with STR, clarithromycin, or moxifloxacin in the mouse footpad model (Table 12.1), the WHO Advisory Group on BU recommended in 2000 to study the efficacy of the RIF/STR combination in humans. Etuaful et al. [118] treated patients with non-ulcerative BU lesions

Table 12.2 Selected results from the screening of diverse compound classes for activity against *M. ulcerans*

Publication	*In vitro* activity	*In vivo* activity in mouse models
Leach and Fenner 1954 [71]	Resistant against isoniazid and thiosemicarbazone	
Dega et al. 2000 [61]		Minocycline, sparfloxacin, and clarithromycin not effective compared to RIF, rifabutin, and amikacin
Thangaraj et al. 2000 [75]	Sparfloxacin inhibited most isolates at 0.25 μg/ml; all isolates were inhibited by amikacin and ciprofloxacin at 1 μg/ml and by ofloxacin at 2 μg/ml	
Bentoucha et al. 2001 [76]		No or limited activity of azithromycin, telitbromycin, sparfloxacin, and levofloxacin
Dhople 2001 [92]	MIC between 0.012 and 0.025 μg/ml for the benzoxacinorifamycin KRM-1648; synergy with ofloxacin	
Dhople 2001 [93]		Bactericidal activity of KRM-1648
Dhople 2001 [94]	Epiroprim most active dihydrofolate reductase inhibitor (MIC 0.5–1 μg/ml); synergy effects with dapsone	
Dhople and Namba 2002 [95]	MIC for sitafloxacin 0.125–0.5 μg/ml	
Ji et al. 2006 [37]	MIC values R207910 < moxifloxacin < STR < RIF < amikacin < linezolid < PA-824	No bactericidal activity with PA-824, some activity with R207910 and linezolid
Dubuisson et al. 2010 [96]	Limited activity of capuramycin analogs against *M. ulcerans*	
Scherr et al. 2012 [50]	Limited activity of antifungal azoles against *M. ulcerans*	
Cappoen et al. 2013 [97]	Low activity of bisbenzaldehydes against *M. ulcerans*	
Cappoen et al. 2014 [98]	Limited activity of diazene derivatives against *M. ulcerans*	
Cappoen et al. 2014 [99]	Activity of 1,3 diaryltriazenes against *M. ulcerans*	
Scherr et al. 2015 [100]	Of several avermectins tested, selamectin had the highest activity (MIC 2–4 μg/ml)	
Omansen et al. 2015 [101]	Limited activity of ivermectin and moxidectin	
Scherr et al. 2016 [88]	Most scaffolds from a library of compounds with activity against *M. tuberculosis* were inactive or only weakly active against *M. ulcerans*	

(nodules and plaques) for 2, 4, 8, or 12 weeks with RIF (10 mg/kg) and STR (15 mg/kg). Five patients received no treatment. Their lesions were excised at enrollment, whereas the lesions of patients who received treatment were excised after completion of drug therapy. While the lesions of the patients who received no treatment and the patients who received only two weeks of drug treatment were culture-positive after excision, all others were negative. These encouraging results motivated further clinical studies. In a case series of 215 patients with BU from Benin in 2003/2004, encouraging results were obtained with eight weeks of RIF/STR combination therapy. Only three patients had a recurrence one year after completion of treatment [119]. An open-label, randomized clinical trial conducted between 2006 and 2008 in Ghana compared RIF/STR treatment for eight weeks with RIF plus intramuscular STR for four weeks followed by RIF plus oral clarithromycin (7.5 mg/kg) for four weeks. After one year, 96% of the patients in the RIF/STR group and 91% in the RIF/STR-clarithromycin group had healed lesions [120]. A pure oral treatment with RIF (10 mg/kg) plus clarithromycin (12 mg/kg) once daily for eight weeks was evaluated in 2007–2009 in 30 patients with BU from Benin [121]. All lesions healed and there were no relapses after 1.5 years. Results of a randomized controlled trial with RIF/clarithromycin are expected to become available in 2019.

M. ulcerans does not grow at the human core body temperature. Meyers et al. showed in the 1970s that this thermosensitivity makes thermotherapy a valid treatment option [122]. Due to the lack of a simple thermotherapy device, this therapeutic approach was not followed up for a long time. However, more recently it has been shown that inexpensive reusable phase-change material devices available as commercial pocket heat pads can be used to trigger suitable local hyperthermia and that heat treatment with these simple rechargeable pads is effective [123, 124]. A combination of chemotherapy and thermotherapy may be an attractive future approach to reduce the duration of specific BU therapy [73].

BU lesions frequently show a transient worsening during treatment [125]. This may be related to the repulsion of necrotic tissue [126] or secondary infection [127], but may occasionally also represent an immune reconstitution inflammatory syndrome [128, 129]. During antibiotic treatment, massive leukocyte infiltration of lesions is observed and ectopic organized lymphoid tissue is developing [130, 131], but this immune reconstitution does not necessarily lead to "paradoxical reactions," since it does not seem to interfere with wound healing in most patients [131]. After successful completion of specific treatment, small BU lesions may close rapidly with the development of a stable scar. Large wounds may require debridement and skin grafting [1].

12.8 Future Directions and Opportunities

BU is one of the infectious diseases designated by the World Health Organization as a neglected tropical disease (NTD). While for some of these diseases, nonprofit drug research and development organizations, such as the Drugs for Neglected Diseases initiative (DNDi) support the development of new drugs,

there are only sporadic efforts toward drug development for BU. When taking the cost of failure into account, development of a new drug is estimated to cost several hundreds of million dollars. Overall funding for BU research and development in 2017 was $2.9 million, with 46% of this amount targeted towards basic research and 42% towards drug development [132]. Within product (drugs, vaccines, and diagnostics) development, each area received less than $1 million. Since overall drug research and development for BU, including the screening of drug candidates, progression from hits to leads, lead optimization, and preclinical and clinical studies, is thus not achievable, repurposing of drugs under development for other diseases, in particular tuberculosis, appears currently to be the only viable approach. Recent evidence indicates that an IPA-based drug, like the tuberculosis development candidate Q203, could both simplify and shorten BU treatment, an assumption that should be tested in human clinical trials.

References

1 Junghanss, T., Johnson, R.C., and Pluschke, G. (2014). *Mycobacterium ulcerans* disease. In: *Manson's Tropical Diseases*, 519–531. Edinburgh: Saunders Ltd.

2 Roeltgen, K. and Pluschke, G. (2015). Epidemiology and disease burden of Buruli ulcer: a review. *Res. Rep. Trop. Med. (Dovepress)* 6: 59–73.

3 Doig, K.D., Holt, K.E., Fyfe, J.A. et al. (2012). On the origin of *Mycobacterium ulcerans*, the causative agent of buruli ulcer. *BMC Genom.* 13: 258.

4 George, K.M., Chatterjee, D., Gunawardana, G. et al. (1999). Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. *Science* 283: 854–857.

5 Stinear, T.P., Seemann, T., Pidot, S. et al. (2007). Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. *Genome Res.* 17: 192–200.

6 Käser, M., Rondini, S., Naegeli, M. et al. (2007). Evolution of two distinct phylogenetic lineages of the emerging human pathogen *Mycobacterium ulcerans*. *BMC Evol. Biol.* 7: 177.

7 Merritt, R.W., Walker, E.D., Small, P.L. et al. (2010). Ecology and transmission of Buruli ulcer disease: a systematic review. *PLoS Negl. Trop. Dis.* 4: e911.

8 Röltgen, K. and Pluschke, G. (2015). *Mycobacterium ulcerans* disease (Buruli ulcer): potential reservoirs and vectors. *Curr. Clin. Microbiol. Rep.* 2: 35–43.

9 Bratschi, M.W., Bolz, M., Minyem, J.C. et al. (2013). Geographic distribution, age pattern and sites of lesions in a cohort of Buruli ulcer patients from the Mapé Basin of Cameroon. *PLoS Negl. Trop. Dis.* 7: e2252.

10 Boyd, S.C., Athan, E., Friedman, N.D. et al. (2012). Epidemiology, clinical features and diagnosis of *Mycobacterium ulcerans* in an Australian population. *Med. J. Aust.* 196: 341–344.

11 O'Brien, D.P., Friedman, N.D., Cowan, R. et al. (2015). *Mycobacterium ulcerans* in the elderly: more severe disease and suboptimal outcomes. *PLoS Negl. Trop. Dis.* 9: e0004253.

12 Diaz, D., Döbeli, H., Yeboah-Manu, D. et al. (2006). Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. *Clin. Vaccine Immunol.* 13: 1314–1321.
13 Röltgen, K., Bratschi, M.W., Ross, A. et al. (2014). Late onset of the serological response against the 18 kDa small heat shock protein of *Mycobacterium ulcerans* in children. *PLoS Negl. Trop. Dis.* 8: e2904.
14 Yeboah-Manu, D., Röltgen, K., Opare, W. et al. (2012). Sero-epidemiology as a tool to screen populations for exposure to *Mycobacterium ulcerans*. *PLoS Negl. Trop. Dis.* 6: e1460.
15 Bieri, R., Scherr, N., Ruf, M.T. et al. (2017). The macrolide toxin mycolactone promotes bim-dependent apoptosis in buruli ulcer through inhibition of mTOR. *ACS Chem. Biol.* 12: 1297–1307.
16 Simmonds, R.E., Lali, F.V., Smallie, T. et al. (2009). Mycolactone inhibits monocyte cytokine production by a posttranscriptional mechanism. *J. Immunol.* 182: 2194–2202.
17 Torrado, E., Fraga, A.G., Logarinho, E. et al. (2010). IFN-gamma-dependent activation of macrophages during experimental infections by *Mycobacterium ulcerans* is impaired by the toxin mycolactone. *J. Immunol.* 184: 947–955.
18 Fraga, A.G., Cruz, A., Martins, T.G. et al. (2011). *Mycobacterium ulcerans* triggers T-cell immunity followed by local and regional but not systemic immunosuppression. *Infect. Immun.* 79: 421–430.
19 Pahlevan, A.A., Wright, D.J., Andrews, C. et al. (1999). The inhibitory action of *Mycobacterium ulcerans* soluble factor on monocyte/T cell cytokine production and NF-kappa B function. *J. Immunol.* 163: 3928–3935.
20 Coutanceau, E., Decalf, J., Martino, A. et al. (2007). Selective suppression of dendritic cell functions by *Mycobacterium ulcerans* toxin mycolactone. *J. Exp. Med.* 204: 1395–1403.
21 Boulkroun, S., Guenin-Macé, L., Thoulouze, M. et al. (2009). Mycolactone suppresses T cell responsiveness by altering both early signaling and posttranslational events. *J. Immunol.*
22 van der Werf, T.S., van der Graaf, W.T., Tappero, J.W., and Asiedu, K. (1999). *Mycobacterium ulcerans* infection. *Lancet* 354: 1013–1018.
23 Ruf, M.T., Bolz, M., Vogel, M. et al. (2016). Spatial distribution of *Mycobacterium ulcerans* in Buruli ulcer lesions: implications for laboratory diagnosis. *PLoS Negl. Trop. Dis.* 10: e0004767.
24 Torrado, E., Fraga, A.G., Castro, A.G. et al. (2007). Evidence for an intramacrophage growth phase of *Mycobacterium ulcerans*. *Infect. Immun.* 75: 977–987.
25 Ruf, M.T., Steffen, C., Bolz, M. et al. (2017). Infiltrating leukocytes surround early Buruli ulcer lesions, but are unable to reach the mycolactone producing mycobacteria. *Virulence* 1–9.
26 World Health Organization, Geneva (2004). Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer).
27 World Health Organization, Geneva (2012). Treatment of *Mycobacterium ulcerans* Disease (Buruli ulcer): Guidance for Health Workers.

28 Dossou, A.D., Sopoh, G.E., Johnson, C.R. et al. (2008). Management of *Mycobacterium ulcerans* infection in a pregnant woman in Benin using rifampicin and clarithromycin. *Med. J. Aust.* 189: 532–533.

29 Johnson, P.D., Hayman, J.A., Quek, T.Y. et al. (2007). Consensus recommendations for the diagnosis, treatment and control of *Mycobacterium ulcerans* infection (Bairnsdale or Buruli ulcer) in Victoria, Australia. *Med. J. Aust.* 186: 64–68.

30 Gordon, C.L., Buntine, J.A., Hayman, J.A. et al. (2010). All-oral antibiotic treatment for buruli ulcer: a report of four patients. *PLoS Negl. Trop. Dis.* 4: e770.

31 O'Brien, D.P., McDonald, A., Callan, P. et al. (2012). Successful outcomes with oral fluoroquinolones combined with rifampicin in the treatment of *Mycobacterium ulcerans*: an observational cohort study. *PLoS Negl. Trop. Dis.* 6: e1473.

32 Couppié, P.; Dufour, J.; Papot, E.; Hotz, C.; Guédénon, F.; Fior, A.; Queuche, F.; Sainte-Marie, D. (2010). *Mycobacterium ulcerans* (Buruli ulcer) disease in French Guiana: development of therapeutic practice in the period 2004-2009. *WHO Annual Meeting on Buruli Ulcer,* Geneva, Switzerland (22–24 March 2010); Vol. 66.

33 World Health Organization, Geneva (2011). Fluoroquinolone use in pediatrics: focus on safety and place in therapy. *Report of the 18th Expert Committee on the Selection and Use of Essential Medicines*.

34 Chan-Tompkins, N.H. (1995). Toxic effects and drug interactions of antimycobacterial therapy. *Clin. Dermatol.* 13: 223–233.

35 Klis, S., Stienstra, Y., Phillips, R.O. et al. (2014). Long term streptomycin toxicity in the treatment of Buruli ulcer: follow-up of participants in the BURULICO drug trial. *PLoS Negl. Trop. Dis.* 8: e2739.

36 Friedman, N.D., Athan, E., Walton, A.L., and O'Brien, D.P. (2016). Increasing experience with primary oral medical therapy for *Mycobacterium ulcerans* disease in an Australian cohort. *Antimicrob. Agents Chemother.* 60: 2692–2695.

37 Ji, B., Lefrançois, S., Robert, J. et al. (2006). *In vitro* and *in vivo* activities of rifampin, streptomycin, amikacin, moxifloxacin, R207910, linezolid, and PA-824 against *Mycobacterium ulcerans*. *Antimicrob. Agents Chemother.* 50: 1921–1926.

38 Ji, B., Chauffour, A., Robert, J. et al. (2007). Orally administered combined regimens for treatment of *Mycobacterium ulcerans* infection in mice. *Antimicrob. Agents Chemother.* 51: 3737–3739.

39 Jansson, M., Beissner, M., Phillips, R.O. et al. (2014). Comparison of two assays for molecular determination of rifampin resistance in clinical samples from patients with Buruli ulcer disease. *J. Clin. Microbiol.* 52: 1246–1249.

40 Marsollier, L., Honoré, N., Legras, P. et al. (2003). Isolation of three *Mycobacterium ulcerans* strains resistant to rifampin after experimental chemotherapy of mice. *Antimicrob. Agents Chemother.* 47: 1228–1232.

41 Owusu, E., Newman, M.J., Kotey, N.K. et al. (2016). Susceptibility profiles of *Mycobacterium ulcerans* isolates to streptomycin and rifampicin in two districts of the Eastern region of Ghana. *Int. J. Microbiol.* 2016: 8304524.

42 Toutous Trellu, L., Nkemenang, P., Comte, E. et al. (2016). Differential diagnosis of skin ulcers in a *Mycobacterium ulcerans* endemic area: data from a prospective study in Cameroon. *PLoS Negl. Trop. Dis.* 10: e0004385.

43 Portaels, F. (2014). Laboratory diagnosis of Buruli ulcer: a manual for health care providers. *World Health Organization, Geneva*.

44 Lavender, C.J. and Fyfe, J.A. (2013). Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. *Methods Mol. Biol.* 943: 201–216.

45 Eddyani, M., Lavender, C., de Rijk, W.B. et al. (2014). Multicenter external quality assessment program for PCR detection of *Mycobacterium ulcerans* in clinical and environmental specimens. *PLoS One* 9: e89407.

46 Aung, T.T., Yam, J.K., Lin, S. et al. (2015). Biofilms of pathogenic nontuberculous mycobacteria targeted by new therapeutic approaches. *Antimicrob. Agents Chemother.* 60: 24–35.

47 Aung, T.T., Chor, W.H.J., Yam, J.K.H. et al. (2017). Discovery of novel antimycobacterial drug therapy in biofilm of pathogenic nontuberculous mycobacterial keratitis. *Ocul. Surf.* 15: 770–783.

48 Franzblau, S.G., Witzig, R.S., McLaughlin, J.C. et al. (1998). Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. *J. Clin. Microbiol.* 36: 362–366.

49 Hamid, R., Rotshteyn, Y., Rabadi, L. et al. (2004). Comparison of alamar blue and MTT assays for high through-put screening. *Toxicol. in Vitro* 18: 703–710.

50 Scherr, N., Röltgen, K., Witschel, M., and Pluschke, G. (2012). Screening of antifungal azole drugs and agrochemicals with an adapted alamarBlue-based assay demonstrates antibacterial activity of croconazole against *Mycobacterium ulcerans*. *Antimicrob. Agents Chemother.* 56: 6410–6413.

51 Marcus, L., Stottmeier, K., and Morrow, R. (1975). Experimental infection of anole lizards (Anolis carolinensis) with *Mycobacterium ulcerans* by the subcutaneous route. *Am. J. Trop. Med. Hyg.* 24: 649–655.

52 Marcus, L., Stottmeier, K., and Morrow, R. (1976). Experimental alimentary infection of anole lizards (Anolis carolinensis) with *Mycobacterium ulcerans*. *Am. J. Trop. Med. Hyg.* 25: 630–632.

53 Walsh, D.S., Meyers, W.M., Krieg, R.E., and Walsh, G.P. (1999). Transmission of *Mycobacterium ulcerans* to the nine-banded armadillo. *Am. J. Trop. Med. Hyg.* 61: 694–697.

54 Addo, P., Adu-Addai, B., Quarty, M. et al. (2006). Clinical and histopathological presentation of Buruli ulcer in experimentally infected grasscutters (Thryonomys swindarius). *Int. J. Trop. Med.* 3.

55 Read, J., Heggie, C., Meyers, W., and Connor, D. (1974). Cytotoxic activity of *Mycobacterium ulcerans*. *Infect. Immun.* 1114–1122.

56 Silva-Gomes, R., Marcq, E., Trigo, G. et al. (2015). Spontaneous healing of *Mycobacterium ulcerans* lesions in the guinea pig model. *PLoS Negl. Trop. Dis.* 9: e0004265.

57 Williamson, H.R., Mosi, L., Donnell, R. et al. (2014). *Mycobacterium ulcerans* fails to infect through skin abrasions in a guinea pig infection model: implications for transmission. *PLoS Negl. Trop. Dis.* 8: e2770.

58 Bolz, M., Ruggli, N., Ruf, M.T. et al. (2014). Experimental infection of the pig with *Mycobacterium ulcerans*: a novel model for studying the pathogenesis of Buruli ulcer disease. *PLoS Negl. Trop. Dis.* 8: e2968.

59 Bolz, M., Ruggli, N., Borel, N. et al. (2016). Local cellular immune responses and pathogenesis of Buruli ulcer lesions in the experimental *Mycobacterium ulcerans* pig infection model. *PLoS Negl. Trop. Dis.* 10: e0004678.

60 Walsh, D.S., Dela Cruz, E.C., Abalos, R.M. et al. (2007). Clinical and histologic features of skin lesions in a cynomolgus monkey experimentally infected with *mycobacterium ulcerans* (Buruli ulcer) by intradermal inoculation. *Am. J. Trop. Med. Hyg.* 76: 132–134.

61 Dega, H., Robert, J., Bonnafous, P. et al. (2000). Activities of several antimicrobials against *Mycobacterium ulcerans* infection in mice. *Antimicrob. Agents Chemother.* 44: 2367–2372.

62 Dega, H., Bentoucha, A., Robert, J. et al. (2002). Bactericidal activity of rifampin-amikacin against *Mycobacterium ulcerans* in mice. *Antimicrob. Agents Chemother.* 46: 3193–3196.

63 Stanford, J.L. and Phillips, R. (1972). Rifampicin in experimental *Mycobacterium ulcerans* infection. *J. Med. Microbiol.* 5: 39–45.

64 Andreu, N., Zelmer, A., Fletcher, T. et al. (2010). Optimisation of bioluminescent reporters for use with mycobacteria. *PLoS One* 5: e10777.

65 Andreu, N., Zelmer, A., Sampson, S.L. et al. (2013). Rapid *in vivo* assessment of drug efficacy against Mycobacterium tuberculosis using an improved firefly luciferase. *J. Antimicrob. Chemother.* 68: 2118–2127.

66 Zhang, T., Li, S.Y., Converse, P.J. et al. (2011). Using bioluminescence to monitor treatment response in real time in mice with *Mycobacterium ulcerans* infection. *Antimicrob. Agents Chemother.* 55: 56–61.

67 Zhang, T., Bishai, W.R., Grosset, J.H., and Nuermberger, E.L. (2010). Rapid assessment of antibacterial activity against *Mycobacterium ulcerans* by using recombinant luminescent strains. *Antimicrob. Agents Chemother.* 54: 2806–2813.

68 Zhang, T., Li, S.Y., Converse, P.J. et al. (2013). Rapid, serial, non-invasive assessment of drug efficacy in mice with autoluminescent *Mycobacterium ulcerans* infection. *PLoS Negl. Trop. Dis.* 7: e2598.

69 Havel, A. and Pattyn, S.R. (1975). Activity of rifampicin on *Mycobacterium ulcerans*. *Ann. Soc. Belg. Med. Trop.* 55: 105–108.

70 Ullmann, U., Schubert, G.E., and Kieninger, G. (1975). Bacteriological investigations and animal experiments with *Mycobacterium ulcerans* (Tübingen 1971) (author's trans.). *Zentralbl. Bakteriol. Orig. A* 232: 318–327.

71 Leach, R.H. and Fenner, F. (1954). Studies on *Mycobacterium ulcerans* and *Mycobacterium balnei*. III. Growth in the semi-synthetic culture media of Dubos and drug sensitivity *in vitro* and *in vivo*. *Aust. J. Exp. Biol. Med. Sci.* 32: 835–852.

72 Feldman, W.H. and Karlson, A.G. (1957). *Mycobacterium ulcerans* infections; response to chemotherapy in mice. *Am. Rev. Tuberc.* 75: 266–279.

73 Krieg, R.E., Wolcott, J.H., and Meyers, W.M. (1979). *Mycobacterium ulcerans* infection: treatment with rifampin, hyperbaric oxygenation, and heat. *Aviat. Space Environ. Med.* 50: 888–892.

74 Portaels, F., Traore, H., De Ridder, K., and Meyers, W.M. (1998). *In vitro* susceptibility of *Mycobacterium ulcerans* to clarithromycin. *Antimicrob. Agents Chemother.* 42: 2070–2073.

75 Thangaraj, H.S., Adjei, O., Allen, B.W. et al. (2000). In vitro activity of ciprofloxacin, sparfloxacin, ofloxacin, amikacin and rifampicin against Ghanaian isolates of *Mycobacterium ulcerans*. *J. Antimicrob. Chemother.* 45: 231–233.

76 Bentoucha, A., Robert, J., Dega, H. et al. (2001). Activities of new macrolides and fluoroquinolones against *Mycobacterium ulcerans* infection in mice. *Antimicrob. Agents Chemother.* 45: 3109–3112.

77 Lefrançois, S., Robert, J., Chauffour, A. et al. (2007). Curing *Mycobacterium ulcerans* infection in mice with a combination of rifampin-streptomycin or rifampin-amikacin. *Antimicrob. Agents Chemother.* 51: 645–650.

78 Ji, B., Chauffour, A., Robert, J., and Jarlier, V. (2008). Bactericidal and sterilizing activities of several orally administered combined regimens against *Mycobacterium ulcerans* in mice. *Antimicrob. Agents Chemother.* 52: 1912–1916.

79 Ji, B., Chauffour, A., Aubry, A. et al. (2009). Impacts of dosing frequency of the combination rifampin-streptomycin on its bactericidal and sterilizing activities against *Mycobacterium ulcerans* in mice. *Antimicrob. Agents Chemother.* 53: 2955–2959.

80 Almeida, D., Converse, P.J., Ahmad, Z. et al. (2011). Activities of rifampin, Rifapentine and clarithromycin alone and in combination against *mycobacterium ulcerans* disease in mice. *PLoS Negl.Trop. Dis.* 5: e933.

81 Almeida, D.V., Converse, P.J., Li, S.Y. et al. (2013). Bactericidal activity does not predict sterilizing activity: the case of rifapentine in the murine model of *Mycobacterium ulcerans* disease. *PLoS Negl.Trop. Dis.* 7: e2085.

82 Converse, P.J., Tyagi, S., Xing, Y. et al. (2015). Efficacy of rifampin plus clofazimine in a murine model of *Mycobacterium ulcerans* disease. *PLoS Negl. Trop. Dis.* 9: e0003823.

83 Chauffour, A., Robert, J., Veziris, N. et al. (2016). Sterilizing activity of fully oral intermittent regimens against *Mycobacterium ulcerans* infection in mice. *PLoS Negl. Trop. Dis.* 10: e0005066.

84 DiMasi, J.A., Hansen, R.W., and Grabowski, H.G. (2003). The price of innovation: new estimates of drug development costs. *J. Health. Econ.* 22: 151–185.

85 Chong, C.R. and Sullivan, D.J. (2007). New uses for old drugs. *Nature* 448: 645–646.

86 Gey van Pittius, N.C., Sampson, S.L., Lee, H. et al. (2006). Evolution and expansion of the Mycobacterium tuberculosis PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions. *BMC Evol. Biol.* 6: 95.

87 Potet, J., Casenghi, M., Charitha, G. et al. (2013). Buruli ulcers: an advocacy agenda to improve its control. In: *Med Sante Trop*, vol. 23, 227–228.

88 Scherr, N., Pluschke, G., and Panda, M. (2016). Comparative study of activities of a diverse set of antimycobacterial agents against *Mycobacterium*

tuberculosis and *Mycobacterium ulcerans*. *Antimicrob. Agents Chemother.* 60: 3132–3137.

89 Marsollier, L., Brodin, P., Jackson, M. et al. (2007). Impact of *Mycobacterium ulcerans* biofilm on transmissibility to ecological niches and Buruli ulcer pathogenesis. *PLoS Pathog.* 3: e62.

90 Pethe, K. et al. (2013). Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat. Med.* 19: 1157–1160.

91 Scherr, N., Bieri, R., Thomas, S.S. et al. (2018). Pethe K. *Nat. Commun.* 9 (1): 5370. https://doi.org/10.1038/s41467-018-07804-8.

92 Dhople, A.M. (2001). In vitro activity of KRM-1648, either singly or in combination with ofloxacin, against *Mycobacterium ulcerans*. *Int. J. Antimicrob. Agents* 17: 57–61.

93 Dhople, A.M. (2001). *In vivo* susceptibility of *Mycobacterium ulcerans* to KRM-1648, a new benzoxazinorifamycin, in comparison with rifampicin. Anti-mycobacterial activity of KRM-1648. *Arzneim.-Forsch.* 51: 501–505.

94 Dhople, A.M. (2001). Antimicrobial activities of dihydrofolate reductase inhibitors, used singly or in combination with dapsone, against *Mycobacterium ulcerans*. *J. Antimicrob. Chemother.* 47: 93–96.

95 Dhople, A.M. and Namba, K. (2002). *In vitro* activity of sitafloxacin (DU-6859a) alone, or in combination with rifampicin, against *Mycobacterium ulcerans*. *J. Antimicrob. Chemother.* 50: 727–729.

96 Dubuisson, T., Bogatcheva, E., Krishnan, M.Y. et al. (2010). *In vitro* antimicrobial activities of capuramycin analogues against non-tuberculous mycobacteria. *J. Antimicrob. Chemother.* 65: 2590–2597.

97 Cappoen, D., Forge, D., Vercammen, F. et al. (2013). Biological evaluation of bisbenzaldehydes against four Mycobacterium species. *Eur. J. Med. Chem.* 63: 731–738.

98 Cappoen, D., Majce, V., Uythethofken, C. et al. (2014). Biological evaluation of diazene derivatives as anti-tubercular compounds. *Eur. J. Med. Chem.* 74: 85–94.

99 Cappoen, D., Vajs, J., Uythethofken, C. et al. (2014). Anti-mycobacterial activity of 1,3-diaryltriazenes. *Eur. J. Med. Chem.* 77: 193–203.

100 Scherr, N., Pluschke, G., Thompson, C.J., and Ramón-García, S. (2015). Selamectin is the avermectin with the best potential for buruli ulcer treatment. *PLoS Negl. Trop. Dis.* 9: e0003996.

101 Omansen, T.F., Porter, J.L., Johnson, P.D. et al. (2015). *In-vitro* activity of avermectins against *Mycobacterium ulcerans*. *PLoS Negl. Trop. Dis.* 9: e0003549.

102 Lim, L.E., Vilchèze, C., Ng, C. et al. (2013). Anthelmintic avermectins kill *Mycobacterium tuberculosis*, including multidrug-resistant clinical strains. *Antimicrob. Agents Chemother.* 57: 1040–1046.

103 Ramón-García, S., Vilchèze, C., Lim, L.E. et al. (2014). Measurements of the *in vitro* anti-mycobacterial activity of ivermectin are method-dependent. *J. Antimicrob. Chemother.* 69: 1723–1724.

104 Ackumey, M.M., Gyapong, M., Pappoe, M. et al. (2012). Socio-cultural determinants of timely and delayed treatment of Buruli ulcer: implications for disease control. *Infect. Dis. Poverty* 1: 6.

105 Tsouh Fokou, P.V., Kissi-Twum, A.A., Yeboah-Manu, D. et al. (2016). Antimycobacterial ingredients from plants used in traditional medicine to treat Buruli ulcer. *Int. J. Mycobacteriol.* 5 (Suppl 1): S204–S205.

106 Tsouh Fokou, P.V., Appiah-Opong, R., Yeboah-Manu, D. et al. (2016). Antimycobacterial potency and cytotoxicity study of three medicinal plants. *Int. J. Mycobacteriol.* 5 (Suppl 1): S206–S207.

107 Tsouh Fokou, P.V., Kissi-Twum, A.A., Yeboah-Manu, D. et al. (2016). *In vitro* activity of selected West African medicinal plants against *Mycobacterium ulcerans* disease. *Molecules* 21: 445.

108 Keumoe, R., Nguembou, M.S., Tsouh, F.P. et al. (2016). Antimycobacterial activity of medicinal plants against the causative agent of buruli ulcer: *Mycobacterium ulcerans*. *Int. J. Mycobacteriol.* 5 Suppl 1: S105.

109 Yemoa, A., Gbenou, J., Affolabi, D. et al. (2011). Buruli ulcer: a review of in vitro tests to screen natural products for activity against *Mycobacterium ulcerans*. *Planta Med.* 77: 641–646.

110 Tsouh Fokou, P.V., Nyarko, A.K., Appiah-Opong, R. et al. (2015). Ethnopharmacological reports on anti-Buruli ulcer medicinal plants in three West African countries. *J. Ethnopharmacol.* 172: 297–311.

111 Tsouh Fokou, P.V., Nyarko, A.K., Appiah-Opong, R. et al. (2015). Update on medicinal plants with potency on *Mycobacterium ulcerans*. *Biomed. Res. Int.* 2015: 917086.

112 Yemoa, A., Gbenou, J., Affolabi, D. et al. (2015). Beninese medicinal plants as a source of antimycobacterial agents: bioguided fractionation and in vitro activity of alkaloids isolated from *Holarrhena floribunda* used in traditional treatment of Buruli ulcer. *Biomed. Res. Int.* 2015: 835767.

113 Claes, P., Cappoen, D., Mbala, B.M. et al. (2013). Synthesis and antimycobacterial activity of analogues of the bioactive natural products sampangine and cleistopholine. *Eur. J. Med. Chem.* 67: 98–110.

114 Andreoli, A., Mou, F., Minyem, J.C. et al. (2015). Complete healing of a laboratory-confirmed Buruli ulcer lesion after receiving only herbal household remedies. *PLoS Negl. Trop. Dis.* 9: e0004102.

115 Fehr, H., Egger, M., and Senn, I. (1994). Cotrimoxazole in the treatment of *Mycobacterium ulcerans* infection (Buruli ulcer) in west Africa. *Trop. Doct.* 24: 61–63.

116 Revill, W.D., Morrow, R.H., Pike, M.C., and Ateng, J. (1973). A controlled trial of the treatment of *Mycobacterium ulcerans* infection with clofazimine. *Lancet* 2: 873–877.

117 Espey, D.K., Djomand, G., Diomande, I. et al. (2002). A pilot study of treatment of Buruli ulcer with rifampin and dapsone. *Int. J. Infect. Dis.* 6: 60–65.

118 Etuaful, S., Carbonnelle, B., Grosset, J. et al. (2005). Efficacy of the combination rifampin-streptomycin in preventing growth of *Mycobacterium ulcerans* in early lesions of Buruli ulcer in humans. *Antimicrob. Agents Chemother.* 49: 3182–3186.

119 Chauty, A., Ardant, M.F., Adeye, A. et al. (2007). Promising clinical efficacy of streptomycin-rifampin combination for treatment of buruli ulcer (*Mycobacterium ulcerans* disease). *Antimicrob. Agents Chemother.* 51: 4029–4035.

120 Nienhuis, W.A., Stienstra, Y., Thompson, W.A. et al. (2010). Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. *Lancet* 375: 664–672.
121 Chauty, A., Ardant, M.F., Marsollier, L. et al. (2011). Oral treatment for *Mycobacterium ulcerans* infection: results from a pilot study in Benin. *Clin. Infect. Dis.* 52: 94–96.
122 Meyers, W.M., Shelly, W.M., and Connor, D.H. (1974). Heat treatment of *Mycobacterium ulcerans* infections without surgical excision. *Am. J. Trop. Med. Hyg.* 23: 924–929.
123 Junghanss, T., Um Boock, A., Vogel, M. et al. (2009). Phase change material for thermotherapy of Buruli ulcer: a prospective observational single centre proof-of-principle trial. *PLoS Negl. Trop. Dis.* 3: e380.
124 Vogel, M., Bayi, P.F., Ruf, M.T. et al. (2016). Local heat application for the treatment of Buruli ulcer: results of a phase II open label single center non comparative clinical trial. *Clin. Infect. Dis.* 62: 342–350.
125 Nienhuis, W.A., Stienstra, Y., Abass, K.M. et al. (2012). Paradoxical responses after start of antimicrobial treatment in *Mycobacterium ulcerans* infection. *Clin. Infect. Dis.* 54: 519–526.
126 Ruf, M.T., Sopoh, G.E., Brun, L.V. et al. (2011). Histopathological changes and clinical responses of Buruli ulcer plaque lesions during chemotherapy: a role for surgical removal of necrotic tissue? *PLoS Negl. Trop. Dis.* 5: e1334.
127 Yeboah-Manu, D., Kpeli, G.S., Ruf, M.T. et al. (2013). Secondary bacterial infections of buruli ulcer lesions before and after chemotherapy with streptomycin and rifampicin. *PLoS Negl. Trop. Dis.* 7: e2191.
128 O'Brien, D.P., Robson, M.E., Callan, P.P., and McDonald, A.H. (2009). "Paradoxical" immune-mediated reactions to *Mycobacterium ulcerans* during antibiotic treatment: a result of treatment success, not failure. *Med. J. Aust.* 191: 564–566.
129 Ruf, M.T., Chauty, A., Adeye, A. et al. (2011). Secondary Buruli ulcer skin lesions emerging several months after completion of chemotherapy: paradoxical reaction or evidence for immune protection? *PLoS Negl. Trop. Dis.* 5: e1252.
130 Schütte, D., Um-Boock, A., Mensah-Quainoo, E. et al. (2007). Development of highly organized lymphoid structures in Buruli ulcer lesions after treatment with rifampicin and streptomycin. *PLoS Negl. Trop. Dis.* 1: e2.
131 Schütte, D. and Pluschke, G. (2009). Immunosuppression and treatment-associated inflammatory response in patients with *Mycobacterium ulcerans* infection (Buruli ulcer). *Expert. Opin. Biol. Ther.* 9: 187–200.
132 Chapman, N., Doubell, A., Oversteegen, L. et al. G-Finder (2018). Neglected Disease Research and Development: Reaching new heights. https://www.policycuresresearch.org/wp-content/uploads/Y11_G-FINDER_Full_report_Reaching_new_heights.pdf (accessed 19 March 2019).

13

Drug Discovery and Development for Leprosy

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13.1 Unmet Medical Needs in the Treatment of Leprosy

Leprosy or Hansen's disease has afflicted humanity since time immemorial [1]. Improvements in hygiene and sanitation and organized initiatives to deploy multidrug therapy (MDT) have reduced the overall prevalence of this infection [2]. Despite these interventions, leprosy persists as a chronic mycobacterial infection that imposes a substantial burden of disease and human suffering in many settings [3, 4]. As a result, the World Health Organization (WHO) considers leprosy a neglected tropical disease (NTD) since it is a symptom of social inequity and poverty [5–9]. By causing neurologic dysfunction, disability and deformity, this NTD promotes underdevelopment by trapping the world's poorest in a cycle of destitution [9].

Leprosy is caused by two mycobacterial species, *Mycobacterium leprae* and *Mycobacterium lepromatosis* [10, 11], which upon entering the human host target specific cells including macrophages, histiocytes, Schwann cells, and endothelial cells [12]. The peripheral neuropathy that happens after invading Schwann cells results in peripheral nerve dysfunction and produces impaired sensation to touch, temperature, and proprioception, causing severe neurologic dysfunction, deformity, and loss of limbs [13–15]. Some individuals develop blindness because of corneal ulcerations secondary to lagophtalmos or due to chronic uveitis [1]. Historically, stigma leads to self-imposed isolation, decreased social involvement, decreased self-worth, and long-term enduring shame [8, 9]. From a societal perspective, individuals with leprosy have been persecuted, incarcerated, isolated, and mistreated simply because of their illness. The stigma of leprosy is as damaging as the infection per se and remains an ongoing challenge in worldwide efforts to control or eliminate this mycobacterial infection [8].

The precise mode of transmission of leprosy remains uncertain but probably involves human-to-human contact through respiratory droplets [16–19]. Recent ecological data suggest that environmental factors such as trauma or skin breaks during soil and water exposures, insect vectors, free-living amoebas, and animal

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reservoirs (armadillos, squirrels, felines, or other animals) influence leprosy transmission [16–20].

The immune response to *M. leprae* by the human host defines the clinical manifestations of leprosy [1, 21]. Clinical classification (Ridley–Jopling staging) includes tuberculoid, borderline (borderline tuberculoid, borderline borderline, and borderline lepromatous) and lepromatous forms (Table 13.1). Leprosy reactions, due to their potential inflammatory impact on the peripheral nerve, constitute an important contributor to peripheral nerve dysfunction characterized as sensory loss and muscle atrophy (Figure 13.1) [13]. Most reactions fall into two main types: reversal reactions (type 1 reaction) and erythema nodosum leprosum (type 2 reaction). These reactions occur in more than half of patients and manifest as acute episodes of inflammatory processes that may occur before, during, and even after completion of effective MDT [22]. Management of leprosy reactions traditionally involves the use of glucocorticoids, thalidomide, and clofazimine to reduce the inflammatory response and prevent further nerve injury [1, 22]. Infection due to *M. lepromatosis* produces diffuse leprosy of Lucio and the Lucio phenomenon [10, 11].

13.2 Current Therapies for Leprosy

13.2.1 Direct-Acting Antibacterial Therapy

For millennia, there have been many natural remedies used to ameliorate the clinical manifestations of leprosy. For example, in India, the *Sushruta Samhita* (600 BCE), an ancient treatise on medicine and surgery written in Sanskrit, recommended treating leprosy (also known as "kushta," meaning "eating away") with oil derived from the chaulmoogra tree [9]. This oil remained the mainstay of leprosy treatment in India until the introduction of sulfones [9]. In 1933, Sister Hilary Ross of the Sisters of Charity and Dr. George Fite of the US Public Health Service established a laboratory for drug testing at the National Hansen's Disease Program in Carville, Louisiana in the United States [22]. Subsequently, Dr. Guy Henry Faget pioneered sulfone drug therapy and demonstrated the efficacy of sulfone drugs, including promin, diasone, and promizole in the treatment of leprosy [22]. Sulfone-based therapy consisting of intravenous promin began by 1941 [2, 23–25]. The use of dapsone replaced promin by 1947 [24]. The inability of *M. leprae* to grow in axenic culture systems impaired the ability to develop anti-leprosy drugs, requiring the assessment of antibacterial activity employing alternative approaches other than the traditional *in vitro* methods [25]. By 1960, the inoculation of *M. leprae* into the mouse footpad allowed the replication of bacilli that offer a model for assessing the antimicrobial activity of compounds against *M. leprae* and determine their bactericidal or bacteriostatic properties [26]. By the mid-1970s, rifampin and clofazimine were introduced to treat cases of leprosy [2, 24, 27–31]. In 1982, in response to concerns of antimicrobial resistance developing in patients receiving dapsone monotherapy and the potential emergence to rifampin, the WHO Study Group on Chemotherapy of Leprosy for Control Programmes recommended the introduction of MDT.

Table 13.1 Clinical staging of leprosy (Hansen's disease) according to the Ridley–Jopling system and WHO categories, types of leprosy reactions, and multidrug therapies.

Ridley–Jopling classification	Tuberculoid (TT)	Borderline tuberculoid (BT)[a]	Borderline borderline (BB)	Borderline lepromatous	Lepromatous
WHO[b] classification	Paucibacillary	Paucibacillary	Multibacillary	Multibacillary	Multibacillary
Type 1 reaction	No	Yes	Yes	Yes	No
Type 2 reaction[c]	No	No	No	Yes	Yes
Clinical features	Skin lesions: single or few hypopigmented macules or plaques with a raised edge, dry, scaly, hairless with hypoesthesia or anesthesia Few peripheral nerves are commonly enlarged	Skin lesions: hypopigmented anesthetic patches that become confluent Moderate nerve involvement May have late neural thickening with asymmetrical anesthesia and paresis	Skin lesions: hypopigmented anesthetic patches, punched-out centers, and raised erythematous borders Multiple nerve involvement with symmetrical thickened nerves Asymmetrical anesthesia and paresis may be present Deformity, amputation, and disability	Skin lesions: widely distributed nodules with diffuse skin infiltration Multiple nerve involvement with symmetrical involvement (thickened nerves) Symmetrical glove and stocking anesthesia Deformity, amputation, and disability	Skin lesions: widely distributed skin lesions: macules, nodules, erythematous papules Diffuse skin infiltration with thickened peripheral nerves Symmetrical glove and stocking anesthesia Deformity, amputation, and disability
Multidrug treatment US NHDP[d]	Dapsone 100 mg/d × 12 mo Rifampin 600 mg/d × 12 mo		Dapsone 100 mg/d × 24 mo Rifampin 600 mg/d × 24 mo Clofazimine 50 mg/d × 24 mo (may substitute with daily minocycline)		

(Continued)

Table 13.1 (Continued)

Ridley–Jopling classification	Tuberculoid (TT)	Borderline tuberculoid (BT)[a)]	Borderline borderline (BB)	Borderline lepromatous	Lepromatous
Multidrug treatment WHO[e)]	Dapsone 100 mg/d × 6 mo Rifampin 600 mg once monthly under supervision × 6 mo		Dapsone 100 mg/d × 12 mo Rifampin 600 mg once monthly under supervision × 12 mo Clofazimine 50 mg/d × 12 mo plus 300 mg every month under supervision × 12 mo		
Alternative drug regimens	Clarithromycin 500 mg daily used as a substitute for clofazimine in children or used as a substitute for other drugs in adults Ofloxacin 400 mg daily may also be used as a substitute for clofazimine only in adults Minocycline 100 mg daily may also be used as a substitute for dapsone in those individuals with intolerance to dapsone (i.e. those with side effects including methemoglobinemia, hemolytic anemia, or severe gastrointestinal intolerance) The combination of rifampin, ofloxacin, and minocycline is sometimes used for single-lesion paucibacillary leprosy or in relapse cases of leprosy				

a) Borderline forms represent a mixture of signs and symptoms of polar forms.
b) World Health Organization (WHO).
c) Management of type 1 reaction (reversal reaction) requires prednisone or prednisolone (40–80 mg daily tapered over a 12- to 20-wk period. Treatment of type 2 reaction (erythema nodosum leprosum) involves the use of prednisone or prednisolone (40–80 mg daily tapered over a 12- to 24-wk period) but sometimes requires a longer taper. Thalidomide at a dose of 200–400 mg in divided doses is sometimes used in combination with corticosteroids to control severe ENL. Clofazimine may be used in those intolerant to corticosteroids or in combination. Other drugs are also employed as steroid-sparing agents including methotrexate or cyclosporine.
d) National Hansen's Disease Program (NHDP), United States.
e) The decision by WHO to recommend MDT in 1982 was largely based on expert opinion and the existing experience at that time of the benefits of MDT in the treatment of tuberculosis.

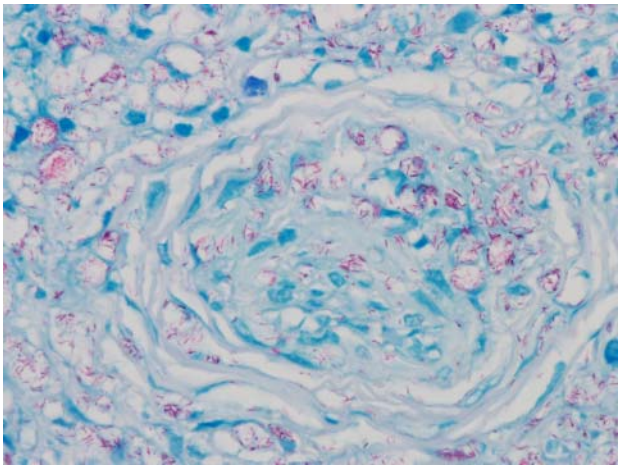

Figure 13.1 Fite-Faraco staining of a skin biopsy from a patient with lepromatous leprosy demonstrating large number of bacilli in cutaneous structures and many bacilli residing inside the peripheral nerve characteristic of leprosy or Hansen's disease, 400×.

First-line drugs included a combination of dapsone, rifampin, and clofazimine for treating patients with multibacillary forms of leprosy, and dapsone combined with rifampin for those with paucibacillary forms (Table 13.2) [27]. In 1991, WHO recommended shortening MDT regimens in order to increase coverage [2, 24]. In addition, in 1998, a single dose of a combination of rifampin, ofloxacin, and minocycline (ROM) was introduced for treating single-lesion paucibacillary forms of leprosy, demonstrating increased adherence and fewer side effects but was also less effective than the standard WHO-recommended therapy [28]. Currently, minocycline and ofloxacin are used as alternative drugs for those with drug intolerances or among relapse cases.

The institution of MDT through active case finding among populations living in endemic communities reduced the prevalence of this infection [2, 4]. Nevertheless, since 2005, the number of reported new cases has remained consistently stable despite continued use of MDT. The number of new cases will reach the 4 million mark by 2020 [4]. Most new cases already have grade 2 neurologic disability by the time of their diagnosis. Since cases of leprosy in children indicate ongoing transmission of *M. leprae* in endemic settings, targeted screening involving school-based surveillance followed by household continuous surveillance increases early detection of new leprosy cases.

The management of leprosy requires the use of MDT in combination with steroids or other anti-inflammatory drugs among those with leprosy reactions [22]. The effectiveness of MDT is measured as the rate of relapse after completing an officially recommended antibacterial regimen. Cases of relapse or reinfection with *M. leprae* are rare [24]. The appearance of new lesions during or after completing MDT is most frequently due to the occurrence of leprosy reactions [22]. Relapse after successful completion of WHO-recommended MDT is rare [24]. Detection of drug resistance with the use of rapid-DNA-based molecular

Table 13.2 Comparison of the two treatment strategies of leprosy: direct-acting antibacterial therapy and host-directed therapy.

Treatment strategy	Direct-acting antibacterial therapy	Host-directed therapy
Goal	Kill or inhibit the growth of *M. leprae* and *M. lepromatosis*	Prevent infection Prevent the occurrence of clinical manifestations Ameliorate clinical manifestations and/or severity of the disease including the occurrence of leprosy reactions
Mechanism(s)	Bactericidal or bacteriostatic mechanisms involving inhibition of DNA transcription or translation by blocking key enzymes or at the ribosomal level, or blockage of cell wall synthesis	Enhance innate immune response (macrophages) Augment acquired cell-mediated immunity Modulate intracellular pathways involved in autophagy Modulate intracellular lipid metabolism in phagocytic cells (i.e. macrophages and histiocytes)
Examples	Dapsone Rifampin Clofazimine[a)] Ofloxacin Minocycline[b)] Clarithromycin Nitazoxanide Other quinolones (moxifloxacin)	Rifampin or dapsone used as chemoprophylaxis among household contacts in cases of multibacillary types of leprosy BCG (Bacillus Calmette-Guerin) vaccine has shown to protect against some forms of leprosy Interferon-gamma-promoting macrophage function Nitazoxanide influencing autophagy Leukotriene inhibitors such as zafirlukast or peroxisome proliferator-activated receptor (PPAR) agonists influencing intracellular pathways involved in the intracellular processing of carbohydrates and lipids

a) Clofazimine likely acts through both treatment strategies, as an antibacterial drug but has also immunomodulatory roles.
b) Minocycline is considered to have also some degree of immunomodulatory activity.

assays may occur among patients having a relapse. However, except for patients who were treated with dapsone monotherapy, the emergence of multidrug resistance has been only rarely identified [24].

13.3 Innovative Therapeutic Strategies

Despite important efforts including the institution of MDT to control or eliminate leprosy, this disease remains an important public health concern that affects mostly impoverished populations living in underdeveloped settings [2]. The use of shorter MDT reduced the prevalence of leprosy but did not end its transmission. Detection of new cases indicates persistent transmission of the infection, with more than 100 countries still reporting cases [3, 4]. Indeed, there are many gaps in our understanding of the epidemiology of leprosy that diminishes our ability to eliminate leprosy transmission by the year 2020 [4]. Furthermore, many patients who are "successfully treated" due to completion of antimicrobial therapy are left with long-term neurologic dysfunction that often ends with limb loss and/or disability [22]. Therefore, leprosy is a chronic infectious disease that urgently needs innovative strategies to eliminate its transmission [32] and preventing nerve injury including (i) advancing our understanding of the epidemiology of leprosy in order to develop elimination tools; (ii) detecting new cases earlier in order to prevent neurologic sequelae; (iii) developing host-directed therapies including drugs, biologicals, or vaccines targeting the prevention of nerve damage and/or the occurrence of leprosy reactions [33–39] (Table 13.3).

13.3.1 Host-Directed Therapy

M. leprae has evolved to adapt by metabolic alterations to survive the intracellular environment of the human host [33]. Once macrophages and histiocytes engulf and internalize *M. leprae* into membrane-bound organelles called phagosomes, *M. leprae* subvert the endosomal–lysosomal fusion [33, 34]. Concomitantly, *M. leprae* produce accumulation of lipid droplets inside macrophages, and some of these lipid compounds are host-derived and some are part of the cell membrane of the leprosy bacillus [34]. As a result, these *foamy cells* or *foamy macrophages* are typically identified in tissue specimens of multibacillary cases [13, 34]. Lipids play two crucial roles in the intracellular survival by providing nourishment to *M. leprae* and evading the immune system: host-derived lipids serve the energy needs of *M. leprae*, whereas its own lipids modulate the immune response [34]. It has been shown that patients with multibacillary leprosy have abundant levels of polyunsaturated fatty acids such as eicosapentaeoic acid, arachidonic acid, and phospholipids. Other important lipid compounds of *M. leprae* cell membrane include phenolic glycolipid A (PGL-1), phthiocerol dimycocerosate, and Man-lipoarabinomannan play crucial roles in the survival and virulence of the leprosy bacillus [34]. Targeting the metabolic strategies employed by the leprosy bacillus, including the roles of lipids in the survival and pathogenesis of leprosy, is important to future drug designing efforts (Table 13.3) [35]. Restoring the autophagy machinery among those with multibacillary leprosy may also prove

Table 13.3 Description of currently available drugs used in the treatment of leprosy, leprosy reactions, and drugs in development.

Class	Drug	Mechanism of action	Described resistance[a)]
Sulfone	Dapsone (diaphenylsufone or DDS)	Prevents the utilization of para-aminobenozic acid (PABA) for the synthesis of folic acid by competitive inhibition for dihydropteroate synthetase Mostly bacteriostatic, but it does have some bactericidal activity	Missense mutation within codons 53 and 55 of the DRDR *folP1* is considered the most common form of antimicrobial resistance due to the initial use of dapsone monotherapy[b)]
Rifamycin	Rifampin	Inhibits the DNA-dependent RNA polymerase by binding on its β-subunit Bactericidal against *M. leprae*	β-subunit of the DNA-dependent RNA polymerase encoded by *rpoB*
Rimnophenazine	Clofazimine[c)]	Not fully elucidated. It reaches high concentrations inside macrophages and binds preferentially to guanine-containing sequences of bacterial DNA Mostly bacteriostatic, but it does have minimal bactericidal activity	Not fully elucidated and appears to be rare given the multiple mechanisms of action of this drug
Fluorinated carboxyquinolone	Ofloxacin	Inhibits DNA replication by inactivating tetramer containing two β-subunits (*gyr*A) and two β-subunits (*gyr*B) of the DNA gyrase	DRDR *gyr*A, *gyr*B
Tetracycline	Minocycline	Inhibits bacterial protein synthesis by binding into the 30s and 50s ribosomal subunits	Not fully studied in *M. leprae* infection probably because of its use mostly as single-dose therapy in combination with rifampin and ofloxacin for single-lesion PB leprosy
Macrolide	Clarithromycin	Inhibits bacterial protein synthesis by binding to the 23SrRNA ribosomal subunit	Missense mutation within the 23SrRNA gene, reducing binding of the drug to ribosomes
Fluoroquinolone	Moxifloxacin DC-159a	Inhibits DNA replication by inactivating tetramer containing two β-subunits (*gyr*A) and two β-subunits (*gyr*B) of the DNA gyrase Moxifloxacin is considered more effective than ofloxacin against leprosy and is considered a bactericidal agent	Not yet evaluated in the treatment of leprosy
2-Acetoloxy-*N*-(5-nitro-2-thiazolyl) benzamide	Nitazoxanide	Induces autophagy in mammalian cells by unblocking the autophagy machinery and processes interrupted in lepromatous forms of leprosy It may also disrupt the membrane potential and intrabacterial pH homeostasis	Not yet evaluated in the treatment of leprosy

Glucocorticoids	Prednisone or prednisolone	Inhibition of NF-kappa β by promoting the synthesis of the inhibitor of Kappa β, reducing the nuclear transcription of genes involved in the production of inflammatory cytokines (IL-1, TNF-α) Used in the treatment of type 1 (reversal reactions) and type 2 reactions (erythema nodosum leprosum)	N/A
Piperidinyl isoindole	Thalidomide	Inhibits the release of TNF-α from monocytes and modulates other cytokine action Used in the treatment of type 2 reactions (erythema nodosum leprosum)	N/A
Methylxanthine derivative	Pentoxiphylline	Inhibits phosphodiesterase affecting intracellular signaling of inflammatory cytokines (TNF) Used in the treatment of type 2 reactions (erythema nodosum leprosum)	N/A
Tumor necrosis factor (TNF) inhibitors [38]	Infliximab Adalimumab etanercept	Inhibits TNF-α receptor and some case reports have shown successful treatment of leprosy reactions with the use of etanercept and infliximab Paradoxically, there have also been associations of these biologic agents unveiling previously subclinical leprosy or trigger type 1 reactions	N/A
T-cell inhibitors	Methotrexate	Blocks proliferation of lymphocytes by inhibiting dihydrofolate reductase, which is essential for purine and pyrimidine base biosynthesis	N/A
	Cyclosporine [39]	It forms a complex with cyclophilin that inhibits the activity of calcineurin, which regulates nuclear translocation of NFAT Both agents used mostly in the treatment of type 2 reactions (erythema nodosum leprosum)	

a) DRDR (drug resistance determining region) of genetic markers of antimicrobial resistance detected with the use of rapid DNA-based molecular assays.
b) The strategy of multidrug therapy relies on the principle that for chronic infections that require prolonged treatment courses, combining effective antimicrobials reduces the emergence of resistance.
c) Macrophages sequestrate clofazimine as crystal-like drug inclusions, leading to an important metabolic disruption in energy homeostasis within macrophages and an increase in interleukin-1 receptor antagonist production.

to be a useful synergistic intervention along with WHO-recommended MDT regimens by promoting the intracellular killing of *M. leprae* [36, 37].

Leukotriene inhibitors such as zafirlukast [40] or peroxisome proliferator-activated receptor (PPAR) [41] agonists may play a potential role by influencing intracellular metabolism of lipids in the treatment of leprosy or leprosy reactions. Finally, sequence-based genomic analysis of *M. leprae* to identify the function of specific mycobacterial proteins is crucial in future efforts to identify newer compounds to treat leprosy or prevent the occurrence or ameliorate the severity of leprosy reactions [42].

13.4 Conclusions

The best form of preventing leprosy and most NTDs is to improve social conditions that predispose individuals to become ill in the first place. However, there is no doubt that we urgently need a better understanding of this ancient disease with the use of modern technologies and application of innovative approaches to relieve human suffering of this unique infectious disease. The availability of anti-leprosy drugs has reduced the overall prevalence of this infection. However, we continue to see a large number of new cases and many of them are left with long-term disability, dysfunction, and deformity. In this context, further research efforts should assess the use of host-directed therapies including the potential use of preventive or therapeutic vaccines [43–45] to reduce the risk of infection or prevent nerve damage. Drug development needs to target the intracellular life of *M. leprae* including interfering with lipid metabolism or promoting intracellular killing of *M. leprae* by promoting the function of phagolysosomes or enhancing the autophagy machinery.

References

1 White, C. and Franco-Paredes, C. (2015). Leprosy in the 21st century. *Clin. Microb. Rev.* 28 (1): 80–94.
2 Smith, C.S., Aerts, A., Saunderson, P. et al. (2017). Multidrug therapy for leprosy: a game changer on the path to elimination. *Lancet Infect. Dis.* 17: e293–e297.
3 Franco-Paredes, C. and Rodriguez-Morales, A.J. (2016). Unsolved matters in leprosy: a descriptive review and call for further research. *Ann. Clin. Microbiol. Antimicrob.* 15 (1): 33.
4 Smith, C.S., van Brakel, W., Gillis, T. et al. (2015). The missing millions: a threat to the elimination of leprosy. *PLoS Neglec. Trop. Dis.* 9 (4): e0003658.
5 Kerr-Pontes, L.R.S., Barreto, M.L., Evangelist, C.M.N. et al. (2006). Socioeconomic, environmental, and behavioural risk factors for leprosy in Northeast Brazil: results of a case-control study. *Int. J. Epidemiol.* 35: 994–1000.
6 Kerr-Pontes, L.R.S., Montenegro, A.C.D., Barreto, M.L. et al. (2004). Inequality and leprosy in Northeast Brazil: an ecological study. *Int. J. Epidemiol.* 33: 262–269.

7 Lockwood, D.N.J. (2004). Leprosy and poverty. *Int. J. Epidemiol.* 33: 269–270.
8 White, C. (2011). Leprosy and stigma in the context of international migration. *Lepr. Rev.* 82 (2): 147–154.
9 Jacob, J.T. and Franco-Paredes, C. (2008). The stigmatization of leprosy in India and its impact on future approaches to elimination and control. *PLoS Negl. Trop. Dis.* 2 (1): e113.
10 Han, X.Y., Seo, Y.H., Sizer, K.C. et al. (2008). A new *Mycobacterium* species causing diffuse lepromatous leprosy. *Am. J. Clin. Pathol.* 130: 856–864.
11 Vargas-Ocampo, F. (2007). Diffuse leprosy of Lucio and Latapí: a histologic study. *Lepr. Rev.* 78: 248–260.
12 Polycarpou, A., Walker, S.L., and Lockwood, D.N. (2013). New findings in the pathogenesis of leprosy and implications for the management of leprosy. *Curr. Opin. Infect. Dis.* 26: 413–419.
13 Scollard, D.M., Dacso, M.M., and Abad-Venida, M.L. (2015). Tuberculosis and leprosy. Classical granulomatous diseases in the twenty-first century. *Dermatol. Clin.* 33: 541–562.
14 Scollard, D.M., Truman, R.W., and Ebenezer, G.J. (2015). Mechanisms of nerve injury in leprosy. *Clin. Dermatol.* 33: 46–54.
15 Scollard, D.M. (2008). The biology of nerve injury in leprosy. *Lepr. Rev.* 79: 242–253.
16 Blake, L.A., West, B.C., Lary, C.H., and Todd, J.R. IV, (1987). Environmental nonhuman sources of leprosy. *Rev. Infect. Dis.* 9 (3): 561–577.
17 Bratschi, M.W., Steinmann, P., Wickenden, A., and Gillis, T.P. (2015). Current knowledge on *Mycobacterium leprae* transmission: a systematic literature review. *Lepr. Rev.* 86: 142–155.
18 Mensah-Awere, D., Bratschi, M.W., Steinmann, P. et al. (2015). Developing strategies to block the transmission of leprosy. *Lepr. Rev.* 86: 156–164.
19 Wheat, W.H., Casali, A.L., Thomas, V. et al. (2014). Long-term survival and virulence of *Mycobacterium leprae* in amoebal cysts. *PLoS Negl. Trop. Dis.* 8 (12): e3405.
20 Balamayooran, G., Pena, M., Sharma, R., and Truman, R.W. (2015). The armadillo as an animal model and reservoir host for *Mycobacterium leprae*. *Clin. Dermatol.* 33: 108–115.
21 Worobec, S.M. (2012). Current approaches and future directions in the treatment of leprosy. *Res. Rep. Trop. Med.* 3: 79–91.
22 Jacob, J.T., Kozarsky, P., Dismukes, R. et al. (2008). Five-year experience with type 1 and type 2 reactions in Hansen's disease at a US travel clinic. *Am. J. Trop. Med. Hyg.* 79 (3): 452–454.
23 National Hansen's (Leprosy) Disease Program. Health Resources and Service Administration. (2018). https://www.hrsa.gov/hansens-disease/history.html (accessed 11 March 2019).
24 Williams, D.L. and Gillis, T.P. (2012). Drug-resistant leprosy: monitoring and current status. *Lepr. Rev.* 83: 269–281.
25 Franco-Paredes, C. (2014). *Infectious Disease and Antimicrobial Agents* (ed. V. Yu). http://www.antimicrobe.org/ (accessed 10 December 2017.
26 Shephard, C.C. (1960). The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. *J. Exp. Med.* 112: 445–454.

27 World Health Organization (1982). World Health Organization Study Group. Chemotherapy of leprosy for control programmes. *WHO Technical Report Series 675*.

28 Lastória, J.C. (2014). Milanez Morgado de Abreu MA, Leprosy: a review of laboratory and therapeutic aspects – Part 2. *An. Bras. Dermatol.* 89 (3): 389–403.

29 Yamaguchi, T., Yokoyama, K., Nakajima, C., and Suzuki, Y. (2016). DC-159a shows inhibitory activity against DNA gyrases of *Mycobacterium leprae*. *PLoS Negl. Trop. Dis.* 10 (9): e0005013.

30 Legendre, D.P., Muzny, C.A., and Swiatlo, E. (2012). Hansen's disease (leprosy): current and future pharmacotherapy and treatment of disease-related immunologic reactions. *Pharmacotherapy* 32 (1): 27–37.

31 Burgos, J., de la Cruz, E., Paredes, R. et al. (2011). The activity of several newer antimicrobial agents against *Mycobacterium leprae* in mice. *Antimicrob. Agents Chemother.* 50 (4): 1558–1560.

32 Steinmann, P., Reed, S.G., Mirza, F. et al. (2017). Innovative tools and approaches to end the transmission of *Mycobacterium leprae*. *Lancet Infect. Dis.* 17: 298–305.

33 Van der Wel, Hava, D., Houben, D. et al. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 129: 1287–1298.

34 Kaur, G. and Kaur, J. (2017). Multifaceted role of lipids in *Mycobacterium leprae*. *Future Microbiol.* 12 (4): 315=335.

35 Trexel, J., Yoon, G.S., Keswani, R.K. et al. (2017). Macrophage-mediated Clofazimine sequestration is accompanied by a shift in host energy metabolism. *J. Pharm. Sci.* 106: 1162–1174.

36 Silva, B.J., Barbosa, M.G., Andrade, P.R. et al. (2017). Autophagy is an innate mechanism associated with leprosy polarization. *PLoS Pathog.* 13 (1): e1006103.

37 Bailey, M.A., Na, H., Duthie, M.S. et al. (2017). Nitazoxanide is active against *Mycobacterium leprae*. *PLoS One* 12 (8): e0184107.

38 Sena, C.B., Salgado, C.G., Tavares, C.M. et al. (2006). Cyclosporine A treatment of leprosy patients with chronic neuritis is associated with pain control and reduction in antibodies against nerve growth factor. *Lepr. Rev.* 77: 121–129.

39 Scollard, D.M., Joyce, M.P., and Gillis, T.P. (2006). Development of leprosy and type 1 leprosy reactions after treatment with infliximab: a report of two cases. *Clin. Infect. Dis.* 43: e19–e22.

40 Vides, E.A., Cabrera, A., Aberu, K.P., and Levis, W.R. (1999). Effect of zafirlukast on leprosy reactions. *Int. J. Lepr. Other Mycobact. Dis.* 67: 71–75.

41 Gupta, M., Mahajan, V.K., Mehta, K.S. et al. (2015). Peroxisome proliferator-activated receptors (PPARs) and PPAR agonists: the 'future' in dermatology therapeutics? *Arch. Dermatol. Res.* 37: 767–780.

42 Turab Navqi, A.A., Ahmad, F., and Md, I.H. (2017). Identification and functional candidates amongst hypothetical proteins of *Mycobacterium leprae* Br4923, a causative agent of leprosy. *Genome* 58: 25–42.

43 Guerrero, G.G., Rangel-Moreno, J., Islas-Trujillos, S., and Rojas-Espinosa, O. (2015). Successive intramuscular boosting with INF-Alpha protects *Mycobacterium bovis* BCG-vaccinated mice against *M. lepraemurium* infection. *BioMed. Res. Int.* 2015: 414027.
44 Singh Setia, M., Steinmaus, C., Ho, C.S., and Rutherford, G.W. (2006). The role of BCG in prevention of leprosy: a meta-analysis. *Lancet Infect. Dis.* 6: 162–170.
45 Richardus, J.H. and Oskam, L. (2015). Protecting people against leprosy: chemoprophylaxis and immunoprophylaxis. *Clin. Dermatol.* 33: 19–25.

Index

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d

e

f

g

h