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Alzheimer's Disease II

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Michael S. Wolfe

Editor

Alzheimer's Disease II

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Preface

Alzheimer's disease (AD) is a devastating neurodegenerative brain disorder that leads to progressive loss of memory and other higher cognitive functions and ultimately death. Age is the greatest risk factor for AD, and therefore longer life spans mean more cases. In the United States alone, over five million people have AD, and worldwide estimates are 20–30 million. These numbers are projected to rise dramatically in the coming decades, creating a crisis that could bankrupt healthcare systems and overwhelm society as a whole.

The need for effective prevention and treatment is urgent, and many laboratories around the world are engaged in research into the root causes and mechanisms of the disease and the discovery of potential therapeutics. Although important gaps remain in our understanding of the molecular basis of AD pathogenesis and progression, considerable progress has been made in this regard.

Two key pathological features are found in the AD brain: amyloid plaques and neurofibrillary tangles. The former is primarily composed of a 42-residue amyloid β -peptide ($A\beta$), while the latter are composed of the microtubule-associated protein tau. While the deposits themselves may only be disease markers, a large body of evidence, particularly from genetics, strongly suggests that these proteins are directly involved in pathogenesis and progression.

The process apparently begins with the overproduction or under-clearance of the $A\beta$, a proteolytic product generated through cleavage of the amyloid precursor protein by the sequential action of β - and γ -secretases. Assembly of the amphipathic $A\beta$ peptide in the form of neurotoxic oligomers and other higher-order forms leads to synaptic toxicity and the triggering, through unknown mechanisms, of tau misfolding and aggregation.

This volume first provides an overview of AD biology and the prospects for developing therapeutics (chapter “Targets and Strategies Toward the Development of Alzheimer Therapeutics”). What follows are specific and detailed medicinal chemical strategies for AD drug discovery and the development of diagnostic tools.

Preventing the formation of neurotoxic A β through inhibition of β -secretase (chapter “The Design, Development, and Evaluation of BACE1 Inhibitors for the Treatment of Alzheimer’s Disease”) or modulation of γ -secretase (chapter “ γ -Secretase Modulators as A β 42-Lowering Pharmacological Agents to Treat Alzheimer’s Disease”) is a leading strategy. As pathological tau is hyperphosphorylated, the responsible kinases are considered important targets (chapter “Inhibitors of Tau-Phosphorylating Kinases”), and because pathological tau is not able to serve its normal function in stabilizing microtubules, small molecules that rescue this loss of function may have therapeutic benefit (chapter “Microtubule-Stabilizing Agents for Alzheimer’s and Other Tauopathies”). Finally, early diagnosis of AD is critical for enrolling the right patients into clinical trials and intervening before disease progression has gone too far, and the development of PET imaging agents for amyloid plaques has led to several approved diagnostic agents (chapter “PET Imaging Agents for Alzheimer’s Disease”).

I would like to thank all the authors who contributed to this volume for their time, thought, and effort in putting together high-quality chapters on these important targets and strategies for AD prevention, treatment, and diagnosis. I also thank all those working on this difficult problem in human health. We all greatly hope that these efforts will speed the day when AD is a scourge of the past.

Lawrence, KS, USA

Michael S. Wolfe

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Targets and Strategies Toward the Development of Alzheimer Therapeutics

Michael S. Wolfe

Abstract Although Alzheimer's disease (AD) is a devastating neurodegenerative disorder affecting tens of millions worldwide, there are no effective disease-modifying therapies and only a handful of symptomatic treatments. Much has been deciphered regarding the molecular basis of AD in the past 25 years, but serious gaps in understanding remain. Aggregation-prone proteins amyloid- β ($A\beta$) and tau are apparently central to disease pathogenesis and progression; however, the neurotoxic forms of both proteins and the connection between the two remain unclear. Genetic mutations in the precursor protein for $A\beta$ and in a protease that produces $A\beta$ from this precursor cause dominantly inherited early-onset AD. In contrast, an allelic variant of apolipoprotein E is a major risk factor for the much more common late-onset AD, and the encoded protein is critical for $A\beta$ clearance from the brain. Tau is mutated in other forms of dementia, and tau pathology correlates better with neurodegeneration and progression than does $A\beta$ pathology. A variety of therapeutic agents are advancing through the pipeline, many targeting $A\beta$ and tau. New symptomatic treatments are still needed for those who have already progressed too far in the disease process. Advances in drug discovery, new diagnostic tools such as $A\beta$ imaging agents, and improvements in clinical trial design increase the likelihood that effective agents that slow or halt the progression of AD will soon be realized.

Keywords Alzheimer's disease, Amyloid, Neurodegeneration, Tau

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Abbreviations

5-HT	5-Hydroxytryptamine or serotonin
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid- β protein precursor
A β	Amyloid- β protein
BACE	β -Site APP-cleaving enzyme
CDK	Cyclin-dependent kinase
CSF	Cerebrospinal fluid
CTF- β or - β	C-Terminal fragment generated by β - or β -secretases
FAD	Familial Alzheimer's disease
FDA	Food and Drug Administration
GSK	Glycogen synthase kinase
MARK	Microtubule affinity-regulating kinase
MCI	Mild cognitive impairment
NMDA	<i>N</i> -Methyl-D-aspartate
PET	Positron emission tomography
PS1 or PS2	Presenilin-1 or presenilin-2
SAD	Sporadic Alzheimer's disease
USP	Ubiquitin-specific protease

1 Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that leads to progressive memory deficiencies and cognitive impairment and ultimately to death within 5–20 years from onset of symptoms. Although many factors such as genetics and lifestyle affect an individual's risk of getting AD, the greatest risk factor by far is age: AD is rare before age 65 and increases dramatically in the years afterward, with a ~40% chance of having AD by age 85. Over 5 million people in the United States and perhaps over 30 million worldwide have AD, with these numbers expected to increase dramatically as demographic changes result in greater numbers of elderly [1].

Much has been learned in the past three decades about the biological basis of the disease, revealing potential means of developing biomarkers for diagnosis, risk assessment, clinical trial enrollment, and drug target engagement, as well as suggesting targets for drug discovery for prevention and treatment. Despite this progress, only a few drugs for AD have been approved, and these are symptomatic treatments that do not affect the underlying progressive neurodegeneration and for this reason soon become ineffective. Indeed, no drugs have been approved for AD since 2003; every clinical candidate brought forward since the approval of the NMDA receptor blocker memantine has failed, and these failures number in the hundreds. The possible reasons for these failures are many, but taken together the inability to identify effective therapeutics suggests insufficient knowledge of disease mechanisms. This overview chapter will summarize the current state of understanding of the biology of the disease along with targets and strategies being pursued to discover effective AD therapeutics, presenting the progress as well as the challenges.

2 AD Presentation and Progression

AD begins with subtle problems with memory, learning, and executive function, termed mild cognitive impairment (MCI). As the disease progresses over the course of years, these problems become more pronounced, with the patient increasingly less able to function without help. Ultimately, family and friends are overwhelmed by the challenges of helping the AD patient meet his or her basic daily needs, preventing the wandering off without notice, and managing the disruptions in the sleep-wake cycle, with professional care in the form of a nursing home being the typical fate for late-stage AD patients. This pattern of disease presentation and progression is the consequence of the relentless loss of synapses and neurons in regions of the brain responsible for learning, memory, and cognition, beginning in the entorhinal cortex, spreading into the hippocampus and the frontal cortex and temporal lobes [2].

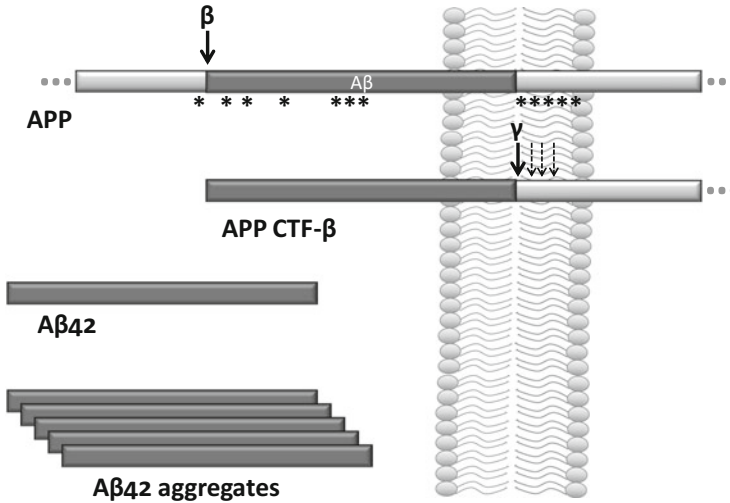


Fig. 1 Proteolytic processing of APP to A β , with subsequent aggregation of A β 42

3 Pathology

Two pathological hallmarks of AD were first described by Alois Alzheimer over a century ago. Silver staining of brain sections taken from a 55-year-old woman who died after developing presenile dementia revealed extra-neuronal deposits of what was dubbed amyloid as well as intraneuronal deposits called neurofibrillary tangles in nerve cell bodies and processes. Decades later, in the 1980s, the extra-neuronal deposits were found to be composed of a 4 kDa peptide called the amyloid- β protein (A β) [3], and the intraneuronal deposits were determined to be composed of a 352–441-residue protein called tau [4, 5]. A third pathological feature also became recognized, neuroinflammation, an apparent consequence of over-activation of non-neuronal cells such as microglia and astrocytes [6].

3.1 Amyloid

A β is a 38–43-residue secreted peptide and the product of proteolytic processing of the A β precursor protein (APP), a type I integral membrane protein of unknown function [7, 8]. The N-terminus of A β is formed through cleavage in the luminal/extracellular region of APP by the membrane-tethered aspartyl protease β -secretase [9], to release the APP ectodomain into the luminal/extracellular space and leave a 99-residue membrane-bound protein stub (APP CTF- β) (Fig. 1). The C-terminus of A β is generated through the action of γ -secretase, an unusual membrane-embedded aspartyl protease complex [10], cutting APP CTF- β within its single

transmembrane domain and releasing the APP intracellular domain (AICD) as the other proteolytic product. Alternative processing of APP by α -secretases ADAM-10 and ADAM-17 [11, 12] within the A β sequence precludes A β production, with the 83-residue protein remnant (APP CTF- α) subsequently cut by γ -secretase to produce an N-terminally truncated form of A β called p3. Although the 40-residue form of A β (A β 40) is the primary proteolytic product, the minor 42-residue variant (A β 42) is much more aggregation prone [13] and is the principle protein found in the cerebral amyloid deposits of AD [14].

Dominantly inherited missense mutations in the gene encoding APP cause familial AD (FAD), with an age of onset before age 60 [15, 16]. These mutations are found in and around the small A β region of the much larger APP. Mutations near the β -secretase cleavage site increase proteolysis by the protease, leading to increased production of all forms of A β . Mutations within the A β region increase the aggregation propensity of A β . Mutations within the transmembrane of APP increase the proportion of A β 42/A β 40. Thus, all FAD-causing mutations in APP increase the tendency of A β to aggregate. Other dominant mutations that cause early-onset FAD are found in the multi-pass membrane proteins presenilin-1 (PS1) [16, 17] and presenilin-2 (PS2) [16, 18], proteins that are the catalytic component of the γ -secretase complex [10, 19]. These mutations also increase the proportion of A β 42/A β 40 [10]. Cleavage of APP CTF- β by γ -secretase, however, is more complicated than originally thought. Initial proteolysis at the ϵ cleavage site near the membrane-cytosolic boundary releases AICD and produces 48- or 49-residue A β (A β 48 or A β 49), which is sequentially trimmed generally every three amino acids by a carboxypeptidase function of the γ -secretase complex [20, 21]. Thus, there are two pathways to secrete A β : A β 49 $\rightarrow$ A β 46 $\rightarrow$ A β 43 $\rightarrow$ A β 40 and A β 48 $\rightarrow$ A β 45 $\rightarrow$ A β 42 $\rightarrow$ A β 38, with this last cleavage releasing a tetrapeptide instead of a tripeptide. Most presenilin mutations increase the proportion of ϵ cleavage giving A β 48, thereby increasing A β 42/A β 40 [22]. These mutations also dramatically reduce the carboxypeptidase function of the γ -secretase complex [23].

Although FAD is clearly associated with altered A β production, the much more common late-onset sporadic AD (SAD) is associated with decreased clearance of A β [24]. Degradation of A β in the brain has been attributed to the metalloprotease neprilysin [25] and insulin-degrading enzyme [26]. Thus, a common feature of AD is increased levels of aggregation-prone forms of A β , either through altered production or clearance. For this reason, A β has been the major target for AD drug discovery, either directly through antibodies or aggregation inhibitors or indirectly through the secretases or A β -degrading enzymes.

The aggregation of A β is closely correlated with AD. However, the amyloid plaques do not correlate well spatially with neurodegeneration [2]. This has led to the focus on soluble A β oligomers as candidate toxic entities responsible for synaptic and neuronal dysfunction [27]. Synthetic A β oligomers as well as natural A β oligomers, secreted from cells or extracted from transgenic AD mouse brain or human AD brain, have been shown to affect dendritic spines, synaptic signaling, and electrophysiological responses, including long-term potentiation associated with memory formation [28]. The search for possible receptors for oligomeric A β

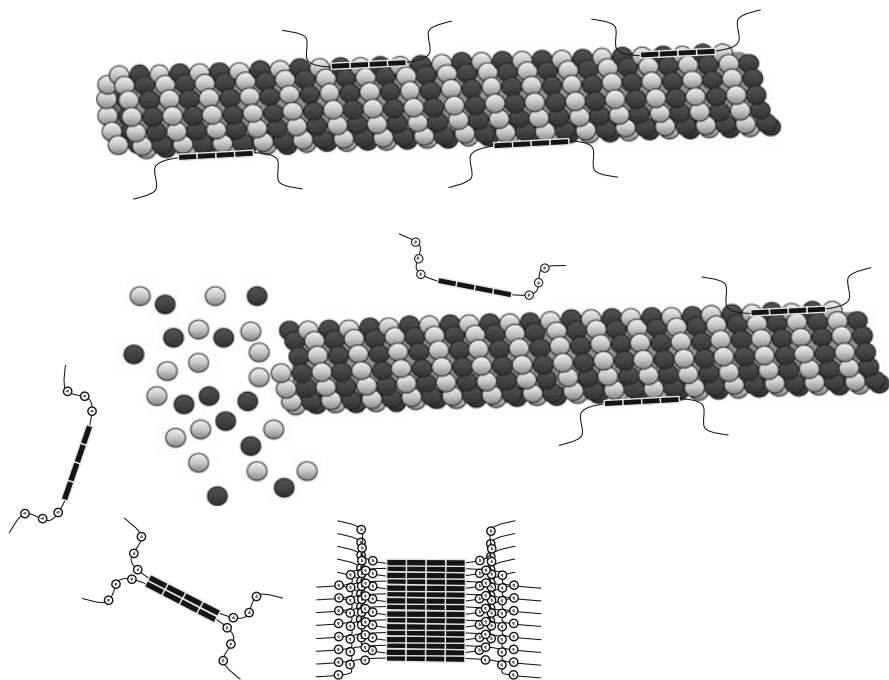


Fig. 2 Destabilization of microtubules upon tau protein release and phosphorylation. Hyperphosphorylation is correlated with tau aggregation and neurofibrillary tangles

has led to the identification of the cellular prion protein as a leading candidate [29], with evidence for downstream effects on NMDA receptors and activation of kinases that phosphorylate tau [30]. Sizes ranging from dimers and trimers [31] to dodecamers [32] and to protofibrils [33] have been suggested as toxic forms of A β , but at this time there is no consensus on which of these, if any, is most likely. Indeed, it has been suggested that there is no single toxic species but rather that a toxic “Ab soup” may be responsible [34].

3.2 *Tau*

Tau, the other aggregation-prone protein in AD, is normally associated with microtubules (Fig. 2) [35]. Although tau can help stabilize microtubules, this function is apparently redundant with other proteins, as knockout of tau is not lethal [36]. Crossing tau knockout mice with APP transgenic AD mouse models does not lead to altered A β production or deposition but results in attenuation of age-dependent learning and memory deficits [37], suggesting that tau is downstream of A β in AD pathogenesis. Dominantly inherited mutations in tau do not cause AD, but they do result in tau pathology, neurodegeneration, and other forms

of dementia [38, 39]. These and other findings suggest that A β is one of a variety of factors that can trigger pathological tau aggregation and consequent neurodegeneration.

Tau protein in the neurofibrillary tangles of AD is hyperphosphorylated, which has led to efforts to identify the responsible kinases and search for selective inhibitors [40, 41]. Although the assumption is that the increased phosphorylation of tau is important for AD pathogenesis, whether the relationship between tau hyperphosphorylation and neurodegeneration is one of cause and effect or only correlative is unclear. Aggregation of tau in the form of filaments dramatically decreases tau turnover and may therefore simply allow more time for kinases to phosphorylate the protein. Even if hyperphosphorylation is part of the pathogenic process, identifying the specific sites that are most important and the responsible kinases has been fraught with complications, especially as phosphorylation of a given site can affect phosphorylation of another site, and more than one kinase is often capable of phosphorylating a given site [42].

Despite the above critique of the role of hyperphosphorylation in AD pathogenesis, it is important to point out that phosphorylation of tau is critical to regulating its interaction with microtubules [43], and dissociation from microtubules increases the tendency of the protein to aggregate [42] (Fig. 2). Moreover, hyperphosphorylation may play a role in AD pathogenesis due to dissociation of tau and consequent destabilization of microtubules. Although knockout of tau in mice is not lethal and does not lead to neurodegeneration [36], compensation may take place in the context of deletion from conception onward. Thus, it would be important to determine the effects of conditionally knocking out the tau gene in the adult mouse brain. Furthermore, even if loss of microtubule-stabilizing function alone does not cause neurodegeneration, it may be a secondary contributor to the primary insult of tau aggregation. For this reason, microtubule-stabilizing agents have been sought for the potential treatment of AD and other “tauopathies” [44].

The aggregation of tau is also closely linked to AD pathogenesis, with neurofibrillary tangle pathology correlating better with neurodegeneration than amyloid pathology does [2]. Moreover, seeds of aberrant forms of tau are now thought to spread tau dysfunction and pathology from neuron to neuron through synapses, that is, through neuronal networks [45, 46]. This mechanism of transmission from neuron to neuron is reminiscent of how prion diseases manifest themselves in the brain. Indeed, the idea that many proteins that misfold and aggregate in neurodegenerative diseases spread through prion-like means is gaining advocates [47]. If true, this could potentially provide new means of intervening pharmacologically.

3.3 Neuroinflammation

The activation of non-neuronal cells, specifically microglial and astrocytes, along with the release of reactive cytokines is also a hallmark of AD pathology [6]. Related to macrophages, microglial are immune cells of the brain, responsible

for scavenging for debris, damaged neurons, and infectious agents. Amyloid plaques are among the brain debris engulfed through phagocytosis by microglial. Chronic over-activation of microglial leads to a state called neuroinflammation, which involves release of pro-inflammatory cytokines such as interferon- γ and interleukin-1 and subsequent tau phosphorylation and neuronal dysfunction. Astrocytes are support cells for neurons, providing critical nutrients and even eliciting signaling through release of the neurotransmitter glutamate. Reactive astrocytes are also found near amyloid plaques and can take up and degrade A β .

4 Pathogenesis and Risk Factors

The leading model for AD pathogenesis is the amyloid hypothesis [28], which posits that elevation of aggregation-prone forms of A β , either through altered production as in FAD or altered clearance as in SAD, results in the formation of toxic A β , perhaps soluble oligomers, that disrupt normal synaptic and neuronal function, trigger changes in tau, and elicit neuroinflammation. These effects ultimately lead to neurodegeneration and over years and decades to the clinical manifestation of AD.

The primary risk factor for AD is age. Incidence of AD before age 60 is rare and associated with dominantly inherited FAD mutations in APP and presenilins. After age 60, the risk increases dramatically, so that by age 85 the chance of having AD is ~40–50% [1]. For late-onset SAD, the most important genetic risk factor is apolipoprotein E: a single copy of the E4 allele increases the risk of AD three- to fourfold, and two copies increase the risk 12–15-fold [48]. These findings suggest that ApoE may be a worthwhile target for drug discovery. Diabetes and cardiovascular disease are also important AD risk factors, raising hopes that dietary and lifestyle changes may reduce lifetime risk to AD [49, 50].

5 Animal Models

Transgenic mice have been by far the primary animal models developed for AD. These have been engineered to overexpress FAD mutant forms of human APP and PS1, leading to age-dependent deposition of amyloid plaques and deficiencies in learning and memory [51]. However, these mice have demonstrated neither tau pathology nor frank neurodegeneration, leading to concerns about whether the observed cognitive deficits are elicited through bona fide AD mechanisms and not simply the consequence of gross overexpression of these proteins. To elicit tau pathology, APP/PS1 transgenic mice have been crossed with mice overexpressing human tau with point mutations that cause the AD-related frontotemporal dementia [52]. This cross has no effect on the amyloid production and deposition, but does increase tau pathology and exacerbate cognitive deficits.

Such AD animal models have been used extensively to test candidate AD therapeutics. However, the translation of promising preclinical candidates to the clinic has not been successful, raising serious concerns about the validity of the animal models, particularly of the transgenic mice that highly overexpress aggregation-prone proteins. The concern about artifacts due to overexpression has led to efforts to generate knock-in mice that express disease-causing mutant forms of APP, PS1, or tau under the control of endogenous promoters, with levels and spatiotemporal distribution that is physiological [53–55].

6 Clinical Trials

The design of clinical trials for AD experimental therapeutics is challenging [56]. First, these trials can be quite lengthy, depending on the goal. For symptomatic treatments, 6-month trial is often sufficient, whereas testing for disease modification requires at least 18 months and hundreds of subjects during phase II and thousands of subjects during phase III. This is because the underlying neurodegeneration – the fundamental aspect that is to be modified – is a slow process; extensive time and large numbers of subjects are needed to distinguish a statistically significant effect between treatment and placebo.

Second, identifying those suitable for enrollment requires tests for memory and cognition that are spaced apart in time to determine that the disease is progressive. This is still not sufficient for distinguishing AD from other dementias; biomarkers such as amyloid PET imaging are required for a clearer diagnosis [57]. The use of amyloid imaging agents is particularly critical for testing the efficacy of anti-A β therapeutic candidates: the enrollment of non-AD dementia subjects has interfered with the clear interpretation of many previous trials for AD clinical candidates.

Third, biomarkers are needed not only to enroll the right subjects but as a measure of drug effectiveness in engaging its target. In addition to PET imaging for A β deposition, CSF A β 42 is a biomarker [58]. Counterintuitively, CSF levels of this peptide are reduced in AD, presumably due its deposition in the brain. Elevated total tau and phosphorylated tau in the CSF are also considered AD biomarkers. Fourth, the optimal point of intervention is critically important but can be difficult to ascertain. For instance, amyloid pathology can begin 25 years before the onset of symptoms [59], and it is unclear if enrolling presymptomatic subjects 1–2 years prior to the emergence of cognitive deficits will be sufficient for testing anti-A β therapeutics.

7 Therapeutic Targets and Strategies

There are many potential points of therapeutic intervention for AD, both for symptomatic and disease-modifying agents. Candidate symptomatic treatments often target a neurotransmitter or its receptors, examples being cholinesterase inhibitors or 5-HT receptor subtype agonists or antagonists. Candidates for disease modification may target A β production, aggregation, or clearance; tau aggregation, loss of microtubule-binding function, or interneuronal transmission; and glial cell over-activation.

7.1 *Symptomatic Treatment*

Cholinesterase inhibitors such as donepezil (Aricept) have been a mainstay of AD therapy for nearly 20 years [60]. These agents boost acetylcholine levels at synapses, allowing for stronger cholinergic signaling. This is important because many of the neurons lost in AD critical to learning and memory are cholinergic. Cholinesterase inhibitors, however, do not affect the underlying neurodegeneration and are thus symptomatic treatments. An advantage of these agents is that they boost acetylcholine levels that are naturally released from the presynaptic terminal; that is, they potentiate the signal that is normally produced in time and space in the brain. However, cholinesterase inhibitors typically become ineffective within 6 months to a year, as more and more cholinergic neurons are lost.

Another strategy for cholinergic stimulation is developing muscarinic and nicotinic agonists to target acetylcholine receptor subtypes [61–63]. Although this could lead to chronic stimulation of these receptors, which could result in intolerable side effects, positive allosteric modulators are typically sought, allowing greater sensitivity or responsiveness to the endogenous neurotransmitter. In this way, spatial and temporal control over receptor activation and synaptic firing is maintained. 5-HT₆ receptor antagonists have also been considered for symptomatic treatment [64], primarily as an add-on with donepezil. Blocking this serotonin receptor subtype enhances acetylcholine and glutamate function. Modulation of glutamate receptors also provides symptomatic treatment; this is the basis of the approved AD drug memantine, which blocks excess signaling from the NMDA glutamate receptor subtype [60, 65].

7.2 *Targeting A β*

Targeting A β with small molecules can be indirect, by preventing its production through β - or γ -secretase, or direct, by blocking its aggregation. Many candidates targeting amyloid, directly or indirectly, have been in clinical trials; however,

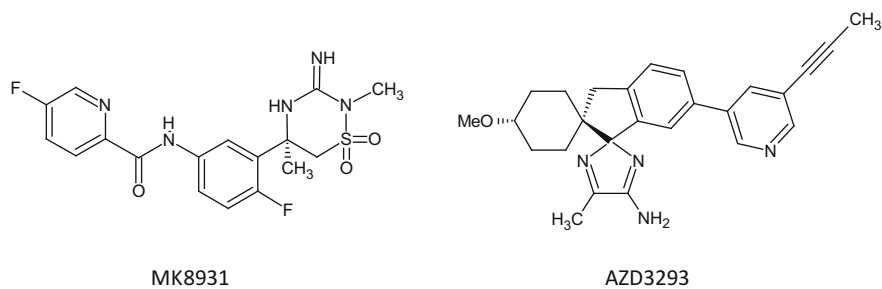


Fig. 3 BACE1 inhibitors in late-stage clinical trials

nothing has yet emerged as an approved drug. One reason for this may be what was mentioned earlier, that A β deposition begins many years, even decades before the onset of symptoms [59]. Clinical trials have typically enrolled subjects with MCI or early AD. Even a prevention trial, enrolling subjects predicted to show symptoms within the span of the trial, may be too late for an anti-A β therapeutic. The process may be tau driven and A β independent by this point, so intervening at the level of A β accomplishes little. Ongoing prevention trials with agents targeting A β are running 5 years, and perhaps there will be some clear benefits of intervening at this stage, several years prior to expected onset of symptoms.

7.2.1 β -Secretase Inhibitors

At present, β -secretase is the top anti-A β target for small-molecule therapeutics, with several compounds in the clinic, some in late-stage trials (Fig. 3), including long-term prevention trials [66]. The enzyme β -secretase, also known as β -site APP-cleaving enzyme 1 or BACE1 [67–71], is encoded by a single gene, and knockout of the BACE1 gene in mice eliminates A β production in the brain [72–74]. Moreover, crossing BACE1 knockout mice with APP transgenic mice rescues learning and memory deficits [75]. BACE1 knockout mice are viable and fertile; however, they show deficiencies in Schwann cell and oligodendrocyte sheathing of neurons [76] and develop cognitive, emotional, and synaptic problems [77], perhaps due to the inability to cleave the alternative substrate neuregulin-1 [76]. However, this occurs upon knockout of BACE1 from conception. Conditional knockout of the gene in adult mice would provide a better indication of the safety of BACE1 inhibition in elderly AD patients.

The β -secretase enzyme has been crystallized and its high-resolution structure determined, both alone and in complex with small-molecule inhibitors [66, 78]. This has allowed structure-based design and refinement of β -secretase inhibitors with high potency [66]. The biggest obstacle to β -secretase inhibitor development, however, has been brain penetration and retention. Potent inhibitors are often large molecules that do not penetrate the brain readily, and even when they do traverse the blood–brain barrier, they are often ejected by P-glycoprotein

[79]. These obstacles, however, have been largely overcome in recent years, with a number of compounds entering into human trials. Among these clinical candidates, Eli Lilly's LY2811376 could effectively block A β production in CSF in healthy volunteers; however, retinal toxicity observed in rodents led to halting human trials [80]. At present, it is unclear if mechanism-based toxicity will be a problem, as β -secretase has a number of other substrates besides APP and neuregulin-1 [81, 82].

7.2.2 γ -Secretase Modulators

The other protease responsible for A β production, γ -secretase, has also been a top target for the development of AD therapeutics [83]. However, as mentioned earlier, knockout of components of the γ -secretase complex blocks cleavage of Notch receptors, preventing essential signaling for cell differentiation, resulting in an embryonic lethal phenotype [84, 85]. Notch signaling is also critical for many cell differentiation events in adulthood. Because of this, inhibitors of γ -secretase cause serious side effects such as immunosuppression, skin lesions, and gastrointestinal distress [86–88]. Efforts to titrate the dose of γ -secretase inhibitors to effectively lower CNS A β levels while avoiding the toxicities due to deficient Notch signaling failed in advanced human trials, even with γ -secretase inhibitors that purportedly possessed selectivity for blocking proteolysis APP over that of Notch [88, 89].

For this reason, the focus with respect to γ -secretase as a target has shifted to modulators [90]. These are compounds that lower the aggregation-prone A β 42 selectively, by specifically stimulating the carboxypeptidase trimming of A β 42 to A β 38 [91]. At effective concentrations, total A β is not lowered, and Notch proteolysis is not affected. These compounds can be very safe and given at high doses [92, 93]. However, potency and brain accessibility have been problems, and at present no γ -secretase modulator is in advanced clinical trials. It should be mentioned that the success of these agents entirely depends on A β 42 being the pathogenic entity in AD. As mentioned earlier, this matter has not been entirely settled.

7.2.3 Aggregation Inhibitors

The identification of compounds that directly bind to A β and A β assemblies to prevent aggregation has been a long-studied strategy for the development of AD therapeutics [94]. The challenge of this approach over blocking the secretases is one of stoichiometry, as inhibiting an enzyme can prevent the production of many A β molecules, while direct binding requires higher levels of compound, with increased potential for off-target effects. Nevertheless, substoichiometric levels of A β -binding compounds have been reported to prevent aggregation [95]. A critical issue here is that the compounds should not prevent deposition of A β while increasing levels of potentially neurotoxic A β oligomers. Other problems with this approach have been demonstrating selectivity and brain penetration. Although

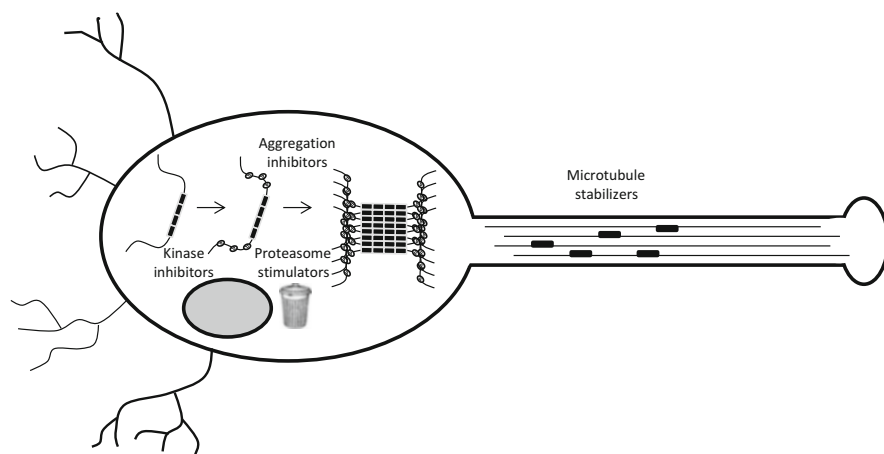


Fig. 4 Strategies for developing therapeutics targeting tau

many compounds have been reported to inhibit A β aggregation, at present none are in advanced human trials.

7.3 Targeting Tau

The deposition of tau in the form of filaments found in neurofibrillary tangles is another canonical feature of AD pathology. As mentioned earlier, tau is critical for the development of learning and memory deficits in APP transgenic mice [37]. This and other evidence suggest that tau is downstream of A β in the AD pathogenic process. As with A β , however, the pathogenic form of tau is not known, which makes it challenging to develop a strategy for developing effective therapeutics that target this protein. Nevertheless, several approaches have been pursued over the years, described below (Fig. 4).

7.3.1 Aggregation Inhibitors

Although the toxic form of tau is not known, its aggregation is closely coupled to pathogenesis, not only for AD but for other neurodegenerative diseases collectively called “tauopathies” [96]. As with A β aggregation inhibitors, the search for tau aggregation inhibitors faces challenges of identifying compounds that are potent, selective, and accessible to the brain. Nevertheless, the dye methylene blue appears to have these properties [97], although the compound has also been reported to have other effects such as enhancing protein degradation through the proteasome and autophagy and regulating gene expression [98–100]. In vivo, methylene blue can prevent tau aggregation as well as cognitive deficits in tau transgenic mice

[101, 102]. Phase II clinical trials were promising but controversial [103], and the compound failed in a recently concluded phase III trial [104, 105].

7.3.2 Kinase Inhibitors

As tau is hyperphosphorylated in AD [106, 107] and phosphorylation leads to dissociation of tau from microtubules and its aggregation [108], considerable effort has gone into identifying responsible kinases and identifying potent, selective, and brain-penetrating inhibitors of these kinases. As mentioned earlier, tau contains many phosphorylation sites, and which sites might be responsible for tau toxicity is unclear [41]. Moreover, a given site may be phosphorylated by more than one kinase, and a given kinase has other substrates besides tau. Nevertheless, several kinases have been particularly implicated in the pathological phosphorylation of tau, including GSK-3 β , CDK5, and MARKs. The development of effective inhibitors has also been reported, including examples of efficacy in transgenic mice [109]. Despite these findings, there are concerns about on-target toxicity, especially with GSK-3 β , which is involved in glucose utilization. At present, no inhibitors of kinases targeting tau are in advanced clinical trials.

7.3.3 Microtubule Stabilizers

Phosphorylation of tau regulates its interaction with microtubules, affecting the role of tau in microtubule stabilization and dynamics [43, 110]. As discussed earlier, the aggregation of tau may be the primary neurotoxic pathway for this protein, but loss of function with respect to microtubules may also be a contributing factor [111]. For this reason, microtubule-stabilizing agents have been sought for the potential treatment of AD and other tauopathies [44]. Such agents have been used as anticancer therapeutics, as microtubules are also critical for mitosis and cell replication. This fact raises the potential concern for microtubule stabilizers: in addition to requiring potency, selectivity, and brain penetration, these compounds must avoid toxic consequences of blocking normal cell replication with chronic administration. Nevertheless, promising compounds have emerged, with one (TPI-287) [112] presently in clinical trials for AD as well as two other tauopathies.

7.3.4 Increasing Clearance

Facilitating the proteolytic breakdown of tau via the proteasome is another strategy for preventing the pathological intraneuronal aggregation [113]. Although stimulation of enzymes is typically challenging, the proteasome has multiple subunits and associated regulatory proteins that open up possibilities for increasing the degradation of misfolded proteins. One example is inhibition of the proteasome component USP14 [114], which normally trims ubiquitin chains from proteins poised for

proteasomal degradation. Thus USP14 inhibits protein degradation, and inhibiting this ubiquitin hydrolase with a small molecule could enhance the intracellular clearance of tau protein. However, in vivo deficiency of USP14 revealed that this enzyme is essential for proper synaptic transmission and development of neuromuscular junctions [115]. In addition, USP14 knockout mice showed no decrease in total tau, but a concerning increase in phosphorylated tau [116]. Thus, USP14 may not be a worthwhile target, although the general idea of stimulating proteasomal degradation of tau still has merit.

7.4 Targeting ApoE

Targeting ApoE has been more challenging than targeting tau or A β . ApoE is apparently involved in A β clearance; however, it remains unresolved whether up- or downregulation of ApoE would be beneficial. Thus, even though transcription factors such as retinoic acid receptors control ApoE gene expression, it remains unclear whether agonists or antagonists to these receptors would be therapeutic or whether activating or blocking these receptors would be safe. Another approach has been to identify compounds that alter the conformation of the AD-associated ApoE4 protein so that its conformation is closer to those of ApoE3 or ApoE2 [117].

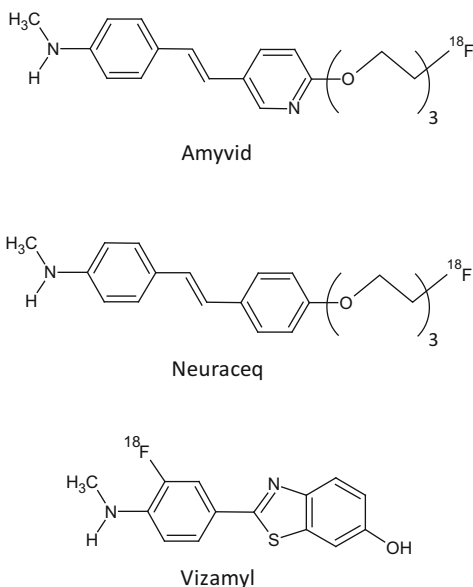
8 Imaging Agents

Critical to proper diagnosis, clinical trial selection, drug target engagement, and efficacy studies are the identification of suitable biomarkers. Toward this end, the development of imaging agents has been the one bright spot for medicinal chemistry for AD, with several agents approved for human use [118, 119]. At present all the approved imaging agents (Fig. 5) target amyloid deposits, although tau imaging agents are in the pipeline. The pharmacokinetic requirements for imaging agents are different than for therapeutic agents; for the former, high brain uptake and fast washout from normal brain are optimal.

8.1 Amyloid

Several positron emission tomography (PET) imaging agents for amyloid deposition have been approved, although only as a way to rule out AD in cases where little or no plaques are present [118, 119]. These agents can support a diagnosis of AD but cannot be used to diagnose AD on their own. Still, this is an important advance, especially for enrolling subjects into clinical trials for candidate AD therapeutics, as one in five patients diagnosed with AD is later found not to have the disease

Fig. 5 Approved amyloid imaging agents



[120]. This is because other forms of dementia (e.g., vascular dementia, frontotemporal dementia) can mimic and overlap with AD clinical presentation. The inadvertent inclusion of non-AD subjects has confounded efforts to determine efficacy of AD clinical candidates. These imaging agents have also proven useful for demonstrating target engagement by experimental therapeutics, for instance, with an anti-A β antibody that was shown to clear amyloid plaques [121]. The approved imaging agents use ^{18}F as the positron emitter, as this isotope has a 110 min half-life, compared with a 20 min half-life for ^{11}C .

8.2 *Tau*

No imaging agents for tau have been approved yet, although a number of PET imaging agents are in development and under clinical investigation [119]. For use in AD diagnosis, these agents require high specificity for tau aggregates over amyloid deposits, as the latter are much more predominant than the former. However, even without high specificity, a tau imaging agent might be useful in the diagnosis of other tauopathies, especially if amyloid deposition has been ruled out with an A β imaging agent. As tau pathology spreads from one region of the brain to another in a fairly predictable course in AD [2] and is more closely correlated with neurodegeneration, both spatially and temporally, than A β pathology is, tau imaging agents may ultimately be more useful for diagnosis and staging of the disease as well as for tracking target engagement and efficacy for anti-tau clinical candidates.

9 Perspective

The understanding of the molecular basis of AD has advanced considerably in the past 25 years. The discovery of genes that cause FAD clearly point to some form of A β as pathogenic. Studies in mice strongly suggest that pathogenic tau lies downstream of A β . However, major gaps remain, including definitive identification of the specific pathogenic forms of A β and tau, the connection between these two aggregation-prone proteins, and the role of risk factor ApoE. Transgenic mice mimic some aspects of AD pathology and presentation; however, positive results with numerous experimental therapeutics in these animals have not translated into any approved AD drugs for human use.

A β remains the top target for small molecules in the pipeline, particularly through inhibition of β -secretase. However, A β deposition can begin over two decades before symptoms, raising concerns that the pathogenic process may become tau driven and A β independent many years before anti-A β agents can be practically given. For this reason, interest in targeting tau is on the rise, with multiple approaches in development. The further development of symptomatic treatments remains important for those who have already progressed to mild-to-moderate AD and may be beyond the reach of disease-modifying therapies. Finally, imaging agents have been a success story for medicinal chemistry, with several approved agents that detect A β deposition in the brain. These imaging agents are already helping to enroll the right subjects for clinical trials and demonstrate target engagement with anti-A β clinical candidates. Better mechanistic understanding, expansion of targets and approaches to drug development, validation of more biomarkers, and refinements in the design of clinical trials should together increase the likelihood that effective therapeutics for AD will soon be realized.

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The Design, Development, and Evaluation of BACE1 Inhibitors for the Treatment of Alzheimer's Disease

Arun K. Ghosh, Emilio L. Cárdenas, and Heather L. Osswald

Abstract Alzheimer's disease (AD) is a very serious public health problem. Currently, there is no effective treatment for AD. Among the many biochemical targets for AD drug development, β -secretase (BACE1, memapsin 2) continues to be a promising drug discovery target for AD therapy. This proteolytic enzyme is a membrane-anchored aspartic acid protease that is responsible for the initial step of amyloid precursor protein (APP) cleavage, leading to the production of neurotoxic amyloid- β (A β) peptides in the brain. Since its identification and structural elucidation in 1999, extensive research efforts have led to the development of many promising classes of inhibitors against this protease. Structure-based design strategies led to the evolution of many small-molecule, peptidomimetic, and nonpeptide BACE1 inhibitors that have now overcome the key development challenges including selectivity and brain penetration. To date, 13 BACE1 drug candidates have been brought to clinical trials, and a number of them have advanced to phase II/III human trials. This chapter illustrates structure-based evolution of various classes of BACE inhibitors. Also, it provides a perspective on BACE1 inhibitor drugs for the treatment of AD patients.

Keywords β -Secretase, Alzheimer's disease, BACE1, BACE1 inhibitor, Drug design, Enzyme inhibitors, Memapsin 2, Nonpeptide inhibitor, Peptidomimetic inhibitor, Structure-activity relationship

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Abbreviations

A β	Amyloid- β protein
AD	Alzheimer's Disease
ADAS	Alzheimer's Disease Assessment Scale
ADAS-Cog	Alzheimer's Disease Assessment Scale-Cognitive Subscale
ADCS	Alzheimer's Disease Cooperative Study
ADCS-ADL	Alzheimer's Disease Cooperative Study-Activities of Daily Living
ADCS-PACC	Alzheimer's Disease Cooperative Study-Preclinical Alzheimer's Cognitive Composite
ADME	Absorption distribution, metabolism, and excretion
APP	Amyloid precursor protein
BACE1	β -Site amyloid precursor protein-cleaving enzyme 1
BACE2	β -Site amyloid precursor protein-cleaving enzyme 2
BBB	Blood-brain barrier
CatD	Cathepsin D
CCS-3D	3-Domain Composite Cognition Score
CDR	Clinical Dementia Rating Scale
CDR-SB	Clinical Dementia Rating Sum of Boxes
CFI	Cognitive Function Instrument

CNS	Central nervous system
CSF	Cerebrospinal fluid
C-SSRS	Columbia-Suicide Severity Rating Scale
ECG	Electrocardiogram
FDA	Food and Drug Administration
HIV	Human immunodeficiency virus
HTS	High-throughput screening
IC ₅₀	50% inhibitory concentration
K _i	Inhibitory constant
MMSE	Mini Mental State Examination
MRI	Magnetic resonance imaging
MWM	Morris water maze
NMDA	<i>N</i> -Methyl D-aspartate
NPI	Neuropsychiatric Inventory
NTB	Neuropsychological Test Battery
PET	Positron emission tomography
Pgp	P-glycoprotein
QT	Q-wave to T-wave interval
sAPP α	Soluble amyloid precursor protein α -fragment
sAPP β	Soluble amyloid precursor protein β -fragment
SAR	Structure-activity relationship

1 Introduction

Alzheimer's disease (AD) has emerged as a serious public health issue affecting millions of elderly people around the globe. AD is an irreversible, progressive, degenerative brain disorder that slowly destroys brain function, leading to cognitive decline, behavioral change, and deterioration of bodily function in later stages. Currently, about 44 million people worldwide are believed to have Alzheimer's disease or a related dementia [1, 2]. This number is expected to grow more than 100 million cases by 2050 [3]. The global cost of current AD patient care is estimated to be a staggering \$605 billion, which is equivalent to 1% of the world's gross domestic product [4]. The situation will get worse as there is no cure or disease-modifying treatment for AD at this time. A number of approved drugs are available only to treat symptoms in AD patients [5]. There is an urgent need for mechanism-based, effective AD therapies that will prevent, delay the onset, slow the progression, or improve AD symptoms.

Currently, five drugs are approved for the treatment of AD and related disorders. These include acetylcholinesterase inhibitors, tacrine (approved 1993), donepezil (1996), rivastigmine (1998), and galantamine (2001) [6, 7]. These drugs act by blocking the process that breaks down the neurotransmitter acetylcholine, a key signaling agent responsible for communication between nerve cells. In AD patients, decreased acetylcholine levels and loss of nerve cells are linked to worsening

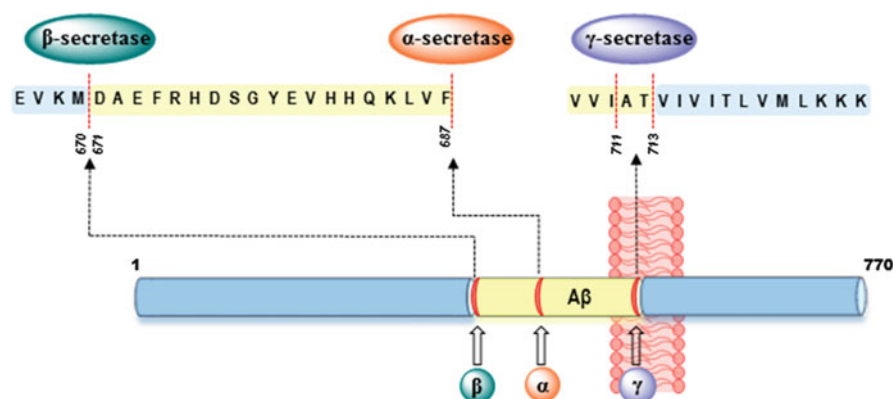


Fig. 1 Processing of APP by α -, β -, and γ -secretase. Cleavage by β -secretase provides the N-terminus of A β , while cleavage by γ -secretase provides the C-terminus of A β . Processing by γ -secretase provides both A β_{40} and A β_{42} by cleaving at residues 711 and 713, respectively. Cleavage by α -secretase occurs in the middle of the A β sequence and precludes the formation of A β

symptoms. Acetylcholinesterase inhibitors prevent breakdown of acetylcholine and help maintain adequate concentration of in the brain. Another Alzheimer's drug, memantine, is an *N*-methyl D-aspartate (NMDA) receptor antagonist, approved in 2004 [8, 9]. The drug regulates the activity of glutamate in the brain. Attachment of the neurotransmitter glutamate to the cell surface of NMDA receptors allows calcium to enter the cell. This is an important event in cell signaling, as well as in learning and memory. However, in AD patients, excess glutamate released from damaged cells leads to overexposure to calcium and accelerates cell damage. Memantine disrupts this chain of events by blocking the NMDA receptors.

AD drug development has been a formidable challenge [5]. A number of current AD drug development efforts are directed towards a breakthrough AD therapy that would treat the underlying disease mechanism and halt or slow down the neuronal cell damage that causes progressive deterioration. One of the major hallmarks of Alzheimer's disease is the accumulation of neuritic plaques containing the 40–42 residue amyloid- β (A β) peptides and neurofibrillary tangles composed of tau protein in the brain [1, 10]. Since a high brain A β level is regarded as an important factor in AD pathogenesis, clinical intervention to reduce its production has become a logical approach for AD therapy development [11, 12]. The origin of neuritic plaques can be attributed to proteolytic processing of amyloid precursor protein (APP) (Fig. 1). More specifically, the sequential cleavage of APP by β -secretase (BACE1, memapsin 2) and γ -secretase yields A β [13, 14]. The two main A β products of APP processing, A β_{40} and A β_{42} , both play a key role in the aggregation of neuritic plaques. While A β_{42} is more prone to aggregation, increasing levels of both peptides are observed early in the pathology of AD, and these rising levels are correlated with the severity of dementia observed in AD patients [15].

The preceding plaques are shown to be neurotoxic and eventually lead to neuronal death [16]. Therapeutic inhibition of β - or γ -secretase has been pursued in AD drug development since these two proteases are the first and second steps in the APP processing leading to $A\beta$ production [11, 17]. Following the cloning and identification of BACE1 in 1999, significant research efforts led to the development of several classes of potent and selective BACE1 inhibitors as a potential treatment of Alzheimer's disease [18, 19]. Herein, we outline structure-based evolution of various classes of BACE1 inhibitors, clinical evaluation of these inhibitors, and their perspective in the treatment of AD.

1.1 The Amyloid Hypothesis of AD

Most AD patients have cases of sporadic AD; however, genetic predispositions to AD can occur within families. While both sporadic and familial AD are pathologically the same, familial AD tends to occur as early-onset AD, with the symptoms appearing as early as age 30 [1]. Familial AD is caused by mutations in either APP or the presenilins responsible for γ -secretase activity. APP mutations result in more efficient cleavage by BACE1, increased propensity for aggregation, or an increase in the more aggregation-prone $A\beta_{42}$ [20]. There are many more presenilin mutations known, which lead to increased γ -secretase processing of APP into pathogenic $A\beta_{42}$ over the more phenotypically normal $A\beta_{40}$. These mutations in early-onset AD and similar pathology of sporadic AD have led to the amyloid hypothesis of AD progression (Fig. 2).

The amyloid hypothesis of AD proposes that the formation of $A\beta$ plaques initiates the flow of outcomes that ultimately leads to dementia in AD patients

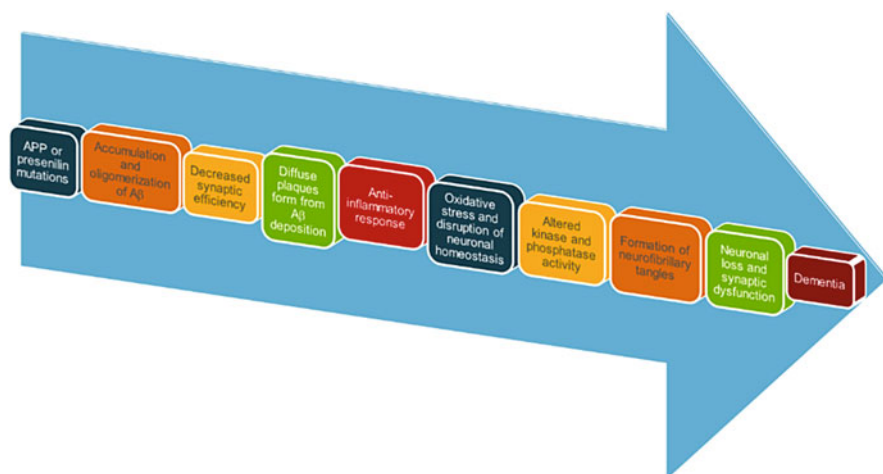


Fig. 2 Progression of events postulated by the amyloid cascade hypothesis

[21]. The accumulation of A β peptides leads to their oligomerization. This event results in decreased activity at the neuronal synapses. These soluble oligomers further associate to form A β plaques. These plaques trigger an anti-inflammatory response causing oxidative stress of the neurons. This can disrupt normal kinase and phosphatase activity, resulting in the hyperphosphorylation of tau protein and subsequent neurofibrillary tangle formation. This adds to abnormal signaling and further synaptic impairment. Ultimately, these events result in neuronal death and dementia in the AD patient [22].

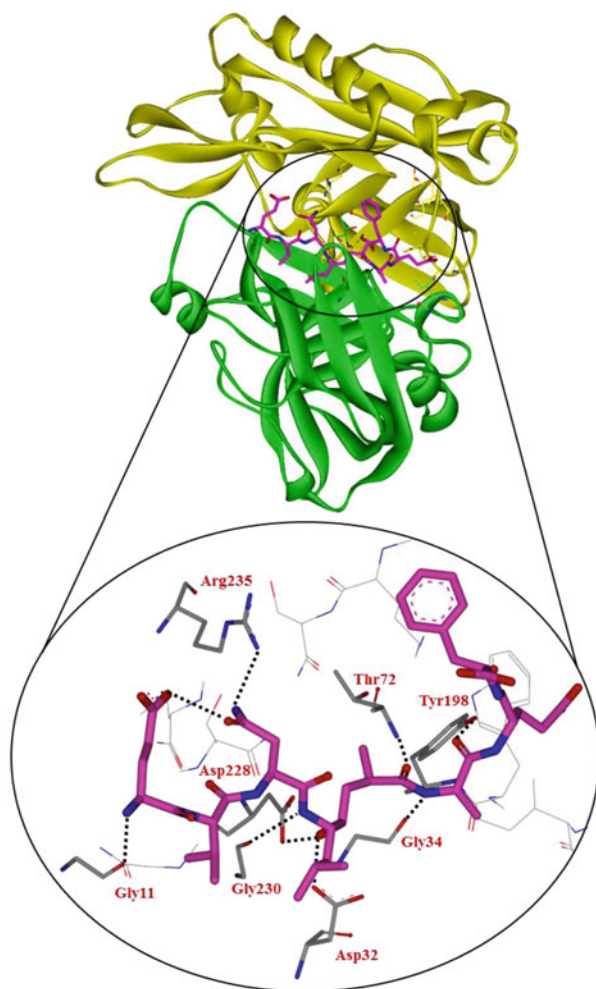
While the occurrence of familial AD and the genetic mutations associated with familial AD are strong arguments for the amyloid hypothesis, some observations remain unexplained [23]. It was once suggested by histological studies that the number of A β plaques in a diseased brain does not directly correlate to the degree of cognitive degeneration in AD patients. However, biochemical and later histological studies have shown that there is a correlation between plaque formation and cognitive decline [15, 23]. While there are further questions regarding the exact neuronal toxicity of A β in vivo, the amyloid hypothesis is still broadly accepted as the general pathological cascade of events in AD [21].

2 BACE1 as a Target for AD

BACE1 is an aspartic acid protease that plays a prominent role in the generation of neurotoxic A β plaques which has been strongly associated with AD pathology (Fig. 3) [24–27]. In light of the initial cloning and further elucidation of the role of BACE1 in AD pathology [26, 28], it has been determined that the inhibition of BACE1 may serve as a promising therapy for AD.

The proteolytic cleavage of APP by BACE1 marks the initial step of amyloidogenesis, which in turn triggers the later events of AD pathogenesis [29–31]. Additionally, the development of successful clinical protease inhibitors (PIs) for HIV protease, another extensively studied aspartic acid protease, has established an invaluable precedent for the development of other aspartic acid protease inhibitors [32]. It was envisioned that knowledge gathered from previous successful aspartic acid protease inhibitors could be utilized to develop effective BACE1 inhibitors. Upon further investigation of the enzyme, it was discovered that BACE1-null mice appeared to be phenotypically normal, with few detectable abnormalities [30, 31, 33]. These mice were viable and fertile and displayed normal tissue histology and blood chemistry. However, upon further analysis of BACE1^{-/-} mice, multiple neuronal phenotypes were discovered, including seizures, memory impairments, reduced myelination, and schizophrenia, among others [34–39]. It is possible that complete cessation of BACE1 activity may lead to unwanted mechanistic side effects. The establishment of a proper therapeutic window would allow for proper inhibition of BACE1 in order to slow or halt the progression of AD while allowing other necessary BACE1 functions to continue.

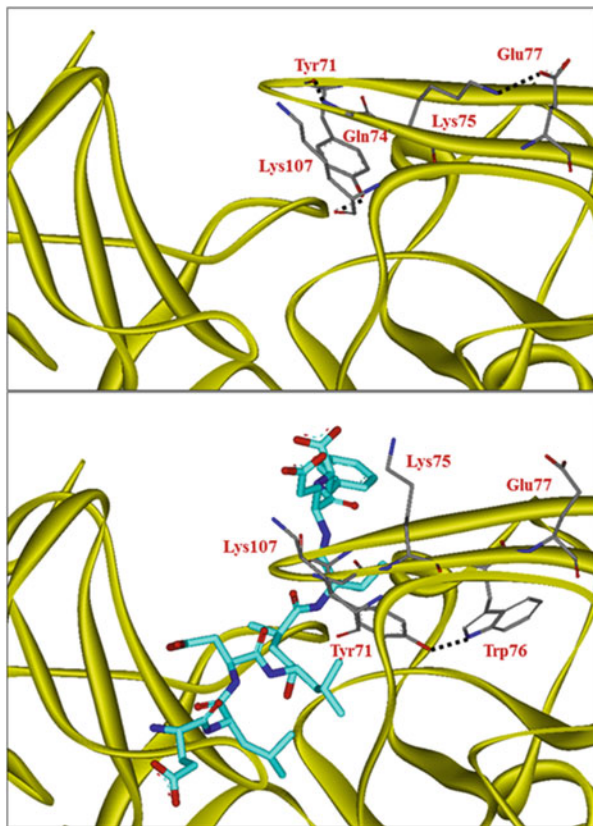
Fig. 3 Overall structure of BACE1 and interactions of inhibitor OM99-2 (**2**) in the BACE1 active site (PDB: 1FKN)



The propensity for membrane penetration of BACE1 inhibitors is vital to clinical success as A β production Leading to AD takes place in the brain [40]. The inability to generate compounds that are permeable through cell membranes and the blood–brain barrier (BBB) would discredit the inhibition of BACE1 as a viable therapy for AD. Validation studies have shown that peptide and sterol linkers have allowed inhibitors to penetrate sites of A β accumulation as well as diminish A β brain levels in AD mice [41, 42]. However, once inhibitors enter into the brain, they face ejection via P-glycoprotein (Pgp). Knockout studies have shown that Pgp-null mice treated with brain-penetrant BACE1 inhibitors resulted in a decline in A β levels, while wild-type mice did not benefit from such treatment [43].

The structural features of BACE1 also provide a challenge when it comes to inhibitor design. The type 1 transmembrane protein is characterized by its

Fig. 4 Flap-open (*top*, apoprotein, PDB: 1SGZ) and flap-closed (*bottom*, bound to OM00-3, PDB: 1M4H) BACE1. Stabilizing hydrogen bonds are shown as *black dashed lines*



expansive catalytic domain which is marked by the centrally located catalytic aspartates Asp32 and Asp228. Free BACE1 features a flap-open conformation that is energetically stable due to the multiple hydrogen bonds in the flap region of the enzyme (Fig. 4, top) [44]. When a substrate is bound, BACE1 assumes a flap-closed conformation, which results in the loss of these optimal hydrogen bonds (Tyr71-Gly74, Lys75-Glu77, Tyr71-Lys107) (Fig. 4, bottom). Interaction with the substrate, along with the formation of a new hydrogen bond between Tyr71 and Trp76, helps to stabilize this conformational shift [44]. In the open conformation, substrates are allowed to enter the active site of the enzyme via a cleft. However, the presence of a bottleneck within the enzyme inherently favors flexible substrates. It is thought that this bottleneck feature may serve as a factor in substrate selectivity [44]. Meanwhile, the somewhat broad specificity in most of the subsites along the active site poses a major hurdle in inhibitor design efforts. X-ray crystal structures of BACE1 validate that the active site is part of a long cleft that serves as a template for potential interactions with designed inhibitors [45, 46].

3 Evolution of BACE1 Inhibitors

BACE1 is an aspartic acid protease which possesses two highly conserved aspartic acid residues in the active site for catalytic cleavage of its peptide substrate, APP. Its catalytic mechanism involves the coordination of a water molecule between the active site aspartates, where one aspartate activates the water molecule for nucleophilic attack on the carbonyl carbon of the substrate scissile site. The resulting tetrahedral intermediate rearranges to provide two peptide fragments. All current aspartic acid protease inhibitor drugs are designed based upon a transition-state mimetic concept which incorporates hydroxyethylene isosteres at the scissile site [19]. Since the catalytic aspartates of BACE1 are similar to other aspartic acid proteases, such as HIV protease and renin, similar principles were utilized for BACE1 inhibitor design at the outset [19]. BACE1 cleaves APP and generates A β mainly in the endosomes of neurons. Therefore, for BACE1 inhibitors to be clinically effective, they must have the necessary properties to cross the BBB and neuronal membranes. Furthermore, clinically relevant inhibitors must exhibit a promising drug-like absorption, distribution, metabolism, and excretion (ADME) profile. Along with good ADME properties, inhibitors must establish selectivity against other aspartic acid proteases. BACE2 is the closest homologue of BACE1 with 64% of the primary structure conserved within both proteases [47]. Cathepsin D (CatD) is a significant off-site target, as it is the most abundant aspartic acid protease found in human cells [48]. Despite many challenges in the design of effective BACE1 inhibitors, there has been enormous progression on this front. Many classes of preclinical inhibitors with impressive potency and selectivity have evolved over the years. As many as 13 drug candidates have entered into clinical development. Herein, we provide a brief overview of the structural evolution of drug-like BACE1 inhibitors.

3.1 Structure-Based Design of Inhibitors

The catalytic properties of BACE1 were thoroughly investigated following its cloning. It has an elongated substrate-binding site that can accommodate up to 11 substrate residues. BACE1 shows a broad specificity. The initial specificity and knowledge of kinetics were obtained from the hydrolysis of several peptides [26, 49]. It is noteworthy that Swedish APP (SEVNL/DAEFR) gets cleaved by BACE1 40-times faster than wild-type APP (SEVKM/DAEFR). Furthermore, specificity studies revealed that alanine is preferred at P1'. This information was used for the design of the first potent substrate-based inhibitors containing seven and eight residues with a nonhydrolyzable Leu-Ala-based hydroxyethylene dipeptide isostere [50]. Inhibitor **1** ($K_i = 36$ nM) is considerably less potent than inhibitor **2** ($K_i = 1.6$ nM), which contains eight residues (Fig. 5). A statin-substituted

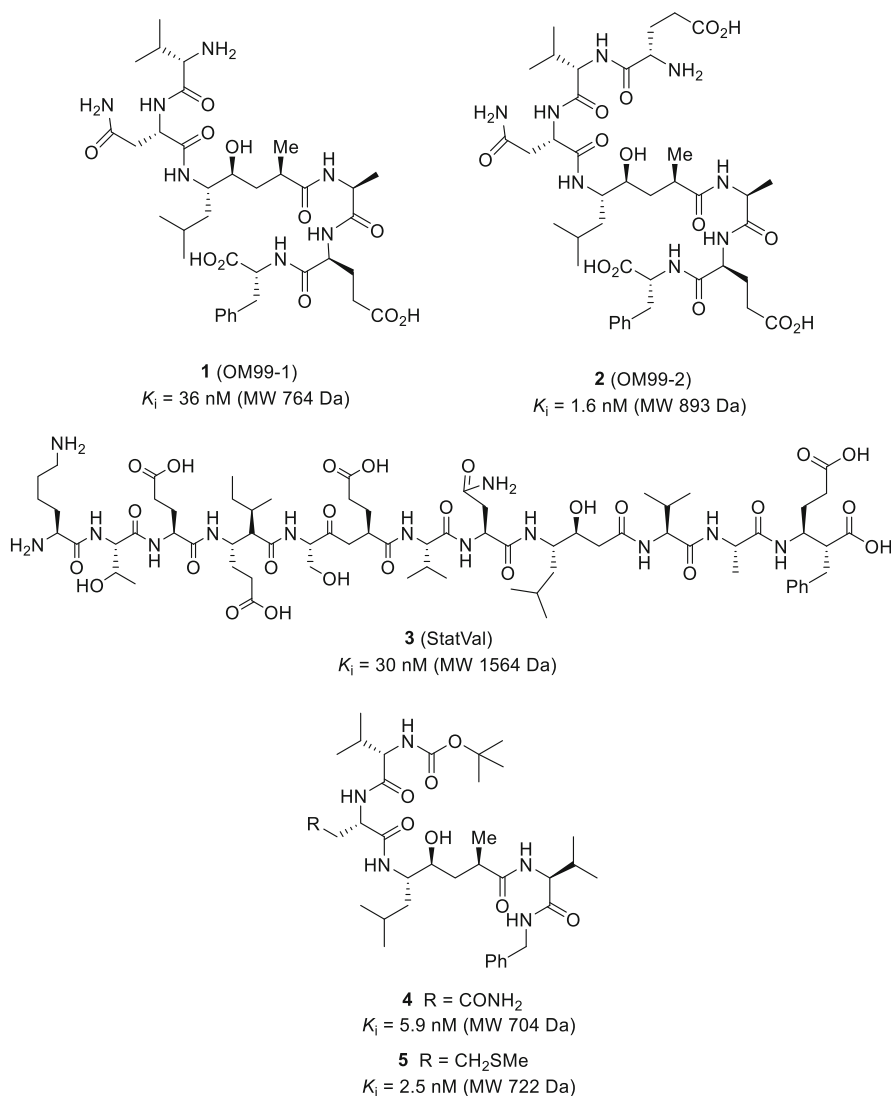


Fig. 5 Structures of pseudopeptidic inhibitors 1–5

inhibitor was designed by incorporating APP residues. Inhibitor **3** ($IC_{50} = 30 \text{ nM}$) shows similar inhibitory activity as inhibitor **1** [25].

The X-ray structure of the protease domain of human BACE1 bound to inhibitor **2** was determined at $1.9 \text{ \AA}$ resolution [45]. This X-ray structure provided critical molecular insight into the ligand-binding site interactions in the active site. The structure revealed that six of the eight residues (P2'–P4) are bound in the active site in an extended conformation. The catalytic aspartates Asp32 and Asp228 form four hydrogen bonds with the hydroxyl group of the transition-state isostere. The

inhibitor also forms ten additional hydrogen bonds throughout the active site including the flap region of BACE1.

The structure shows a rare kink in the S2' subsite where the P2' carbonyl forms a hydrogen bond with the Tyr198 hydroxyl group. Therefore, this interaction may be exploited for selectivity design. Interestingly, both the P3'- and P4'-side-chain residues do not form any specific interactions with BACE1, suggesting that these ligands can be further optimized. Based upon this molecular insight, inhibitor **4**, with an *N*-benzyl amide in place of P3' and P4' residues, showed potent BACE1 activity [51]. The inhibitor makes a number of critical hydrogen bonding interactions in the S2–S4 subsites. These include a hydrogen bond between P2-Asn with Arg235 in the S2 subsite and an intramolecular hydrogen bond with P4-Glu and P2-Asn, which may facilitate hydrogen bonding with Arg235. Structure-based replacement of the P2 ligand resulted in a number of potent inhibitors with reduced molecular weight and peptidic features. Inhibitor **5** is nearly as potent as inhibitor **2** [51].

3.2 Design of Peptidomimetic Inhibitors

The design of substrate-based inhibitors and their X-ray structural studies enabled structure-based design of numerous highly potent peptidomimetic inhibitors with reduced molecular weight and drug-like properties. A variety of dipeptide isosteres, including hydroxyethylene, hydroxyethylamine, and statins, were integrated into the design of these inhibitors. A number of previous reviews cover the evolution of these inhibitors in detail [11, 32]. Herein, we provide a brief highlight of peptidomimetic inhibitors that have addressed specific issues such as reduction of peptidic characteristics, improved cellular and in vivo properties, and design of selective inhibition which are critical to drug development.

3.2.1 Hydroxyethylene-Based Inhibitors

Extensive research efforts in the development of peptidomimetic BACE1 inhibitors led to compounds with molecular weights of 550–650 Da. A number of BACE1 inhibitors have shown good selectivity against other human aspartic acid proteases, particularly against two of the eight aspartic acid proteases deemed necessary, BACE2 and CatD. BACE2 selectivity may be critical, as it has similar specificity as BACE1. The other aspartic acid protease, cathepsin D, is highly abundant in cells, and therefore lack of selectivity will greatly reduce drug concentration of BACE1 inhibitors.

Structure-based design of highly selective inhibitors is shown in Fig. 6. Compound **6** exhibited enhanced selectivity while maintaining the potency of previous inhibitors ($K_i = 0.3$ nM) [52]. It displayed an impressive 436-fold selectivity over CatD and 1,186-fold selectivity over BACE2. The elucidation of a **6**-bound X-ray

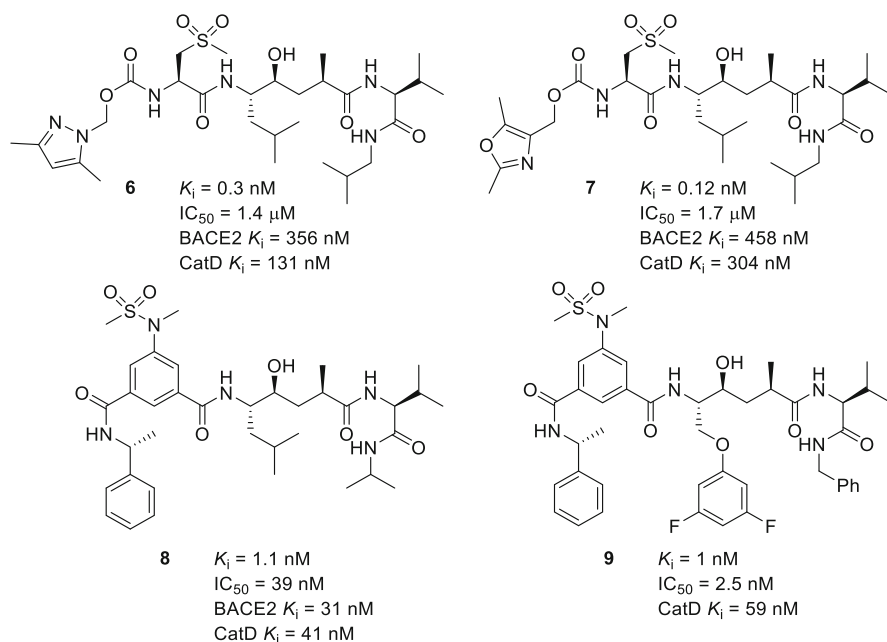
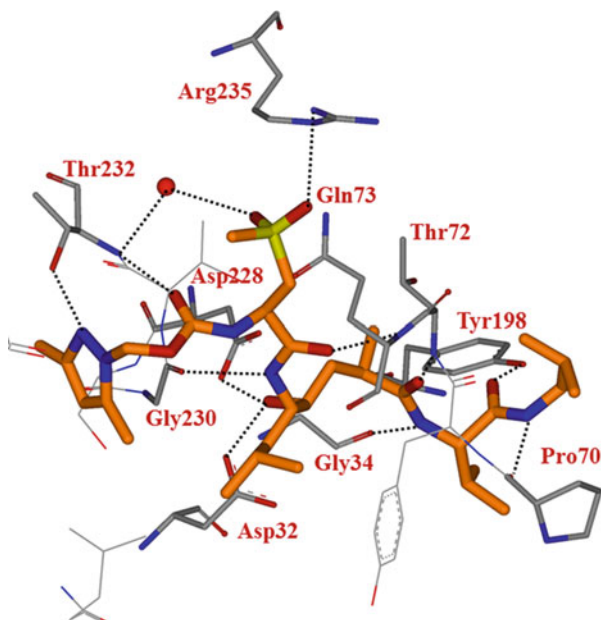


Fig. 6 Structures and activity of hydroxyethylene-based inhibitors **6–9**

crystal structure of BACE1 provided important insight of this observed selectivity. As shown in Fig. 7, the key interactions were observed between Thr232 and a nitrogen within the pyrazole heterocycle, while methyl substituents located on the heterocycle were found to also make interactions within the hydrophobic S3 subsite. The P2 sulfone made other significant hydrogen bonding interactions with Arg235 and a water-mediated hydrogen bond to Thr232 [52]. Substitution of the pyrazolylmethyl ring with the corresponding oxazolylmethyl ligand furnished compound **7**, which was found to have improved potency ($K_i = 0.12 \text{ nM}$) as well as significantly improved selectivity against BACE1 (3,800-fold over BACE2 and 2,500-fold over CatD) [52]. Though inhibitors **6** and **7** were able to display enhanced selectivity, they exhibited IC_{50} values in the micromolar range ($1.4 \text{ }\mu\text{M}$ and $1.7 \text{ }\mu\text{M}$, respectively) in Chinese hamster ovary cells [52]. Since A β IC_{50} and BBB penetration are important features for viable AD therapies, further design modifications must be made to improve subcellular membrane permeability.

In an effort to improve cellular inhibitory activity, inhibitor **8** was designed by incorporating isophthalamide derivatives within the S2 subsite [53]. Inhibitor **8** displayed a K_i of 1.1 nM and a cellular IC_{50} of 39 nM in Chinese hamster ovary cells. Molecular modeling showed that the *N*-methylsulfonamide substituent on the P2 isophthalamide fit nicely in the S2 site. This ligand can make extensive hydrogen bonding interactions with Asn233, Ser325, and Arg235. The model also revealed that the (*R*)- α -methylbenzylamide moiety at the P3 site makes favorable hydrophobic contacts in the S3 pocket [53]. Intraperitoneal injections of 8 mg/kg of

Fig. 7 X-ray crystal structure of **6**-bound BACE1 (PDB: 2G94)



compound **8** reduced plasma A β ₄₀ in Tg2576 transgenic mice by 30% after 8 h. Inhibitor **8** also displayed a 28-fold selectivity over BACE2 and 37-fold selectivity over CatD [53]. Inhibitor **9** featuring a difluorophenyl ether moiety as the P1 ligand proved to be potent with an enzymatic IC₅₀ of 2.5 nM [54].

3.2.2 Hydroxyethylamine-Based Inhibitors

Hydroxyethylamine transition-state mimics have been widely utilized in the design of several FDA-approved HIV-1 protease inhibitors [55, 56]. BACE1 inhibitors with hydroxyethylamine isosteres have been investigated extensively. An early example of a pseudopeptide-based inhibitor containing a hydroxyethylamine isostere is compound **10** in Fig. 8 [57]. This pseudopeptidic inhibitor incorporated a P1 leucine side chain and a transition-state hydroxyl group with an (*S*)-configuration. Further structure-activity relationship studies revealed that a P1-phenylalanine side chain and a (*R*)-hydroxyl configuration are preferred by the BACE1 active site. Inhibitor **11**, with a phenylalanine P1 side chain and a (*R*)-hydroxyl configuration, showed good BACE1 inhibitory activity as well as A β cellular inhibitory activity [58]. It exhibited a 15-fold selectivity over BACE2, 500-fold selectivity over CatD, and greater than 3,000-fold selectivity over renin.

Inhibitor **12** incorporated a lipophilic 3-methoxybenzyl group as the P1 ligand and a phenylalanine side chain as the P1' ligand. This inhibitor exhibited excellent properties, showing an enzymatic *K*_i of 1.8 nM and cellular IC₅₀ of 1.0 nM [59, 60]. An X-ray crystal structure of **12**-bound BACE1 was determined at 2.05 Å resolution.

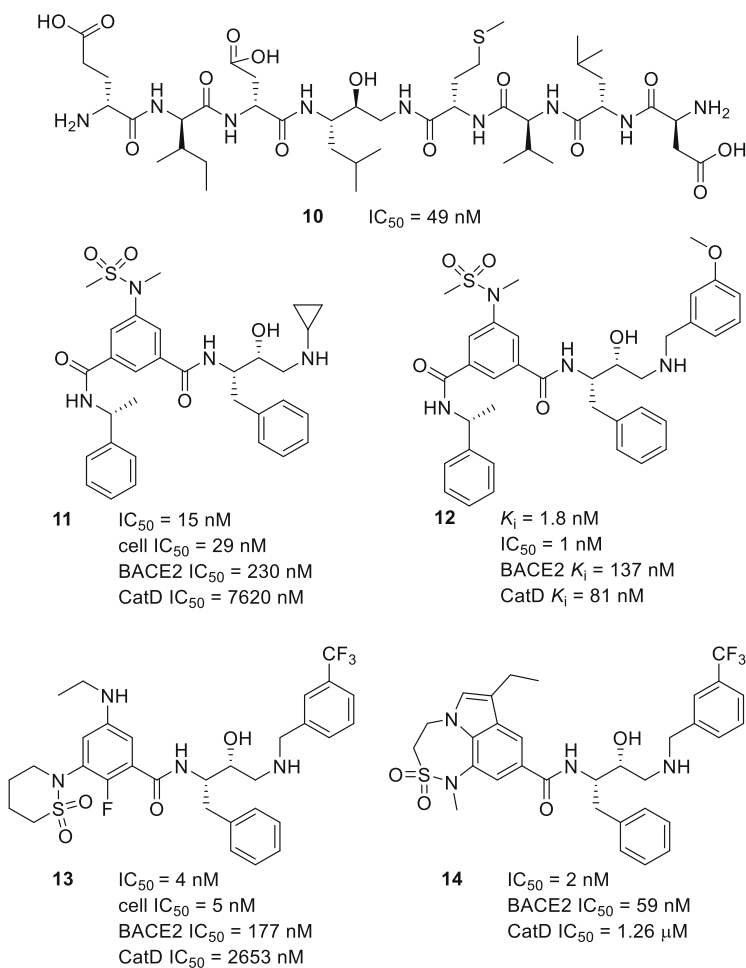
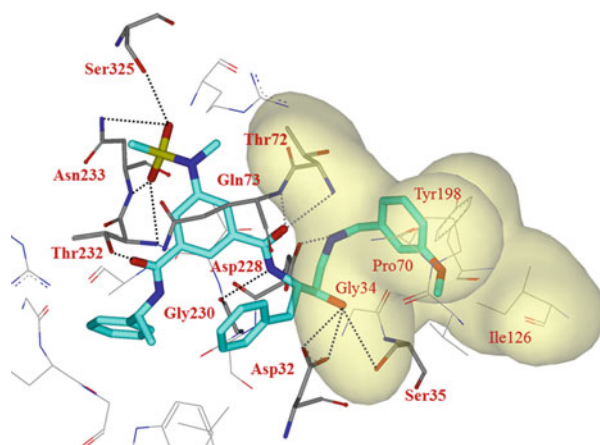


Fig. 8 Structures and activity of hydroxyethylamine-based inhibitors **10–14**

As shown in Fig. 9, incorporation of the P1' methoxybenzylamine resulted in extensive hydrophobic interactions with Gly34, Ile126, Tyr198, Pro70, Tyr71, and Thr72. The transition-state hydroxyl group formed two hydrogen bonds with Asp32; the other catalytic aspartic acid, Asp228, was bound by the hydroxyethylamine nitrogen. Both oxygens of the sulfonamide moiety of the P2 isophthalamide group formed a network of hydrogen bonds with Ser325, Asn233, and Thr232 [60].

Inhibitor **12** showed a 23-fold selectivity over CatD and 39-fold selectivity over BACE2. An intraperitoneal administration of this inhibitor with a dose of 8 mg/kg in Tg2576 mice resulted in a 65% decline of $A\beta_{40}$ levels [60]. An intravenous administration of 4 $\mu\text{g/g}$ ^{14}C -radiolabeled **12** in rats showed the presence of 16% of the inhibitor in the brain over 24 h, demonstrating the BBB

Fig. 9 X-ray crystal structure of **12** in the BACE1 active site (PDB: 2VKM)



penetrability of inhibitor **12** [59]. Further in vivo cognitive studies were carried out by installation of an osmotic pump in transgenic Tg2576 mice. The inhibitor was administered at 33.4 $\mu\text{g/g/day}$. Four experiment groups at varying ages and treatment for varying lengths of time were investigated. The first cohort began treatment with 5.5-months old and sustained treatment for 6.7 months. Cognitive performance, as measured by the Morris water maze (MWM), did not improve at 1.5 or 4.6 months. However, at 6.7 months, the treated group performed significantly better than the control [59]. In older mice (aged 16 months), the administration of inhibitor **12** did not provide any significant cognitive rescue after 4.4 months of treatment, despite the reduction of $\text{A}\beta$ levels similar to that of younger cohorts [59]. These results indicated that even a partial reduction of $\text{A}\beta$ production and neuritic plaque was sufficient to rescue cognitive decline in younger transgenic AD mice. There were no observable signs of toxicity or evidence for the accumulation of unprocessed APP [59].

Inhibitor **13**, with a six-membered sultam, showed good BACE1 IC_{50} and cellular $\text{A}\beta$ activity [61]. The *meta*-ethylamine located in the S3 site provided optimum selectivity and potency with a 44-fold selectivity over BACE2 and a 663-fold selectivity over CatD. A fluorine at the C2 position of the isophthalic aryl ring improved potency in cells expressing both wild-type and Swedish-mutant APP ($\text{IC}_{50} = 5 \text{ nM}$ and 40 nM , respectively) [61]. Inhibitor **13** showed modest oral bioavailability in transgenic mice studies. The administration of oral doses of 250 mg/kg twice daily showed as much as a 23% reduction in $\text{A}\beta_{42}$ levels within the diseased mouse brain. When dosed alongside a Pgp inhibitor, $\text{A}\beta_{42}$ reduction increased to 55% [61]. Tricyclic sultam derivatives, as represented by inhibitor **14**, were developed in order to optimize the metabolic stability of inhibitors. This inhibitor showed a BACE1 IC_{50} value of 2 nM and modest selectivity over BACE2 and CatD [62].

3.2.3 Carbinamine-Based Inhibitors

Further structural modifications of traditional transition-state isosteres were pursued in order to optimize the BBB penetration of inhibitors. As shown in Fig. 10, potent carbinamine-derived inhibitors were designed and evaluated. Inhibitor **15**, containing a primary amine, showed good BACE1 activity and excellent selectivity against renin [63]. As shown in Fig. 11, the primary amine functionality presumably interacts with the catalytic aspartates in the active site. The two oxadiazole nitrogens likely form hydrogen bonds with the backbone of Gln73 and Thr72.

Further optimization of ligand binding led to the evolution of inhibitor **16**, where both the P2 and P3 ligands were modified to improve potency and cellular permeability [64]. It exhibited excellent potency, yet its selectivity against BACE2 was

Fig. 10 Structures and activity of carbinamine-based inhibitors **15–17**

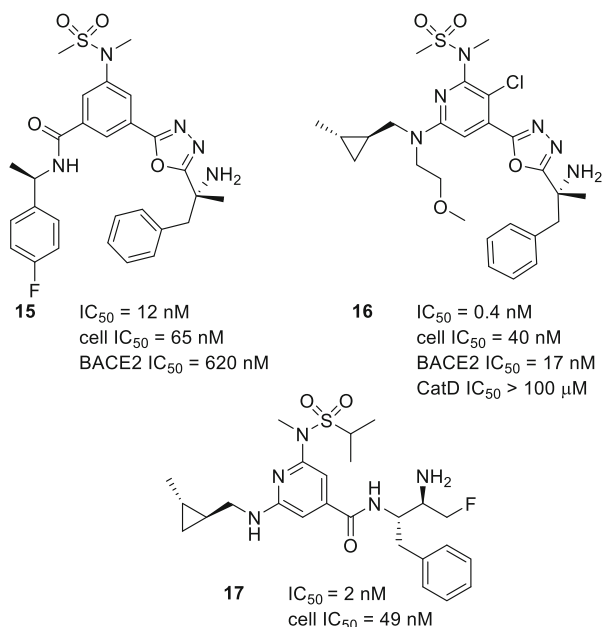
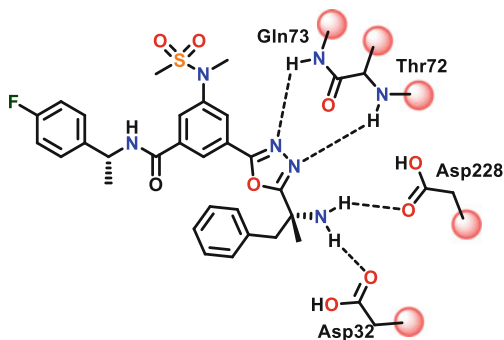


Fig. 11 Binding mode of BACE1 inhibitor **15**



moderate (42-fold). However, it showed enhanced selectivity against CatD. Intraperitoneal administration of **16** at a 100 mg/kg dose resulted in a 26% decline of postinjection A β_{40} levels. Further, time- and dose-dependent studies in rhesus monkeys showed a 65% reduction of plasma A β_{40} levels after 4 h, a return of levels after 8 h, and a complete recovery of A β_{40} after a 24-hour period. Inhibitor plasma concentration was determined to be 3.8 μ M. The oral bioavailability of the inhibitor was found to be poor. However, when dosed with pharmacokinetic booster ritonavir, the bioavailability of the inhibitor improved to 83% [64, 65].

Inhibitor **17** incorporated the 2,6-diaminoisonicotinamide core of inhibitor **16**. It showed potent BACE1 activity and cellular A β inhibitory activity of 49 nM [66]. In vivo studies in transgenic mice using 50 mg/kg i.v. bolus showed decreased A β_{40} levels in the brain by 34% at 3 h post-dose [66]. Pharmacokinetic evaluation in rats showed a high clearance and volume of distribution (Cl = 45.8 mg/min/kg and Vd = 3.9 l/kg). Unfortunately, the half-life of inhibitor **17** in vivo was very short, and the bioavailability was poor ($t_{1/2}$ = 1.6 h and %F = 13%) [66].

3.2.4 Reduced Amide-Based Inhibitors

X-ray structure-based design of BACE1 inhibitors with a reduced amide isostere provided very potent and selective inhibitors. As shown in Fig. 12, inhibitor **18** showed cellular A β inhibitory activity of 22 nM [67]. Subsequent optimization of ligand binding, particularly in the S1' subsite, led to very potent and highly selective BACE1 inhibitors. As shown in inhibitor **19**, an (*S*)-hydroxyl group was incorporated to form hydrogen bonds with substituents in the S1' subsite [68]. It exhibited a 7,000-fold selectivity against BACE2 and 250,000-fold selectivity against CatD. The corresponding inhibitor with a (*R*)-configuration on the P1' hydroxyl group was significantly less potent and selective. The X-ray crystal structure of **19**-bound BACE1 was determined at 2.2 Å resolution. As shown in Fig. 13, the reduced amide isostere formed two tight hydrogen bonds with the active site aspartates. The allo-threonine hydroxyl group is within proximity to form a hydrogen bond with the Tyr198 side chain hydroxyl group. Since this interaction is absent in inhibitor **18**, the enhanced potency and selectivity of inhibitor **19** may be due to specific interactions with Tyr198. Further structural modification of the P2 ligand with a 7,6,5-tricyclic indole resulted in very potent and selective inhibitors as represented in inhibitor **20**. No in vivo studies were reported for these inhibitors.

3.2.5 Macrocyclic Peptidomimetic Inhibitors

The large active site of BACE1 provides an opportunity to design macrocyclic BACE1 inhibitors with a prearranged bioactive conformation. As shown in the X-ray structure of substrate-based inhibitor **2**-bound BACE1 (Fig. 1), the S1–S3 subsites are open in nature. Thus, macrocyclic rings formed between the P1 ligand

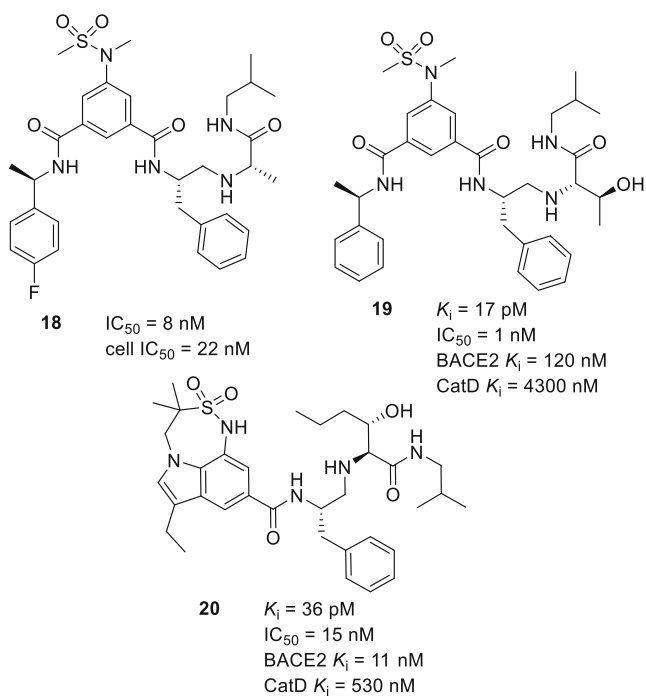


Fig. 12 Structures and activity of reduced amide-based inhibitors **18–20**

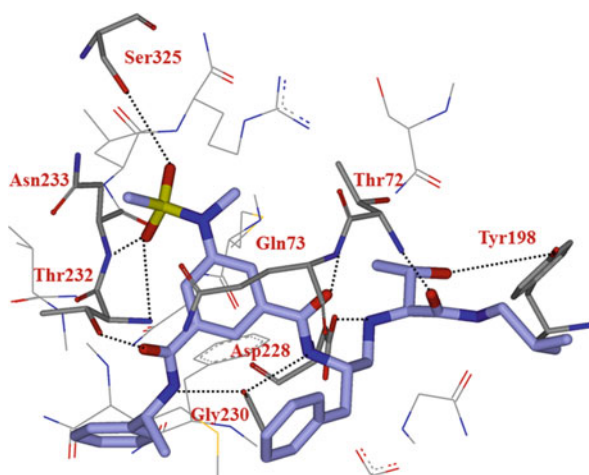


Fig. 13 X-ray crystal structure of inhibitor **19**-bound BACE1 (PDB: 4GID)

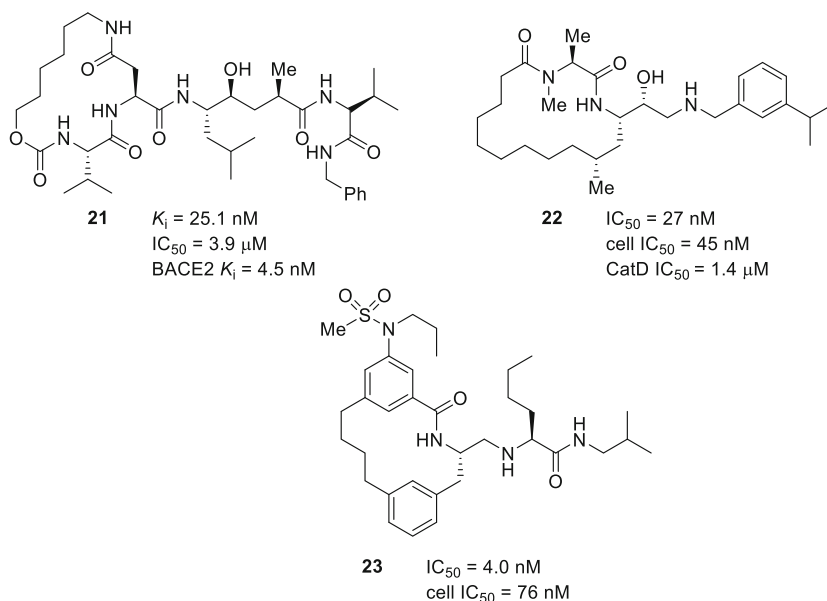


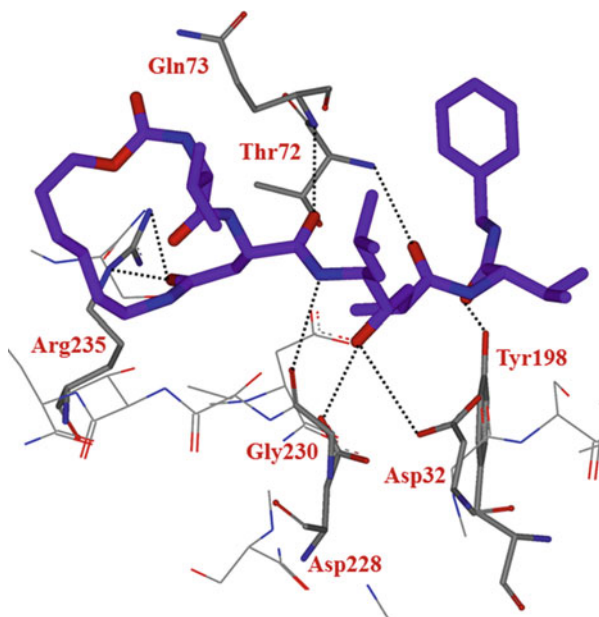
Fig. 14 Structures and activity of peptidomimetic macrocyclic-based inhibitors **21–23**

and the nitrogen of a P2 amide can be accommodated. Macrocyclic inhibitors may lead to more selective inhibitors as the bond rotations are restricted.

Initial examples took advantage of the X-ray structural insight provided by the substrate-based inhibitor **2**-bound BACE1, as shown in Fig. 14. Inhibitor **21**, with a 16-membered macrocycle containing an amide and urethane functionality, showed good BACE1 enzymatic activity [69]. Cyclic inhibitors were more potent than acyclic inhibitors. Also, studies showed that ring sizes 14–16 can be accommodated. An X-ray structure of inhibitor **21**-bound BACE1 showed key ligand-binding site interactions. As shown in Fig. 15, the Leu-Ala dipeptide isostere binds similarly to inhibitor **2**. Furthermore, the P2 asparagine on the macrocycle is able to interact with Arg235 in the S2 subpocket. This class of inhibitors, however, did not show improved cellular activity or selectivity against BACE2.

Inhibitor **22**, with a hydroxyethylamine isostere, showed improved cellular permeability [70]. There was also a modest improvement in selectivity, showing a 63-fold selectivity over CatD. Macrocyclic inhibitors with a reduced amide isostere were designed to bolster the pharmacokinetic profile. The 14-membered macrocyclic inhibitor **23** showed good BACE1 activity and cellular A β inhibitory potency [71]. The inhibitor exhibited a much improved Pgp ratio of 5.5. Further, in vivo studies showed that a 100 mg/kg dose of **23** cleared brain A β_{40} levels by 25% in transgenic mice, while compound concentrations in the brain were found to be approximately 1.1 μM [71].

Fig. 15 X-ray crystal structure of **21**-bound BACE1 (PDB: 1XS7)



3.3 Nonpeptide Inhibitors

In general, the large molecular size and a high degree of peptidic features of peptidomimetic inhibitors led to limitations in the development of effective BACE1 inhibitor drugs. The relatively large active site of BACE1 and the need for efficient brain penetration of BACE1 inhibitors made the design task very challenging. For these reasons, the development of small-molecule and nonpeptide BACE1 inhibitors with different chemotypes has evolved. A number of high-throughput screening approaches have been pursued. These approaches have identified a wide variety of nonpeptidic scaffolds for BACE1 inhibitor design (Fig. 16). Structure-based optimization led to the development of BACE1 inhibitors that are smaller in molecular size and nonpeptidic in nature with improved pharmacokinetic properties and BBB penetration. A number of recent reviews covered this development in detail leading to the discovery of potent and selective BACE1 inhibitors [11, 72]. Herein, we plan to provide a brief outline.

3.3.1 Acyl Guanidine-Based Inhibitors

Acyl guanidine-based BACE1 inhibitors were discerned by high-throughput screening (HTS). The acyl guanidine core shown in Fig. 17 makes interactions with the catalytic aspartic acids (Asp32 and Asp228) of BACE1 [73]. As shown in Fig. 18, inhibitor **24** with an α -substituted 2,4-biphenylpyrrole showed an enzyme

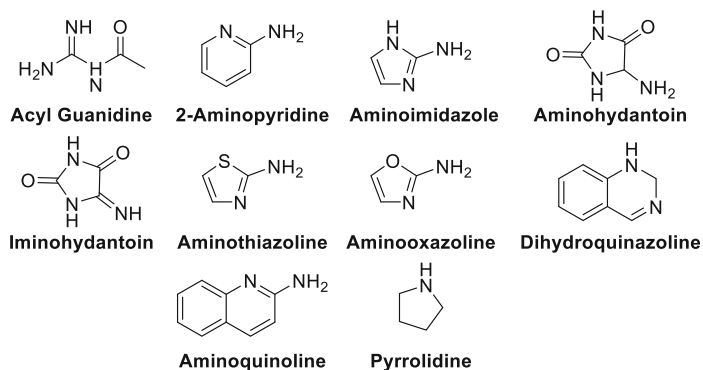


Fig. 16 Common scaffolds for nonpeptide BACE1 inhibitors

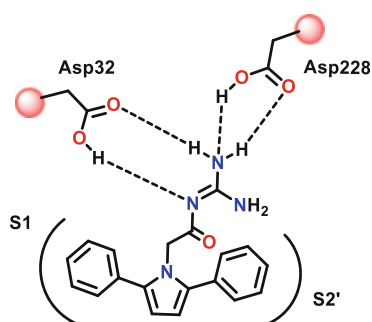


Fig. 17 Binding mode of inhibitor **21**

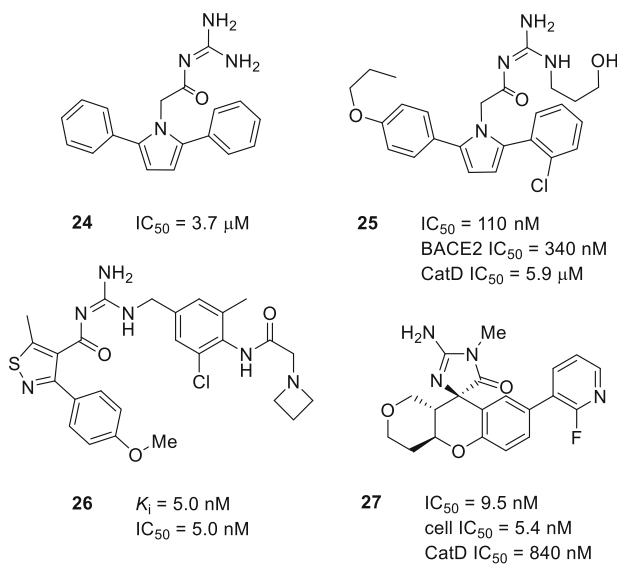


Fig. 18 Structures and activity of acyl guanidine-based inhibitors **24–27**

IC₅₀ of 3.7 μ M [73]. An inhibitor **24**-bound BACE1 X-ray crystal structure revealed that the inhibitor resides in an open conformation in the active site due to stabilizing interactions between Tyr71 and the π -system of the diarylpyrrole ligand (Fig. 17). Furthermore, the biphenyl ligand resides in both the S1 and S2' sites with the P1 phenyl positioned directly toward an unoccupied S3 subsite. To optimize ligand binding in the S1–S3 regions, a *p*-propyloxyphenyl moiety was examined in place of the unsubstituted aryl ring. Similarly, access to potential hydrogen bonding interactions in the S1' pocket was promoted through the functionalization of one of the guanidine nitrogens with a propyl alcohol group. This resulted in a substantial improvement in potency (IC₅₀ 110 nM) for inhibitor **25** (Fig. 18). These biaryl pyrrole-based inhibitors show low levels of selectivity over other human aspartyl proteases.

Inhibitor **26**, with an isothiazole derivative, was optimized after the discovery of the scaffold by HTS. This inhibitor exhibited excellent BACE1 affinity (K_i = 5.0 nM, Fig. 18) [74]. In vivo viability was evaluated with dose-dependent studies in mouse models. Plasma A β ₄₀ levels were found to be diminished, while brain and CSF A β ₄₀ levels were maintained during the treatment. This suggests that the inhibitor is a substrate for Pgp. However, further improvement is necessary for better BBB penetration.

A series of chromane-based spirocyclic acyl guanidine-derived BACE1 inhibitors were developed. Inhibitor **27** showed very good BACE1 and A β cell inhibitory activity [75]. The inhibitor displayed a high efflux ratio. However, it was able to reduce CSF A β ₄₀ levels from 53% to as high as 63% over multiple species [75].

3.3.2 2-Aminopyridine-Based Inhibitors

Small-molecule nonpeptide BACE1 inhibitors have been developed based upon the 2-aminopyridine scaffold. BACE1 bound to 2-aminopyridine-based inhibitors adopts a flap-open conformation that results from the displacement of Tyr71 to a position above the pyridine scaffold. In silico screening of varying sites and degree of substitution on the pyridine ring led to the identification of many lead structures. Among the initial hits, 2,6-substituted aminopyridine **28** displayed a modest potency with an IC₅₀ of 25 μ M (Fig. 19) [76]. A 2,3-substituted aminopyridine derivative **29** showed BACE1 IC₅₀ of 690 nM. This lead structure was then optimized for the S1–S3 hydrophobic pockets. The biaryl-substituted pyrrole substituent was incorporated to improve the cellular potency of inhibitors. Inhibitor **30** displayed an improved cellular potency (EC₅₀ = 440 nM) (Fig. 19) [77]. Furthermore, it exhibited >100-fold selectivity against BACE2 and >500-fold selectivity against CatD. The aminopyridine moiety of inhibitor **30** binds to the catalytic aspartic acids, while the pyrimidine moiety forms a hydrogen bond with Ser229 (Fig. 20).

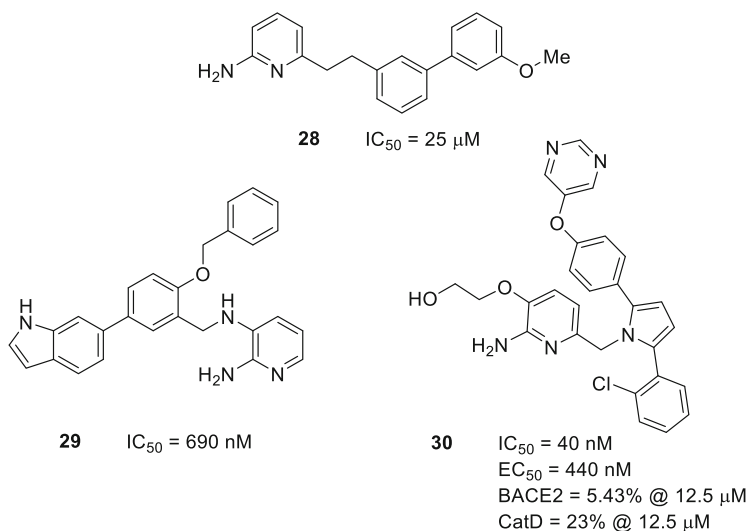


Fig. 19 Structures and activity of 2-aminopyridine-based inhibitors **28–30**

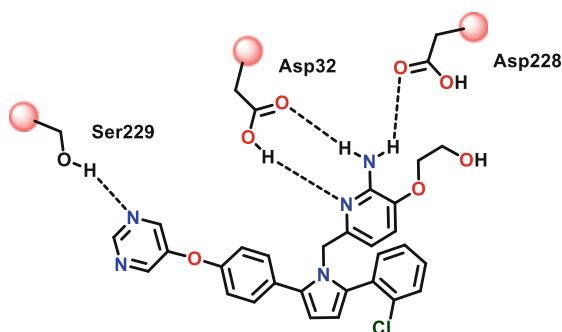


Fig. 20 Binding mode of inhibitor **30**

3.3.3 Aminoimidazole-Based Inhibitors

Potent and selective BACE1 inhibitors emerged based upon the aminoimidazole heterocyclic scaffold. As shown in Fig. 21, HTS initially yielded dibenzyl-substituted imidazole **31** as a weak inhibitor. Molecular modeling studies showed that the amino group of the imidazole heterocycle was responsible for binding with the catalytic aspartic acid residues. Subsequent optimization of the scaffold led to the design of conformationally constrained **32** with an enzymatic IC_{50} of 63 nM [78]. This increase in potency has been attributed to the additional hydrophobic interactions with the flap region of BACE1 as a result of the constrained indane moiety. This inhibitor showed a good Pgp efflux ratio of 3.6, suggesting viable brain penetration.

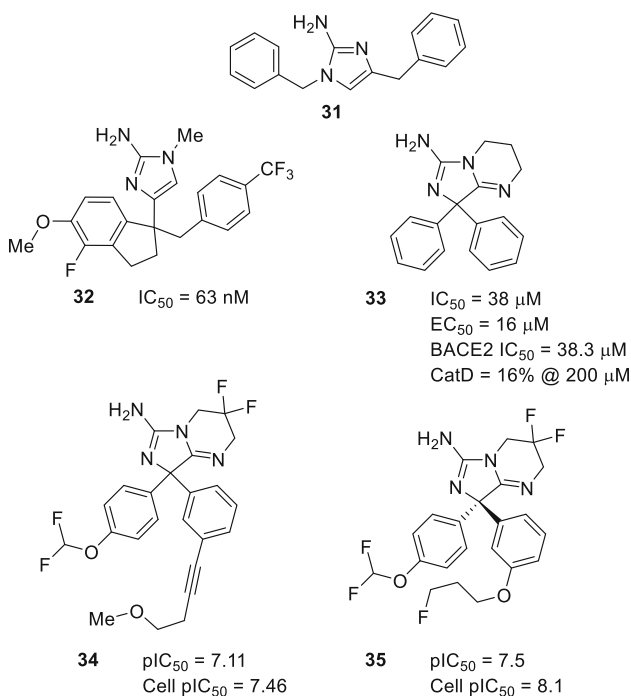
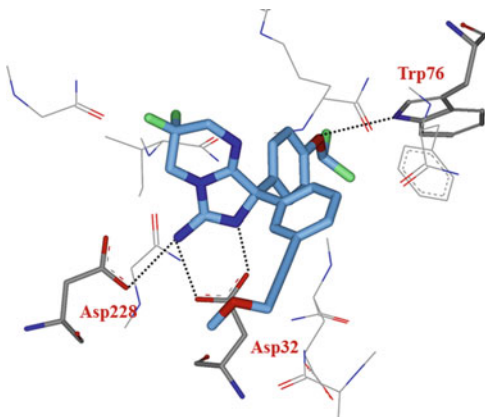


Fig. 21 Structures and activity of aminoimidazole-based inhibitors **31–35**

Further evolution of a bicyclic aminoimidazole scaffold has provided inhibitors with better brain efficacy and overall potency. The initial hit compound **33**, with a bicyclic aminoimidazole core and a biphenyl moiety, exhibited an IC_{50} in the high micromolar range [79]. The removal of the biphenyl motif and functionalization of the aryl rings resulted in potent inhibitors with better cell membrane permeability and Pgp efflux profiles. Inhibitor **34**, with a *p*-difluoromethyl ether substitution on one of the phenyl rings and an *m*-alkynyl substituent on the other ring, displayed good potency in both enzymatic and cellular assays with pIC_{50} values of 7.11 and 7.46, respectively [80]. Cell membrane permeability, as determined by a Caco-2 assay, was $8.4 \times 10^{-6} \text{ cm/s}$, and the efflux ratio was 3.5, indicating potential for BBB penetration. An X-ray crystal structure of **34**-bound BACE1, shown in Fig. 22, provided insight into the ligand-binding interactions of this inhibitor [80]. The structure was determined at 2.0 Å resolution. The aminoimidazole moiety interacted with the catalytic Asp32 and Asp228 residues, binding in a flap-open conformation. This allows Trp76 to be in position for hydrogen bonding to the oxygen of the *p*-difluoromethyl ether. The alkynyl substituent of the second ring extends into the S3 pocket. Replacement of the alkyne chain of inhibitor **34** with a fluorinated propyl ether resulted in inhibitor **35**. This optically active inhibitor showed similar potency as **34** [80]. Interestingly, the efflux ratio of **35** was enhanced to a value of 0.8. Further, in vivo assessment in a mouse model using

Fig. 22 X-ray crystal structure of **34** in the enzyme active site (PDB: 4ACX)



oral coadministration of **35** with a Pgp inhibitor resulted in reduction of brain and plasma A β levels by 17% and 76%, respectively [80].

3.3.4 Aminohydantoin-/Iminohydantoin-Based Inhibitors

Identification of initial hit compound **36** with an aminohydantoin scaffold led to the development of potent and selective BACE1 inhibitors. As shown in Fig. 23, inhibitor **36** displayed an enzymatic IC₅₀ of 3.4 μ M [81]. Subsequent optimization of the hydrophobic core led to inhibitor **37** with an improvement in potency. X-ray structural studies of **37**-bound BACE1 revealed that the catalytic aspartic acid residues were participating in hydrogen bonding interactions with the amino moiety and the N-H nitrogen of the pharmacophore. Furthermore, the phenylpyridine moiety extends into the S3 pocket to make an interaction with Ser229 through a water bridge. Interactions with the Trp76 within the S2' pocket were made possible through the *p*-methoxybenzyl moiety. Inhibitor **37** exhibited good BACE1 activity with an IC₅₀ of 10 nM and a cellular EC₅₀ of 20 nM (Fig. 23) [81]. Furthermore, it displayed greater than 80-fold selectivity against BACE2, CatD, and other aspartic acid proteases. In vivo studies in a transgenic mouse model showed that a 100 mg/kg oral dose of inhibitor **37** cleared 69% of plasma A β ₄₀ [81].

Further exploration of an aminocyclohexyl group as the S1 ligand resulted in inhibitor **38**. This inhibitor displayed a low nanomolar BACE1 activity (IC₅₀ = 22 nM) and a 1136-fold selectivity against CatD [82]. As shown in Fig. 24, the X-ray crystal structure of **38**-bound BACE1 revealed a unique binding mode in the active site. The quinoline group unexpectedly occupies a solvent-exposed region of the active site and makes additional hydrophobic interactions with Ile110 and Lys107. Additionally, it forms a hydrogen bonding interaction with the backbone carbonyl of Phe108. The inhibitor was able to reduce plasma A β ₄₀ by 65% and 55% following oral and subcutaneous administration in rats. Brain A β ₄₀, however, was not reduced when dosed in a mouse model. In whole cell assays, the

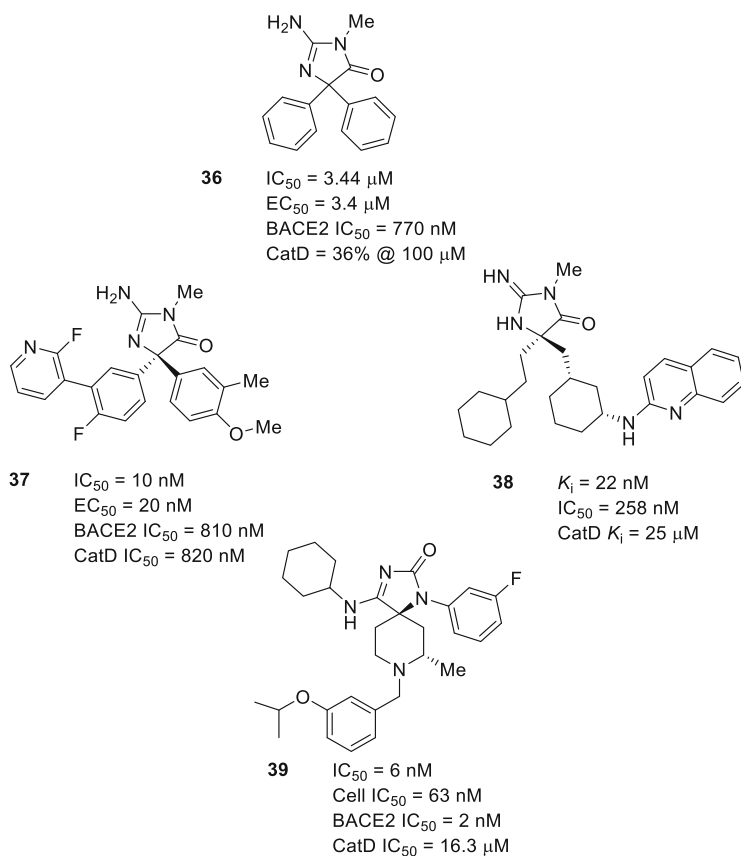


Fig. 23 Structures and activity of aminohydantoin-/iminohydantoin-based inhibitors **36–39**

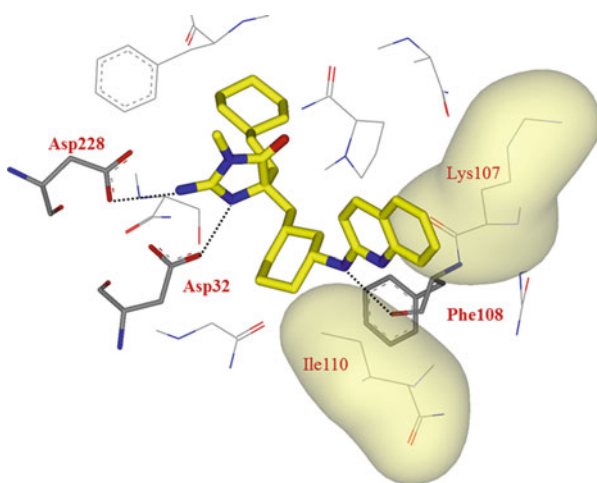


Fig. 24 X-ray crystal structure of **38**-bound BACE1 (PDB: 4R95)

inhibitor showed a cellular EC_{50} of 258 nM. This was further improved to an EC_{50} of 110 nM upon coadministration with a Pgp inhibitor [82].

The development of a closely related spirocyclic piperidine resulted in potent inhibitor **39** [83]. The X-ray crystal structural studies revealed that the binding mode of the inhibitor results in a slightly larger pocket, which can be further optimized. The inhibitor showed no selectivity against BACE2 (IC_{50} = 2 nM) but shows good selectivity against CatD (IC_{50} = 16.3 μ M). It also showed improved cellular potency of 63 nM and a Pgp efflux ratio of 4.4.

3.3.5 Aminothiazoline- and Aminooxazoline-Based Inhibitors

The development of BACE1 inhibitors with an aminothiazoline scaffold was based upon the initial hit compound **40** [84]. As shown in Fig. 25, inhibitor **40** displays a modest enzymatic potency of 41.2 μ M. Initial X-ray studies of **40**-bound BACE1 revealed that meta-substitution of the phenyl moiety could maximize interactions within the S3 subsite. Also, the meta-substituted amide group allows the aromatic ring to adopt a nearly planar conformation through a hydrogen bonding interaction with Gly291. Extensive SAR studies led to inhibitor **41**, which shows good BACE1 and cellular A β inhibitory activity [84]. However, inhibitor **41** turned out to be a good Pgp substrate. It shows 10% reduction of A β_{40} levels with an oral dose of 30 mg kg^{-1} .

Inhibitors with related aminooxazoline scaffolds displayed good BACE1 inhibitory and cellular activity. Inhibitor **42** incorporates a trifluoromethyl group on the oxazoline heterocycle. It shows a BACE1 IC_{50} of 12 nM and cellular A β inhibitory activity of 2 nM [85]. The inhibitor showed good selectivity against other common aspartic acid proteases with IC_{50} levels greater than 200 μ M. In vivo studies of

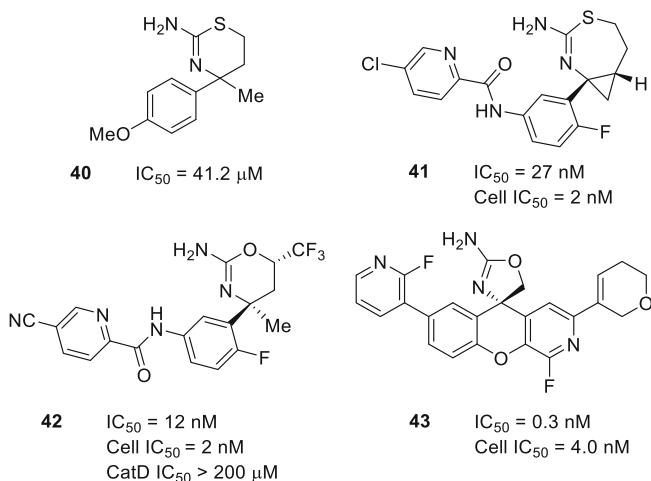
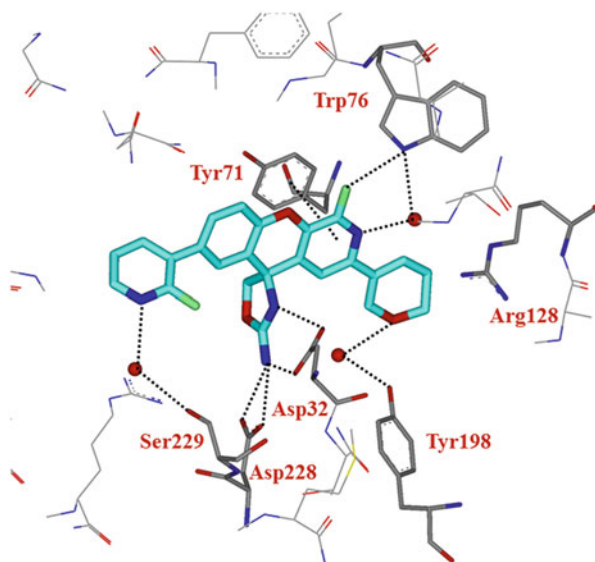


Fig. 25 Structures and activity of aminooxazoline- and aminothiazoline-based inhibitors **40–43**

Fig. 26 X-ray crystal structure of **43** in the BACE1 active site (PDB: 4WTU)



inhibitor **42** with an oral dose as small as 1 mg/kg showed reduction of $A\beta_{40/42}$ levels for up to 24 h. It also displayed a favorable pharmacokinetic profile [85].

Small-molecule BACE1 inhibitors including a xanthene core with an amino-oxazoline head group were explored. Xanthene derivatives generally exhibited good potency but showed high efflux activity. Further modification of the xanthene core and its substituents were pursued in order to develop drug-like inhibitors [86]. Inhibitor **43** with a 3-aza-2-fluoroxanthene scaffold showed excellent BACE1 and cell inhibitory potency [87]. As shown in Fig. 26, an X-ray crystal structure of inhibitor **43**-bound BACE1 revealed that the 3-azaxanthene core formed a crucial hydrogen bonding interaction with Trp76. The oxygen of the dihydropyran also formed a hydrogen bonding interaction with Tyr198. The nitrogen of the pyridine engaged in an additional hydrogen bonding interaction with Ser229. Inhibitor **43** was orally administered to a number of species and showed a robust reduction of central nervous system (CNS) $A\beta_{40}$ levels [87].

3.3.6 Dihydroquinazoline-Based Inhibitors

BACE1 inhibitors containing dihydroquinazoline scaffolds were developed after identification of fragment hit **44** (Fig. 27) [88]. The X-ray structure of **44**-bound BACE1 shows that the inhibitor adopts a hairpin conformation (Fig. 28), enabling the inhibitor to occupy the subsite specific S1 pocket. The X-ray crystal structure reveals a vacant hydrophobic S1' pocket available for optimization of ligand binding. Incorporation of a cyclohexyl group with (*S*)-configuration led to inhibitor **45** [88]. Presumably, the cyclohexyl ligand fills in the S1' hydrophobic pocket. As a result, this inhibitor showed an enzymatic K_{i50} of 11 nM. The inhibitor displayed

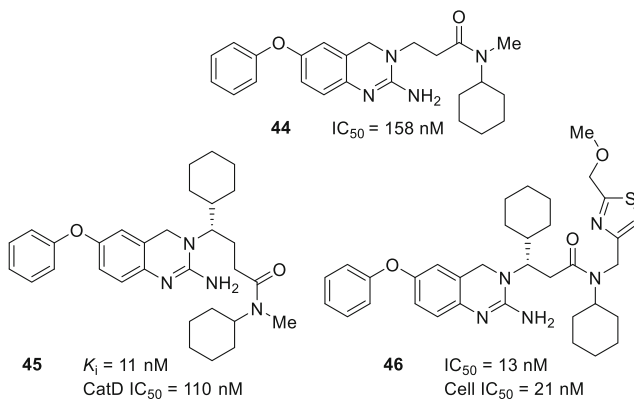


Fig. 27 Structures and activity of dihydroquinazoline-based inhibitors **44–46**

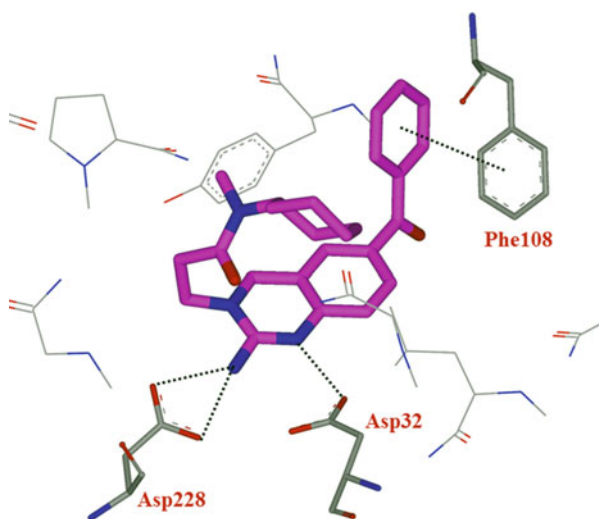


Fig. 28 X-ray crystal structure of **44**-bound BACE1 (PDB: 2Q11)

modest selectivity against common aspartyl proteases such as renin ($IC_{50} = 2.7 \mu\text{M}$) and CatD ($IC_{50} = 0.11 \mu\text{M}$). Oral administration of inhibitor **45** in rats (30 mg/kg) exhibited as much as 70% reduction of $A\beta_{40}$ plasma levels. However, an efflux ratio of 12.2 suggests poor brain permeability for this inhibitor. Further optimization of the ligand binding led to the design of inhibitor **46** [89]. In this inhibitor, a methoxy-methyl thiazole was incorporated in place of the methyl amide. Inhibitor **46** exhibited good BACE1 enzymatic inhibitory activity ($IC_{50} = 13 \text{ nM}$) and cellular $A\beta$ inhibitory activity ($IC_{50} = 21 \text{ nM}$).

3.3.7 Aminoquinoline-Based Inhibitors

Initial fragment-based screening efforts identified aminoquinoline **47** with BACE1 K_d of 900 μM [90]. Initial SAR studies showed that incorporation of an aryl ring at the 6 position of the aminoquinoline may lead to improved potency. Further structural modifications with the introduction of a *N*-cyclohexyl propionamide at C3 of the quinoline ring led to inhibitor **48** (Fig. 29) [90]. This inhibitor showed good BACE1 IC_{50} . Further structural studies suggest that a longer and bulkier alkyl chain could potentially extend further into the $\text{S2}'$ subsite. Inhibitor **49** incorporated an (*R*)- α -methyl on the side chain and a chloropyridyl group to improve pharmacokinetic properties [90]. Inhibitor **49** displayed good activity with a BACE1 IC_{50} of 11 nM and cellular IC_{50} value of 80 nM. It also exhibited an efflux ratio of 3.1. In vivo studies with a 60 mg/kg dose administered subcutaneously in rats showed reduction of $\text{A}\beta_{40/42}$ CSF levels by 42% postinjection. However, inhibitor **49** showed rapid clearance from human and rat microsomes. The X-ray structural studies with **49**-BACE1 complex showed that the aminoquinoline formed a tight interaction with flap residue Tyr71, which orients the quinoline and amino nitrogens toward the catalytic Asp32 and Asp228 residues (Fig. 30) [90].

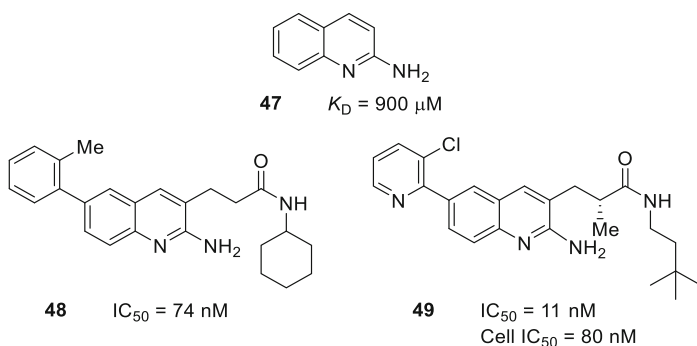


Fig. 29 Structures and activity of aminoquinoline-based inhibitors **47–49**

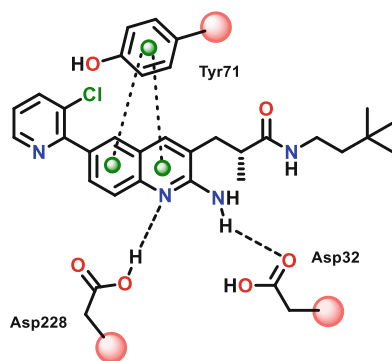


Fig. 30 Binding mode of inhibitor **49**

3.3.8 Pyrrolidine-Based Inhibitors

BACE1 inhibitors containing a pyrrolidine scaffold were identified by HTS. As shown in Fig. 31, initial compound **50** showed a low inhibitory activity of 240 μM [91]. SAR studies and further optimization resulted in inhibitor **51**, which showed BACE1 inhibitory activity of 29 nM. Its cellular activity was 570 nM (IC_{50}), and the inhibitor displayed no selectivity against BACE2 but >200-fold selectivity over CatD [91]. An inhibitor-bound X-ray crystal structure revealed that the pyrrolidine scaffold formed two hydrogen bonds with the catalytic aspartic acid residues. The *cis*-2,4-substituted piperidine ligand nicely occupied a pocket below the flap region (Fig. 32).

3.3.9 Macrocyclic Nonpeptide Inhibitors

The development of macrocyclic nonpeptide inhibitors was carried out in an effort to improve the inhibitors' properties. As mentioned earlier, the dihydroquinazoline-based inhibitors adopt a tight hairpin conformation [88]. A macrocyclic inhibitor may provide stabilization of the bioactive hairpin conformation, leading to an enhancement in potency. As shown in Fig. 33, macrocyclic inhibitor **52** displayed a BACE1 K_i of 5 nM [92]. The cellular A β inhibitory activity was also improved to 7 nM (IC_{50}) compared to the acyclic inhibitor. Further evaluation revealed that

Fig. 31 Structures and activity of pyrrolidine-based inhibitors **50** and **51**

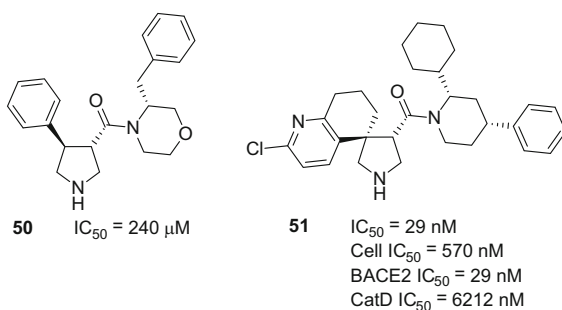


Fig. 32 Binding mode of inhibitor **51**

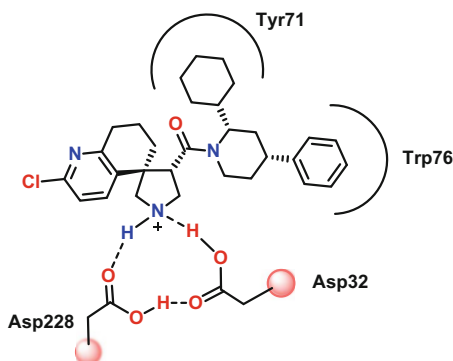


Fig. 33 Structures and activity of nonpeptidic macrocyclic-based inhibitors **52** and **53**

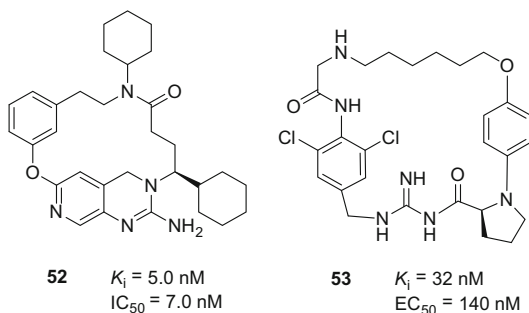
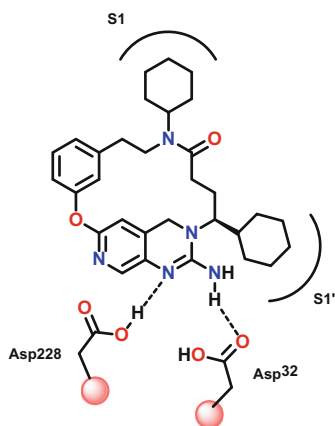


Fig. 34 Binding mode of inhibitor **52**



inhibitor **52** is a Pgp substrate and in vivo studies did not show reduced brain A β in animals. Structural studies suggested that the cyclohexyl moieties occupied the vacant S1 and S1' pockets of the BACE1 active site (Fig. 34).

Macrocyclic inhibitors based upon the acyl guanidine-derived scaffold have been investigated. A representative example is inhibitor **53**, which features a 26-membered macrocycle with an acyl guanidine core, displaying good BACE1 ($IC_{50} = 32 \text{ nM}$) and cellular A β inhibitory activity ($EC_{50} = 140 \text{ nM}$) [93]. Inhibitor **53** also showed good selectivity against multiple aspartic acid proteases, including 700-fold selectivity against CatD. Furthermore, the inhibitor showed a diminished efflux ratio of 2.3. In vivo studies with wild-type mice involving subcutaneous dosing of 40 mg/kg showed a 74% reduction of plasma A β levels and a peripheral drug concentration of 3.8 μM 5 h postinjection. However, A β brain levels were found to be nearly identical to the control levels [93].

4 Clinical Evaluation of BACE1 Inhibitors

The discovery of a clinically translatable BACE1 inhibitor has proven to be a difficult challenge for the medicinal chemistry community. While many highly potent inhibitors have been synthesized and evaluated preclinically, there are many more barriers an inhibitor must cross to be considered clinically viable. Further, a drug intended to treat a brain disorder experiences additional hurdles it must overcome. First, an inhibitor must have the ability to penetrate the BBB. This requires a proper balance of hydrophobicity and hydrophilicity. Also, upon crossing the BBB, Pgp efflux can eject the inhibitor, precluding any therapeutic effect. This efflux can be avoided by carefully designing compounds with minimal recognition by Pgp.

Additionally, due to the chronic nature of AD, any approved therapy would be a maintenance drug, which patients must take for the remainder of their lives. Because of this, accumulation of the drug in body tissues and subsequent toxicity must be monitored and addressed. Further, selectivity over non-pathological aspartic acid proteases is critical, especially in the case of chronic therapy. Selectivities over systemic enzymes such as BACE2 and CatD provide an additional challenge due to the similarity of the proteolytic mechanism and substrate specificity. Unique pockets in each enzyme must be exploited to provide this necessary selectivity.

A final challenge is the balance of inhibitor size. The enzyme has a rather large active site. It has been a difficult challenge to develop compounds large enough to fill the active site and bind efficiently, yet small enough to cross the BBB, cell membrane, and endosomal membrane to inhibit BACE1 at the vesicle. Generally, large peptidomimetic inhibitors tend to have low oral bioavailability, low half-lives, and poor BBB penetration. However, small inhibitors may not adequately fill the enzymatic active site, resulting in poor affinity for the enzyme. Despite these challenges, potential inhibitors have been pursued in clinical trials (Fig. 36). Thirteen drugs have been brought to clinical trials in the United States, six of which have progressed into phase II or III clinical trials and five are currently being tested in the clinic (Figs. 35 and 36)

4.1 Clinical Evaluation of CTS21166

The structure of CTS21166 has not been publically disclosed. This compound, developed by CoMentis, was the first BACE1 inhibitor to be brought to clinical trials. The 8-month phase I trial began in June 2007 and tested CTS21166 in 56 healthy adult males to evaluate the safety profile of single-ascending doses as well as the pharmacokinetics and major plasma metabolites [94]. Intravenous injections of 7.5–225 mg were well tolerated and showed gradual clearance amenable to once-daily dosing across all doses. At 225 mg, plasma A β levels were

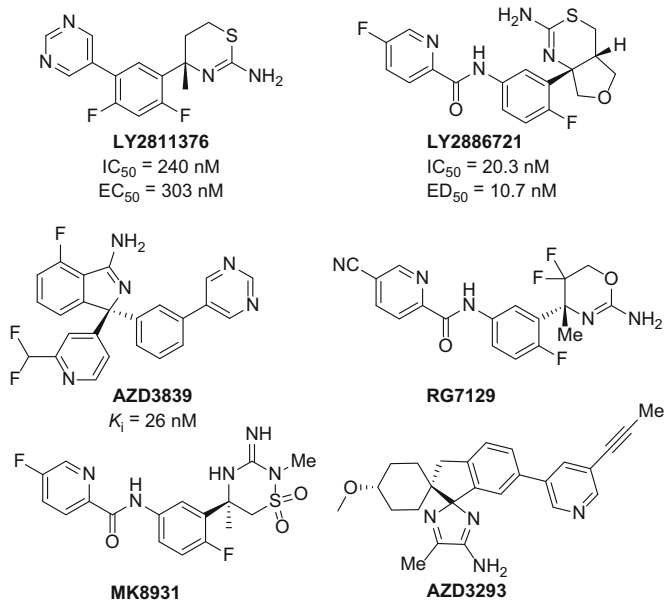


Fig. 35 Known structures of BACE1 clinical drug candidates

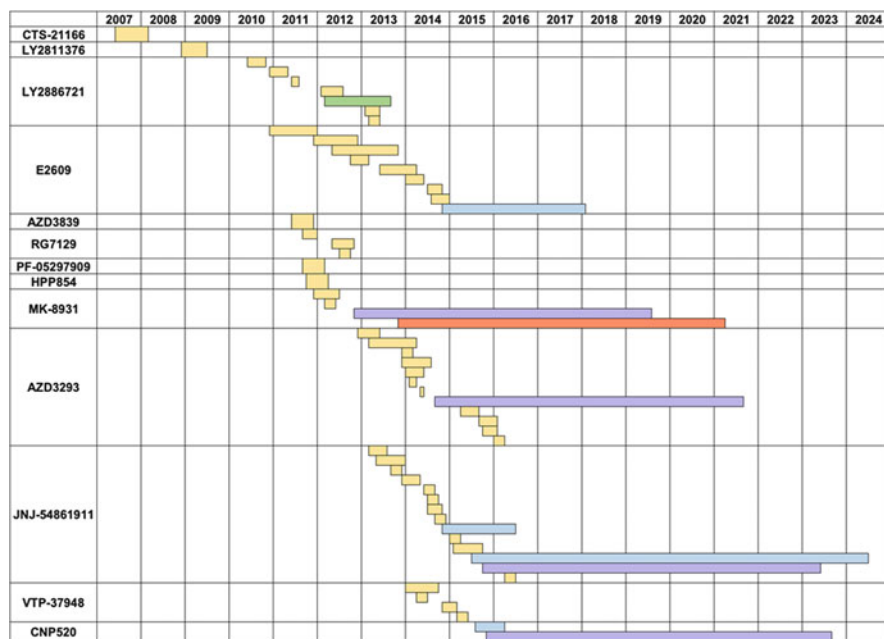
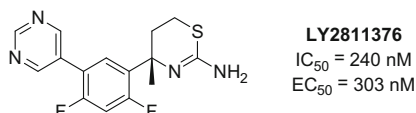


Fig. 36 Timeline of BACE1 inhibitor clinical development. Phase I = yellow; phase I/II = green; phase II = blue; phase II/III = purple; phase III = red

reduced by up to 80% at 3 h postinjection versus placebo [95, 96]. While CTS21166 passed phase I trials, its clinical development was not continued.

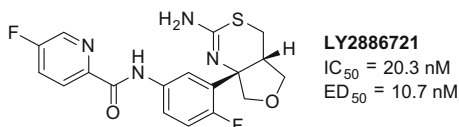
4.2 Clinical Evaluation of LY2811376



LY2811376, Eli Lilly's first clinical BACE1 inhibitor, began phase I clinical trials in 2009. It is a part of the aminothiazoline class of nonpeptidic inhibitors. The inhibitor was given to 61 healthy men and women of non-child-bearing potential to investigate the safety and tolerability at single doses from 5 to 500 mg as oral capsules [97]. LY2811376 was well tolerated with no serious adverse effects reported. Maximum plasma concentrations were reached 2 h post-dose with an average half-life of 40 h. Cerebrospinal fluid (CSF) concentrations reached maximal levels at about 5 h post-dose [98]. Dose-dependent reduction of A β ₄₀ and A β ₄₂ was observed in both the plasma and CSF. At 90 mg, an 80% reduction in A β ₄₀ was observed at 7 h after administration, with no full recovery to pre-dose concentrations within 120 h after dosing. In the CSF, up to a 54% decrease in A β was observed with a statistically significant decrease being observed 7 h after dosing and a maximum reduction at 12–14 h post-dose [98].

In preparation for phase II studies, a rat toxicology study was performed alongside phase I clinical studies. During the 3-month preclinical work, it was observed that autofluorescent granules were accumulating in the retinal epithelium, neurons, and glial cells at doses greater than 30 mg/kg. Based on this data, clinical trials of LY2811376 were immediately terminated. It was hypothesized that the retinal toxicity may be due to off-target effects against other aspartic acid proteases such as BACE2 or CatD. Fortunately, no clinically significant retinal pathology was present in any of the clinical trial participants in follow-up exams [98].

4.3 Clinical Evaluation of LY2886721



It was more than a year before the next clinical candidate was tested in human subjects. LY2886721, developed by Eli Lilly and Company, became the first BACE1 inhibitor to reach phase II trials [99]. Like LY2811376, it is a part of the aminothiazoline class of nonpeptide inhibitors. A total of six phase I studies tested the safety and pharmacology of LY2886721 in 155 subjects. Trials began in June

2010 with a single-ascending dose study in healthy adult males and females, administered as oral capsules in variable doses ranging from 1 to 200 mg or placebo. Concentrations of LY2886721 and $A\beta_{40}$ were measured up to 8 days post-dose in the plasma and up to 36 h post-dose in the CSF [100]. Another study investigated the tolerability of multiple-ascending doses of 5–35 mg or placebo administered as oral capsules in 42 healthy adults. Subjects were monitored for clinically significant effects as well as concentrations of LY2886721 and $A\beta_{40}$ in the plasma and CSF [101].

An open-label study with radioactive ^{14}C -LY2886721 in eight healthy males was used to determine metabolism and excretion pathways [102]. An additional single- and multiple-dose study was performed on healthy volunteers before proceeding to phase I/II studies in Alzheimer's patients [103]. LY2886721 was evaluated in 128 patients with mild cognitive impairment or mild Alzheimer's disease. Doses of 15, 35, or 70 mg or placebo were administered orally once daily for 26 weeks. Concentrations of $A\beta_{40}$ and $A\beta_{42}$ were measured at 12 and 26 weeks in both the CSF and plasma. Other cognitive tests were performed at 26 weeks including a Neuropsychological Test Battery (NTB), Alzheimer's Disease Assessment Scale (ADAS), Clinical Dementia Rating (CDR) Scale, and Mini Mental State Examination (MMSE) [104]. Concurrently, additional phase I studies were performed to evaluate two formulations of LY2886721 and assess safety and side effects in Alzheimer's patients and high-dose tolerability in healthy volunteers [105]. However, in June 2013, Lilly voluntarily halted the phase II trial and all further clinical development of LY2886721 due to abnormal liver biochemistry observed in subjects. It is believed that the abnormalities are not related to the BACE1 mechanism [106]. No clinical trial results of LY2886721 have been published.

4.4 Clinical Evaluation of E2609

Eisai Inc. brought clinical candidate E2609 to trial, starting with an initial phase I safety study beginning in December 2010. The structure of E2609 has not been publically disclosed. Unpublished preclinical work showed that E2609 lowered $A\beta$ levels in the CSF and plasma of rats, guinea pigs, and non-human primates [107]. In the first single-ascending dose study, E2609 was dosed orally in healthy adult volunteers up to 800 mg or placebo and in healthy elderly volunteers at 50 mg or placebo. Subjects were monitored for adverse effects, and pharmacokinetic parameters were assessed in plasma and urine [108]. Adverse effects were reported in 21.8% of subjects treated with E2609 compared to 16.7% dosed with placebo. No serious adverse effects were reported, demonstrating that E2609 was well tolerated in all dosing groups. Plasma concentration of E2609 was dose dependent, with a half-life of 15.9 h at 800 mg. Maximal reduction of plasma $A\beta$ occurred from 6 to

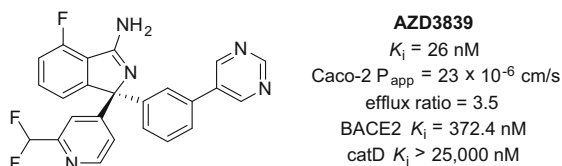
24 h after administration with decreases ranging from 56.3% at 5 mg to 91.0% at 800 mg. At up to 144 h post-dose, an 800 mg dose still afforded a 45% reduction of A β in the plasma [109].

During a multiple-ascending dose study, healthy adult volunteers received 25–400 mg or placebo administered orally once daily for 14 days. Subjects were monitored for adverse effects, as well as A β concentration in the plasma and CSF [110]. E2609 continued to be well tolerated in multiple doses. Plasma levels of E2609 were dose dependent, with a constant concentration achieved at 7 days. Concentration of A β in the plasma was significant, with reduction greater than 85% at the lowest dose. Assessment of CSF showed that at 100 mg, A β_{40} and sAPP β were reduced by 82.1% and 94.2%, respectively, while sAPP α levels increased by 128.6%, indicating enhanced processing by α -secretase due to the inhibition of BACE1 [111].

Other phase I studies included a safety and pharmacodynamic study in 65 mild AD patients given a single oral dose ranging from 5 mg to 400 mg [112] and a bioavailability and absorption assessment in 18 healthy males [113]. Utilizing ^{14}C -radiolabeled compound, the metabolism and excretion pathway of E2609 was investigated in 113 healthy males at a single 100 mg oral dose [114]. To investigate potential drug-drug interactions, 195 healthy adults were dosed with E2609 in the presence or absence of itraconazole, an antifungal and cytochrome P450 3A4 inhibitor; rifampin, an antibiotic and hepatic cytochrome P450 inducer; digoxin, a treatment for heart conditions; and donepezil, an acetylcholinesterase inhibitor used for palliative care in Alzheimer's patients [115]. To assess pharmacokinetic parameters, a single oral dose study in Japanese and white healthy males was performed monitoring plasma concentrations and parameters for up to 10 days [116]. Another drug-drug interaction study was performed on 60 healthy adult volunteers to investigate the effect of moxifloxacin with E2609. Moxifloxacin is an antibiotic which has shown to prolong the QT interval when dosed with some other medications. In this study, particular attention was given to changes in the QTc interval and ECG recordings [117].

In November 2014, Eisai Inc., in collaboration with Biogen, began a phase II study of E2609 in patients with early AD and mild-to-moderate dementia due to AD. An estimated 700 patients will be administered a low, middle, or high dose of E2609 or placebo orally once per day for 18 months. End points will be evaluated via change in derived Alzheimer's Disease Composite Score (ADCS) at 18 months, and change in hippocampal atrophy as determined by MRI, and CSF A β levels [118]. Clinical evaluation of this compound is ongoing, and the current phase II study is estimated to be completed in January 2018.

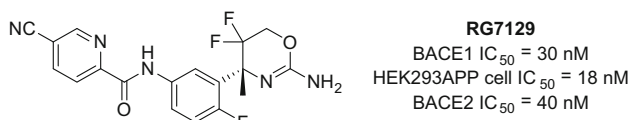
4.5 Clinical Evaluation of AZD3839



In June 2011, AstraZeneca brought clinical candidate AZD3839 into phase I trials. AZD3839 was developed from an aminopyrimidinone NMR hit. Subsequent structural optimization led to the aminoisoindole derivative that was ultimately assessed in the clinic [119]. In preclinical studies, AZD3839 displayed an enzymatic K_i of 26.1 nM/L and a 14-fold and 1,000-fold selectivity over BACE2 and CatD, respectively [119]. In mice, the brain concentration of AZD3839 reached maximum concentration at 30 min and returned to baseline levels at 4.5 h when dosed at 80 $\mu\text{mol/kg}$. When the dose was doubled, the brain exposure elongated to about 8 h. $A\beta_{40}$ levels decreased 30% and 50%, respectively [119]. Guinea pigs were given an oral dose of either 100 or 200 $\mu\text{mol/kg}$. Lasting up to 8 h, guinea pigs which received 200 $\mu\text{mol/kg}$ had 20–60% lower brain $A\beta_{40}$ levels than the control. The lower-dose group showed a brain $A\beta_{40}$ reduction of 20–30% after 1.5–4.5 h post-dose. CSF $A\beta_{40}$ levels in the high-dose group were reduced by 50% at 3 h and 40% at 8 h; however, the reduction was not statistically significant. Plasma $A\beta_{40}$ levels were reduced by 30–80% for both dose classes over the entire test period [119]. In a non-human primate model using cynomolgus monkeys, an intravenous 20 $\mu\text{mol/kg}$ dose of AZD3839 reduced CSF levels of $A\beta_{40}$, $A\beta_{42}$, and sAPP β between 3 and 12 h of exposure. A lower dose of 5.5 $\mu\text{mol/kg}$ did not show any significant effect on biomarker levels. This is proposedly due to low exposure at this concentration, as CSF concentration of AZD3839 peaked at 15 min and decreased tenfold at 3 h [119].

Based on this preclinical data, AZD3839 was brought to phase I clinical studies. A single-dose safety and tolerability study was performed in 72 healthy male volunteers given oral doses ranging from 1 to 300 mg or placebo. Subjects were monitored for adverse effects, as well as for plasma AZD3839, $A\beta_{40}$, and $A\beta_{42}$ levels [120]. AZD3839 was well tolerated at all doses, with 31% of the test group reporting at least one mild adverse event, compared to 39% in the placebo group. A dose-dependent elongation of the QT interval was observed, with an average prolongation of 20 ms at the highest dose of 300 mg. AZD3839 lowered plasma $A\beta_{40}$ and $A\beta_{42}$ in a dose-dependent manner. The maximum reduction of plasma $A\beta_{40}$ and $A\beta_{42}$ was 56% and 39%, respectively [121]. This phase I study was completed in November 2011, and AZD3839 was not brought forward into further clinical studies.

4.6 Clinical Evaluation of RG7129



Hoffmann-La Roche brought inhibitor RG7129 (RO5508887) to clinical trials in September 2011. It is a member of the aminooxazoline class of nonpeptidic inhibitors. Preclinical evaluation showed that RG7129 was potent against BACE1 (IC₅₀ = 30 nM) but was not selective over BACE2 [122]. Single-ascending oral doses were administered to 49 healthy male volunteers. Plasma and urine levels of RG7129 were monitored from 1.5 to 144 h post-dose. Plasma levels of A β ₄₀ and A β ₄₂ were measured in the plasma over the same time frame. The effect of food on the pharmacokinetics of RG7129 and incident of adverse events were also monitored [123]. A secondary study evaluated biomarker levels in the plasma and CSF as well as RG7129 concentrations and adverse effects, in 42 healthy male subjects [124]. A third multiple-ascending dose phase I trial investigated the safety and tolerability of RG7129 over prolonged exposure. Subjects were monitored for the occurrence of adverse effects as well as plasma levels of biomarkers and RG7129 [125]. Results of these studies have not been published or disclosed. In October 2013, Hoffmann-La Roche ended the clinical development of RG7129 but did not provide a clinical explanation for its cessation.

4.7 Clinical Evaluation of PF-05297909

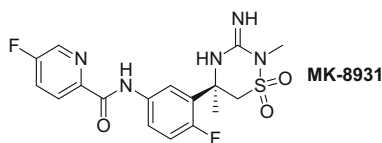
Pfizer brought its first small-molecule BACE1 inhibitor to clinical trials in September 2011; the structure has not been released. PF-05297909 was administered orally as a single dose to 51 healthy volunteers. A thorough pharmacokinetic study was of interest; plasma and CSF levels of biomarkers, in vivo IC₅₀ estimation, half-life, and CSF concentration of PF-05297909 were determined [126]. At all doses, 25–325 mg, PF-05297909 was well tolerated and displayed sufficient plasma concentrations and A β reduction. Unfortunately, this plasma exposure and reduction was not translated to the CSF, and no significant pharmacokinetic or pharmacodynamic effect was observed [127]. Clinical development of PF-05297909 was halted in light of these phase I results.

4.8 Clinical Evaluation of HPP854

High Point Pharmaceuticals developed inhibitor HPP854, which was determined to have significant preclinical activity worthy of translation into a clinical setting.

Phase I trials of HPP854 began in October 2011. HPP854 was administered orally once daily over 28 days in ascending doses to seven patients with mild cognitive impairment or mild AD. Patients were monitored for adverse events, plasma concentration of HPP854, and CSF concentration of A β [128]. Upon the conclusion of the phase I study in March 2012, High Point Pharmaceuticals terminated the clinical development of HPP854. No clinical results or inhibitor structure has been released.

4.9 Clinical Evaluation of MK-8931



MK-8931 (verubecestat) was developed by Merck. It was first brought to clinical trials in the United States in December 2011. Initial clinical studies conducted in healthy human patients consisted of a single-ascending dose and multiple-ascending dose study. Volunteers were given a placebo, a single dose of 20–550 mg, or 10–250 mg once daily for 14 days. Subjects were monitored for adverse effects and CSF biomarker concentrations. MK-8931 was well tolerated across all dosing levels in both single and multiple dosing cohorts. CSF A β_{40} levels were reduced by 25–61% in a dose-dependent manner in the single-dose study; the multiple-dose cohort showed a dose-dependent reduction of A β_{40} ranging from 32 to 94% on day 14 [129]. Exposure of MK-8931 was dose dependent in plasma and CSF with maximum plasma concentrations at 1–4.5 h and maximum CSF concentrations at 4–6 h with a half-life of 14–22 h [130].

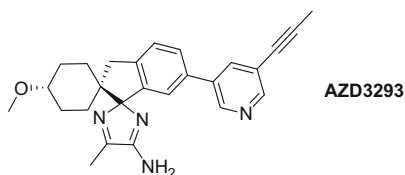
A phase I study of 32 patients with mild-to-moderate AD was performed to evaluate the safety and pharmacokinetics of MK-8931 in diseased patients. Participants received a 12, 40, or 60 mg oral dose of MK-8931 or placebo once daily for 7 days and were monitored for A β_{40} and sAPP β levels in the CSF [131]. An additional study in renal impaired patients evaluated whether renal disease changed the pharmacokinetics of MK-8931. Patients with mild, moderate, or severe renal impairment as well as healthy control groups were given a single oral dose of MK-8931 and monitored for plasma concentration of MK-8931 over 120 h, renal clearance of MK-8931, and excretion in urine [132].

MK-8931 was subsequently brought into a two-part phase II/III trial. The first part consisted of a long-term safety study in which approximately 400 mild-to-moderate Alzheimer's patients were dosed orally, once daily with 12 or 40 mg for 78 weeks or 60 mg for 13 weeks and then 40 mg for the remaining 65 weeks. Efficacy was measured by change in Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) score and Alzheimer's Disease Cooperative Study-Activities of Daily Living (ADCS-ADL) score. Secondary outcome

measurements included change in Clinical Dementia Rating Sum of Boxes (CDR-SB) score, Neuropsychiatric Inventory (NPI) score, MMSE score, hippocampal volume, CSF tau concentration, and brain amyloid concentration. Contingent upon the first safety trial, patients would receive the 12 or 40 mg dosages, once daily by mouth up to 260 weeks with the same primary and secondary end point examinations [133].

In December 2013, it was announced that the Data Monitoring Committee recommended continuation into part two of the phase II/III trials based on the data collected during the safety phase of the study [134]. This study is expected to conclude in July 2019. Concurrently, Merck announced the beginning of a phase III study in early AD patients. Patients will receive 12 or 40 mg MK-8931 once daily for 104 weeks. Upon satisfactory completion of part one, a second part will continue the trial for an additional 260 weeks at 12 or 40 mg. Efficacy will be measured via change in 3-domain Composite Cognition Score (CCS-3D), ADCS-ADL score, hippocampal volume, time to progression of probable AD dementia, CSF tau, and composite cortical amyloid standard uptake value ratio as determined by PET imaging [135]. This trial is currently recruiting participants, with an estimated enrolment of 1500. The study is estimated to be completed in March 2021.

4.10 Clinical Evaluation of AZD3293



AstraZeneca brought this new compound to phase I clinical trials after their termination of AZD3839. AZD3293 is a member of the aminoimidazole class of nonpeptide inhibitors. A single-ascending dose trial of AZD3293 was completed in healthy male and female volunteers. AZD3293 was dosed ranging from 1 to 1000 mg and administered as an oral solution or placebo. Subjects were monitored over 10 days for adverse effects, variation in vital signs, and Columbia-Suicide Severity Rating Scale (C-SSRS). Plasma concentration of AZD3293 and biomarkers were also evaluated [136]. A two-part multiple-ascending dose study was performed on 47 subjects. Part one consisted of healthy elderly volunteers who were dosed with an oral solution of AZD3293 starting at 5 mg upward. Patients were monitored for adverse events, variation in vital signs and C-SSRS scores, as well as plasma and CSF concentrations of biomarkers and AZD3293. Part two consisted of mild-to-moderate AD patients demonstrating AD symptoms at least 6 months prior to the study. Subjects were dosed with an oral solution of AZD3293 and were examined for the same efficacy end points as part one [137].

Potential drug-drug interactions were investigated. AZD3293 was administered to healthy adult volunteers as an oral solution alongside itraconazole, diltiazem, and midazolam. Subjects were monitored for the effects of coadministration on pharmacokinetic parameters of AZD3293 [138]. Pharmacokinetic considerations were assessed in a study of healthy Japanese volunteers who received single doses as an oral solution, 15, 50, and 150 mg for young patients and 1, 15, and 50 mg for elderly patients. A multiple-dose cohort was also established. Subjects were monitored for adverse effects, pharmacokinetic concentrations of AZD3293, and $A\beta_{40}$ and $A\beta_{42}$ concentrations [139]. A study of the effect of AZD3293 on the QT interval was performed in 52 healthy male volunteers. A cohort of each high-dose and low-dose AZD3293 and a moxifloxacin and placebo cohort were given a single dose and monitored for changes in the QT interval as well as inhibitor plasma concentrations and incident of adverse events [140]. Additional phase I studies included a bioavailability assessment of the oral solution versus two different tablet formulations [141]; a study of the effect of food on these tablet formations [142]; an absorption, metabolism, and elimination study with ^{14}C -AZD3293 [143]; and drug-drug interaction studies with simvastatin, midazolam, and donepezil [144], warfarin [145], and dabigatran [146].

In September 2014, AstraZeneca announced it would collaborate with Eli Lilly to develop AZD3293 further and advance into phase II/III studies [147]. This study is currently recruiting participants with an estimated enrolment of 2,202 patients with mild AD. Participants will be given either a 20 or 50 mg dose of AZD3293 (LY3314814) orally, once daily for 104 weeks. Efficacy will be measured primarily by the change in CDR-SB score. Alternative measures include change in ADAS-Cog13 score, ADCS-ADL score, CDR score, NPI score, CSF $A\beta_{42}$ concentration, amyloid PET imaging, whole brain volume, and others [148]. This study is ongoing and results are expected in August 2021.

4.11 Clinical Evaluation of JNJ-54861911

In March 2013, JNJ-54861911 was brought into clinical development by Janssen Research and Development. Structural information for JNJ-54861911 has not been released. Healthy adult volunteers across eight cohorts totaling 56 subjects were given a single dose of JNJ-54861911 ranging from 1 to 160 mg or placebo, administered orally. Patient CSF and blood plasma were assessed for concentration of JNJ-54861911 and $A\beta$ at up to 96 and 36 h post-dose, respectively [149]. A similar study was performed in healthy elderly volunteers. Seventy participants were given a 3–80 mg dose of JNJ-54861911 as an oral suspension, once daily for 14 days. Patients were monitored for adverse effects, while plasma and CSF samples were taken at multiple time points to assess concentration and exposure of JNJ-54861911 as well as $A\beta$ levels [150]. To better understand the pharmacokinetic parameters of JNJ-54861911 and the effect food may have on these parameters, a study was performed in which healthy elderly male volunteers received a single 25 mg dose as

an oral suspension under fasted conditions, a single 25 mg dose as an oral solid formulation under fasted conditions, and a single 25 mg dose as an oral solid formulation under fed conditions with 6 days between each dose and in varying orders of dosing. Bioavailability, absorption, half-life, elimination, and other pharmacokinetic parameters were examined using plasma concentration at discrete time points [151].

A proof-of-mechanism study was performed in early Alzheimer's patients. Forty-five participants were administered a 10 or 50 mg dose as an oral tablet once daily for 28 days. Exposure to JNJ-54861911 and biomarker levels in plasma and CSF were evaluated at discrete time points [152]. Other phase I studies included a study of pharmacokinetic considerations in healthy Japanese males [153] and at-risk asymptomatic Japanese participants [154]; drug-drug interaction studies with clarithromycin and itraconazole [155], caffeine and midazolam and tolbutamide [156], and rosuvastatin and metformin [157]; a study of the effects on QT intervals [158]; and the evaluation of the pharmacokinetic parameters of an alternative oral formulation [159].

Phase II trials for JNJ-54861911 began in November 2014. In a study of 114 participants with early AD, patients received a 10 mg, 50 mg, or placebo oral tablet once daily for 6 months. Safety and tolerability is the primary end point, with adverse events being monitored; however, exposure of JNJ-54861911 in the plasma and CSF as well as concentration of A β , sAPP β , and sAPP α in the CSF and plasma will also be examined [160]. This study is expected to conclude in June 2016. A similar extended study began in July 2015. Patients received a once-daily, oral dose of either 10 mg or 25 mg for 96 weeks or longer until termination of study due to safety issue or registration of JNJ-54861911 [161]. The study is estimated to conclude in June 2024.

A phase II/III study began in October 2015 with an estimated 1,650 asymptomatic at-risk participants. Subjects will be administered 10 mg, 50 mg, or placebo once daily by mouth for 54 months. The primary outcome measurement will be the change in AD Cooperative Study-Preclinical Alzheimer's Cognitive Composite (ADCS-PACC) score, while secondary efficacy end points include change in CFI score and others, incident of adverse events, and concentration of JNJ-54861911 and biomarkers in the plasma and CSF [162]. This study is currently recruiting participants and is expected to be ongoing until May 2023.

4.12 Clinical Evaluation of VTP-37948

Vitae Pharmaceuticals discovered preclinical BACE1 inhibitor VTP-37948 (BI 1181181). This was licensed and developed clinically by Boehringer Ingelheim beginning in January 2014. The structure of VTP-37948 has not yet been disclosed. In phase I development, 65 healthy male volunteers were given a single dose of VTP-37948 in ascending cohorts. The study investigated safety and tolerability as well as pharmacokinetic parameters in three different formulations both with and

without food by assessment of plasma and urine concentrations [163]. Another phase I study assessed pharmacokinetic parameters in healthy males via CSF and plasma analysis after single oral doses [164]. VTP-37948 was determined to be safe across all doses, with a half-life of 16–19 h; CSF A β levels could be lowered by 80% [165]. A multiple-ascending dose study was planned for healthy adult and healthy elderly volunteers. The trial began in September 2014, with participants receiving an oral dose once daily for 10 days [166]. In February 2015, it was announced that Vitae Pharmaceuticals and Boehringer Ingelheim were voluntarily terminating the clinical development of VTP-37948 due to negative skin reactions in some participants [167]. A drug-drug interaction study was withdrawn before participants could be recruited [168]. No further development or data for VTP-37948 has been reported.

4.13 Clinical Evaluation of CNP520

In partnership with Amgen, Novartis has examined CNP520 in phase II clinical trials as a single agent and in phase II/III clinical trials alongside immunotherapy CAD106. The structure of CNP520 has not yet been disclosed. The initial phase II trial was performed in healthy elderly volunteers. Four cohorts were administered a dose of CNP520 orally, once daily for 13 weeks. An additional cohort was given a placebo control. Patients were monitored for adverse effects as well as CNP520, A β_{40} , and A β_{42} levels in the CSF and CNP520 concentrations in the plasma [169]. The study recently ended in March 2016. However, results have not yet been disclosed. A study of at-risk patients is currently recruiting participants to study CNP520 alongside immunotherapy CAD106 when administered separately. Participants will be given only CNP520 or CAD106 or a placebo, but not multiple investigational treatments. Volunteers given CNP520 will be dosed orally, once daily for 60 months. The study is expected to enroll 1340 participants and be completed in August 2023 [170].

5 Conclusions and Outlook

Since the cloning and characterization of BACE1 more than one and a half decades ago, BACE1 continues to be an exciting target for drug development against Alzheimer's disease. To date, several inhibitor drugs have now entered into clinical development for potential new treatment of Alzheimer's disease. This review highlighted design, development, and clinical evaluation of BACE1 inhibitors for the treatment of AD. The early design of BACE1 inhibitors involved replacing the scissile amide bond of the APP β -site with nonhydrolyzable transition-state isosteres. Subsequent determination of the X-ray crystal structure of these substrate-based inhibitors and BACE1 complexes provided necessary drug design

templates and greatly facilitated the structure-based design of drug-like peptidomimetic BACE1 inhibitors. The structural evolution of BACE1 inhibitors has been very impressive. The availability of X-ray structural information of BACE1 with a variety of inhibitors along with past experiences of successful drug design against other aspartic acid proteases, such as renin and HIV protease, expedited the design and discovery of many new classes of BACE1 peptidomimetic inhibitors with therapeutic potential. Several BACE1 inhibitor drugs showed promising results in animal models, as they reduced the amyloid plaque in the brain and rescued cognitive deficits. The first peptidomimetic drug candidate, CTS21166, was evaluated in phase I human clinical trials. The reported results indicated that the drug was well tolerated, and A β reduction was achieved with BACE1 drug treatment.

Despite significant progress with peptidomimetic BACE1 inhibitors, AD drug development brought a different set of medicinal chemistry challenges. BACE1 has a large substrate-binding site which makes it difficult to design a small inhibitor that inhibits the enzyme with desirable drug-like properties. Compounds need to have sufficient lipophilic features to penetrate both plasma and intracellular membranes and gain access to the BACE1 active site in the brain. This requires inhibitor size to be around 550 Da with reduced susceptibility to Pgp-mediated efflux in order to cross the blood–brain barrier efficiently. Furthermore, the high homology among aspartyl proteases makes it particularly important to have high selectivity over cathepsin D, renin, and especially BACE2.

The X-ray structural knowledge of peptidomimetic inhibitors and extensive high-throughput screening efforts ultimately paved the way for the evolution of small-molecule nonpeptide BACE1 inhibitors. Many structural classes have now evolved with novel heterocyclic scaffolds such as acyl guanidine, aminopyridine, and aminoimidazole with unique modes of inhibition. Several optimized inhibitors from these classes have been shown to cross the BBB, reach the brain, and reduce brain A β in transgenic AD mice. As many as 13 such inhibitor drugs with favorable pharmacological properties have entered into clinical development, and two of these drugs have now advanced to phase IIa/b clinical trials with human AD patients. A successful clinical outcome would provide the long-awaited proof for the “amyloid hypothesis” in AD therapy. The accumulation of knowledge of BACE1 and its functions through the years still supports BACE1 as a viable target for Alzheimer’s disease drug development. The progress in the field allows much optimism that β -secretase inhibitor drug treatment of Alzheimer’s disease may soon become a reality.

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γ -Secretase Modulators as A β 42-Lowering Pharmacological Agents to Treat Alzheimer's Disease

Douglas S. Johnson and Martin Pettersson

Abstract γ -Secretase is an intramembrane aspartyl protease comprised of four essential subunits including presenilin, nicastrin (NCT), anterior pharynx defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2). The amyloid precursor protein (APP) is cleaved sequentially by β -secretase and γ -secretase to generate A β peptides including neurotoxic A β 42 monomers and oligomers that are believed to be key pathological species in AD. Familial Alzheimer's disease (FAD) mutations in presenilin and APP increase the relative proportion of A β 42. γ -Secretase modulators (GSMs) have been discovered that bind to presenilin and selectively modulate γ -secretase proteolytic activity. Importantly, GSMs have the opposite effect on the A β cleavage profile as compared to FAD mutations, namely they decrease the relative proportion of A β 42. This review will discuss the initial discovery of GSMs and the recent progress leading to the development of GSMs with improved drug-likeness. These efforts have culminated in GSMs that are currently undergoing proof-of-mechanism studies in the clinic, which is a significant step forward toward testing the amyloid hypothesis.

Keywords A β 42, Alzheimer's disease, Amyloid precursor protein, Medicinal chemistry, Presenilin, γ -Secretase, γ -Secretase modulator

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1 Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder and is the most common form of dementia. It results in difficulty remembering newly learned information, memory loss, disorientation, mood and behaviour changes, and eventually difficulty speaking and walking. The pathological hallmarks of AD include deposition of amyloid- β peptides ($A\beta$) in the brain parenchyma [1], formation of paired helical filaments (PHFs) of tau aggregates [2], and ultimately neuronal loss. Genetic linkage studies and positional cloning efforts revealed that rare mutations in amyloid precursor protein (APP), PSEN1, and PSEN2 cause early onset familial AD (EO-FAD) [3]. The vast majority of these mutations (>200) increase the $A\beta_{42}/A\beta_{40}$ ratio, and this relative increase in $A\beta_{42}$ may promote aggregation leading to oligomers and amyloid fibrils. This convergence of disease pathology and genetics led to the amyloid cascade hypothesis, which suggests that a central event leading to AD is the aggregation of amyloid- β peptides ($A\beta$), especially $A\beta_{42}$, which forms toxic soluble oligomers and deposited $A\beta$ in amyloid plaques [4, 5]. Therefore pharmacological approaches to reduce the levels of $A\beta$ peptides have garnered much attention in the field [6–8].

APP is initially cleaved by β -secretase BACE1 to generate $sAPP\beta$ and β -CTF (also referred to as C99), which is cleaved by γ -secretase in the membrane to form APP intracellular domain (AICD) and multiple $A\beta$ peptides (Fig. 1). γ -Secretase initially cleaves APP β -CTF at the ϵ -site at the membrane/cytosolic border to generate $A\beta_{49}$ and/or $A\beta_{48}$, which undergo successive stepwise cleavage every three to four amino acids to generate shorter forms which are released from the

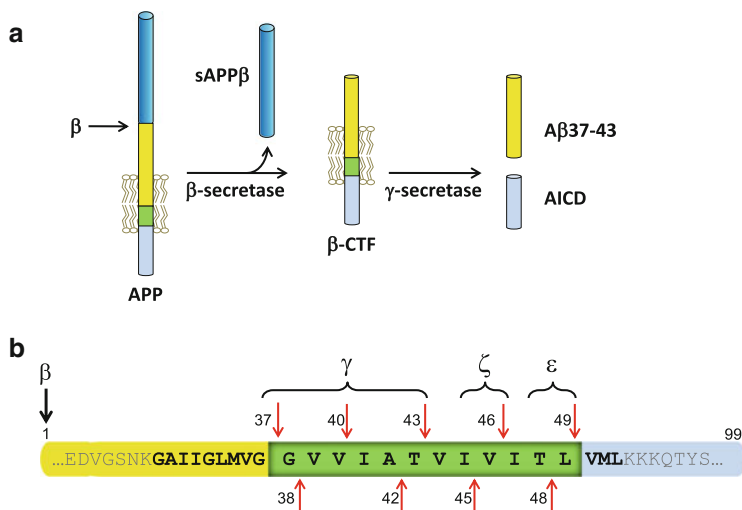


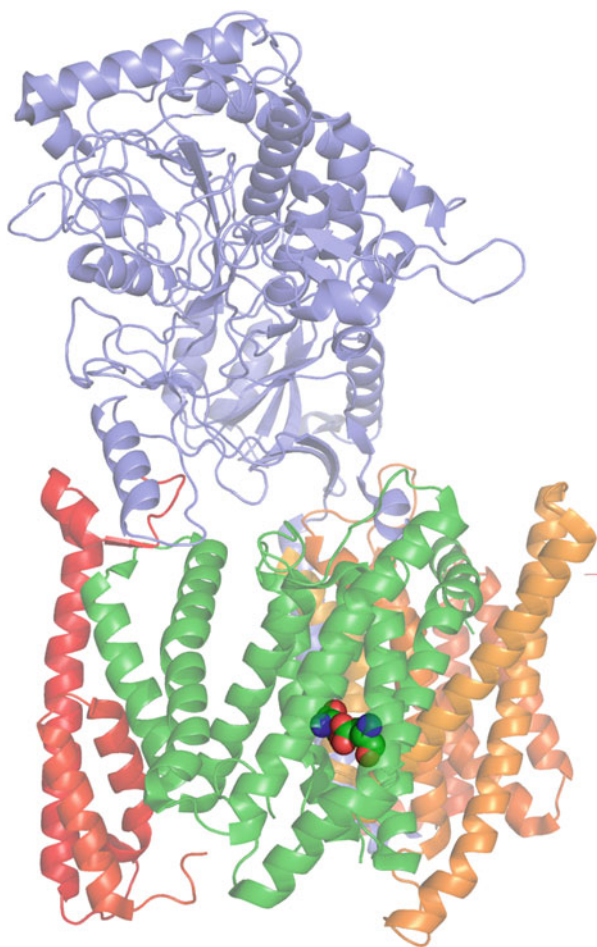
Fig. 1 (a) Sequential cleavage of amyloid precursor protein (APP) by β - and γ -secretases and corresponding products. (b) Sequence of the membrane and nearby regions of the APP β -CTF substrate. Vertical red arrows show locations of γ , ζ , and ϵ cleavages by γ -secretase

membrane and secreted. It has been proposed that γ -secretase cleavage occurs along two product lines with A β 49 leading to A β 46, A β 43, A β 40, and A β 37, and A β 48 leading to A β 45, A β 42, and A β 38 [9]; however, this is probably oversimplified as recent studies have shown that the product lines can cross and the initial γ -secretase cleavage does not always define subsequent product lines [10–12].

There are several lines of evidence that suggest that A β 42 is a key toxic species contributing to AD. First, although A β 42 is a minor A β species, it is the earliest and most predominant form deposited in amyloid plaques in humans [13] and transgenic mouse models [14, 15]. In part, this is because A β 42 is more hydrophobic and more prone to aggregation [16]. Consistent with this early work, recent atomic force microscopy studies show that oligomers of A β 42, but not of A β 40, seed fibril formation [17]. In addition to the importance of A β 42, it is also important to consider the ratio of longer A β species to shorter A β species, where the A β 42/A β 40 ratio has received the most attention to date (although with the advent of γ -secretase modulators (GSMs) that elevate A β 38 and/or A β 37, these species will need to be factored in as well). Analysis of the A β profiles for several familial Alzheimer's disease (FAD) PS mutations suggests that the A β 42/A β 40 ratio, rather than the total amount of A β , correlates better with the age of onset of FAD [18]. It has been suggested that a potential reason for the importance of the A β 42/A β 40 ratio could be that A β 40 plays a protective role by sequestering the more toxic A β 42, thereby preventing further oligomerization of A β 42. In fact, several *in vitro* studies support this hypothesis and show that A β 40 can inhibit the aggregation of A β 42 in an A β 42/A β 40 ratio-dependent manner [19, 20]. Other studies indicate that A β 40 and A β 42 influence their respective aggregation properties where A β 40 inhibits A β 42 aggregation and A β 42 stimulates A β 40 aggregation [21–23]. Also small increases in the A β 42/A β 40 ratio have been shown to exhibit increased synaptotoxicity. In addition, transgenic models that express A β 40 or A β 42 have been developed, and mice expressing A β 42 accumulate insoluble A β 42 and develop amyloid plaques, whereas the mice expressing A β 40 do not develop amyloid pathology [24]. Furthermore, A β 42, but not A β 40, has been shown to induce tau phosphorylation, aggregation, and cleavage [25].

The γ -secretase complex is an intramembrane aspartyl protease and consists of four essential subunits including presenilin (PS), nicastrin (NCT), presenilin enhancer 2 (Pen-2) and anterior pharynx defective 1 (Aph-1) [26]. PS is the catalytic subunit and during assembly of the complex, it undergoes endoproteolysis to produce a heterodimer composed of PS1-NTF and PS1-CTF, which constitutes the active form of the enzyme. An atomic structure of human γ -secretase has been determined by cryo-electron microscopy (Fig. 2) [27, 28]. This structure allows visualization of all 20 transmembrane domains and the interactions between the four components that make up the γ -secretase complex. γ -Secretase has been a highly sought-after target to inhibit A β production, and there has been considerable success developing potent brain-penetrant γ -secretase inhibitors (GSI) with several advancing to human clinical trials. Unfortunately, both semagacestat [29] and avagacestat [30] failed in Phase III and Phase II clinical trials, respectively, and patients receiving the GSIs actually trended toward a worsening of cognition and increased incidence of gastrointestinal adverse events and skin cancer compared with those who received placebo [31]. γ -Secretase has many

Fig. 2 The γ -secretase complex cryo-EM structure (5FN2) [27]. Nicastrin (NCT) is shown in *blue*, anterior pharynx defective 1 (Aph-1) in *orange*, PS1 in *green* and presenilin enhancer 2 (Pen-2) in *red*. The active site aspartic acids are shown as *spheres*



substrates in addition to APP [32], most notably Notch, and it is thought that at least some of the side effects result from inhibition of Notch cleavage [33]. In addition, γ -secretase inhibition results in accumulation of the β -CTF of APP, and this has been proposed as another potential reason for the detrimental side effects of GSI [34]. Given the safety concerns with inhibition of γ -secretase, alternative approaches to modulate γ -secretase activity have been a topic of significant interest and will be described in the next section.

2 γ -Secretase Modulators

GSMs were discovered in 2001 when a subset of non-steroidal anti-inflammatory drugs (NSAIDs) were found to specifically lower A β 42 and increase A β 38, albeit with weak potency [35]. The first generation of NSAID GSMs include sulindac sulfide, indomethacin, (*R*)-ibuprofen, and (*R*)-flurbiprofen (tarenflurbil) (Fig. 3). The A β 42 lowering was independent of the cyclooxygenase activity [35, 36] and *R*-flurbiprofen selectively lowered A β 42 levels in a broken cell γ -secretase assay (A β 42 IC₅₀ ~200–300 μ M) suggesting a direct but weak interaction with γ -secretase [37]. Importantly, these NSAIDs did not inhibit the cleavage of substrates, and the intracellular domains of APP (AICD) and Notch (NICD) were still formed. Despite the weak potency and poor brain penetration, tarenflurbil was advanced into clinical studies, but it did not slow cognitive decline in patients with mild AD in a phase 3 clinical trial [38, 39].

GSMs have several unique and attractive features that suggest that they could be potential therapeutics for AD [7]. They cause: (1) selective decrease of A β 42 or simultaneous decrease of A β 42 and A β 40; (2) increase in shorter A β species (A β 38/A β 37); although the implication of elevating A β 37 or A β 38 in AD is unknown, it is believed that the shorter forms are less prone to aggregation and less pathogenic than A β 42 [17]; (3) no significant effect on the total amount of A β produced; (4) no accumulation of APP β -CTF; (5) no plasma A β rebound; and (6) preserve ϵ cleavage of γ -secretase substrates and, therefore, do not affect the production of APP- and Notch-ICDs (AICD and NICD). Notably, the effect of GSMs on A β peptides is opposite that of many FAD presenilin mutations [40–43], which increase the relative proportion of A β 42 [3]. Furthermore, GSMs increase the levels of shorter peptides (i.e. A β 37 and A β 38), and there is some evidence suggesting that these shorter species could actually inhibit the aggregation of A β 42. Therefore, many pharmaceutical companies have invested heavily in this area, and several different classes of GSMs have been discovered as potential disease-modifying agents for the treatment of AD. These classes can be divided into three categories: NSAID-derived acid GSMs, non-NSAID heterocyclic GSMs, and natural product-derived GSMs as described below. There is some controversy whether NSAID GSMs bind to γ -secretase and/or to the substrate, APP [44, 45], but chemical biology studies

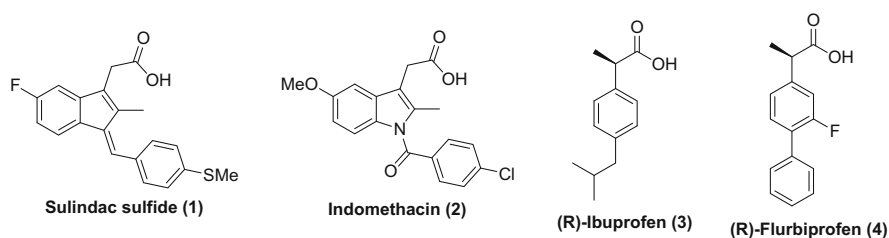


Fig. 3 Non-steroidal anti-inflammatory drug (NSAID) γ -secretase modulators (GSMs)

have shown that second generation GSMs bind directly to PS1-NTF [46–52], although the different classes appear to have distinct binding sites [51].

2.1 Acid γ -Secretase Modulators

Chiesi has developed a series of flurbiprofen analogs with improved potency for lowering A β 42. This culminated in CHF5074 (**5**) which lowered A β 42 with an IC₅₀ between 3 and 41 μ M (Fig. 4) [53]. In a Phase I study looking at the pharmacokinetics and pharmacodynamics of CHF5074 (200, 400, and 600 mg/day), no lowering of A β levels in both plasma and cerebrospinal fluid (csf) was detected. However, it was found that the soluble CD40 ligand, a marker of microglia activation, was lowered in a dose-dependent fashion [54]. So it appears that the compound may be acting as a microglial modulator rather than a GSM. Because of the weak potency and poor brain penetration of early NSAID-derived compounds, a key goal has been to develop GSM compounds with improved potency and brain availability. Several additional groups have also explored this chemical space and were able to generate compounds with A β 42 IC_{50s} less than 200 nM by adding additional substituents to the phenyl acetic acid core of flurbiprofen (Fig. 4). For example, EnVivo has reported EVP-0015962 (**6**) which decreased A β 42 with an IC₅₀ of 67 nM [55]. Consistent with the profile of a GSM, A β 38 was increased while A β total, APP β -CTF, and AICD/NICD generation were not changed. Chronic treatment of EVP-0015962 in Tg2576 mice (20 and 60 mg/kg/day) resulted in brain concentrations of 2.5 and 8.3 μ M, respectively, with corresponding

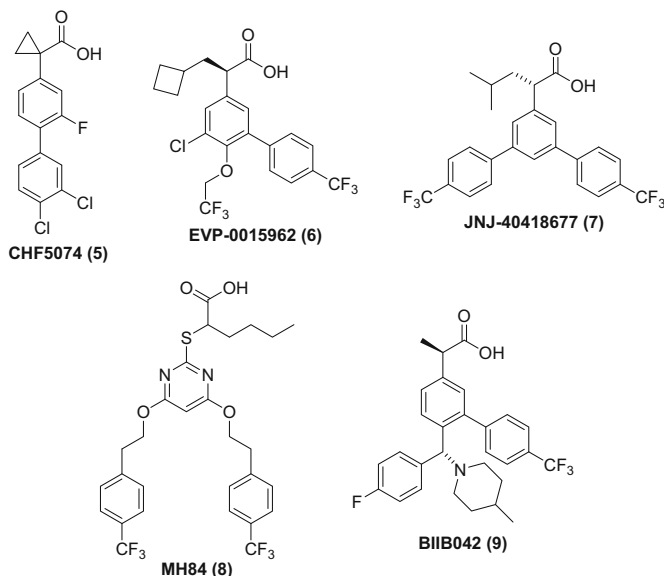


Fig. 4 Aryl NSAID-derived acid GSMs

reduction of cytosolic, membrane-bound, and aggregated A β 42. In addition, amyloid deposition and cognitive deficits were reduced. Janssen added a second trifluoromethylphenyl substituent to give JNJ-40418677 (**7**), a brain-penetrant phenyl acetic acid GSM. JNJ-40418677 reduced A β 42 secretion in cells and primary rat cortical neuronal cultures with an IC₅₀ of approximately 200 nM and did not change levels of total A β or Notch processing [56]. Acute treatment with JNJ-40418677 selectively reduced brain A β 42 levels in wild-type mice with a concomitant increase in A β 38. Chronic administration in Tg2576 mice from 6 to 13 months of age resulted in dose-dependent reduction in amyloid plaque load. Intriguingly, all A β species in soluble and deposited fractions were reduced, which deviates from a typical GSM profile and merits further investigation. Another series of aryl carboxylic acid GSMs, exemplified by MH84 (**8**), has been shown to have PPAR γ agonist activity [57]. However, these compounds are very lipophilic and the potency is weak so they have not advanced beyond being tool compounds. Biogen has also published on a series of aryl acid GSMs that merge the core of flurbiprofen with features present in GSM-1 (**10**, Fig. 5). For example, BIIB042 (**9**) lowered A β 42 in CHO cells with an IC₅₀ of 170 nM and increased A β 38 with an EC₅₀ of 150 nM [58]. A reduction of brain A β 42 was observed when BIIB042 was administered to mice, rats, and cynomolgus monkeys.

Merck made a significant advance by replacing the core aryl ring of previous NSAID-derived GSMs with a piperidine ring (Fig. 5). Then they optimized the substituents to generate a potent series of piperidine acetic acid GSMs, exemplified by GSM-1 (**10**) [59, 60]. GSM-1 has an A β 42 IC₅₀ value of 120–348 nM and selectively reduced A β 42 and increased A β 38, while the levels of total A β and AICD did not

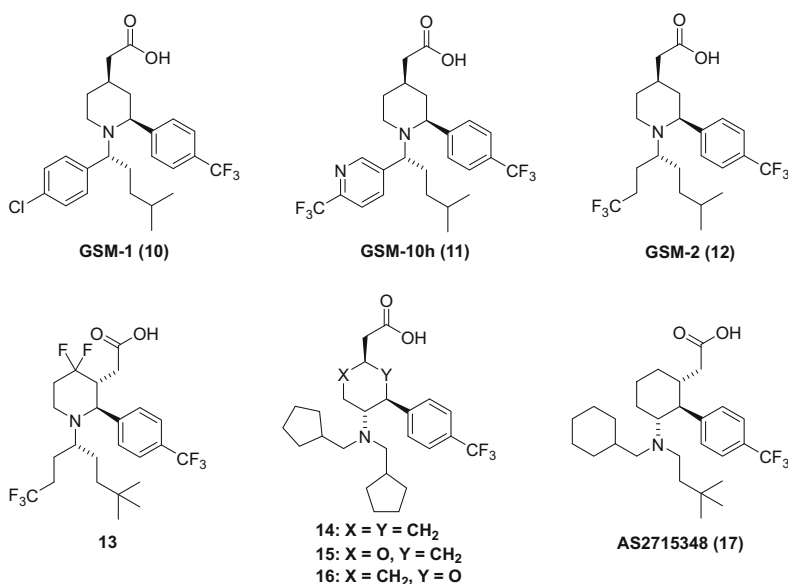


Fig. 5 Piperidine and aminocyclohexane acid GSMs

change. More recently, GSK reported GSM-10h (**11**), which is a pyridyl analog of GSM-1 with lower lipophilicity and similar potency ($A\beta_{42}$ IC_{50} = 300 nM) [61]. GSM-10h increased $A\beta_{38}$ and had no effect on total $A\beta$ levels indicating that the rise in $A\beta_{38}$ compensates for the decrease in $A\beta_{42}$. GSM-10h demonstrated excellent bioavailability and good central nervous system (CNS) penetration and decreased $A\beta_{42}$ in plasma, CSF, and brain of rats. In contrast to GSIs, GSM-10h did not result in accumulation of β -C-terminal fragment (β -CTF) and did not cause $A\beta$ rebound [62]. In addition, Astellas has reported the pharmacological profile of another piperidine acetic acid GSM referred to as GSM-2 (**12**) [34]. GSM-2 selectively lowered $A\beta_{42}$ with an IC_{50} of 65 nM, while increasing $A\beta_{38}$ (EC_{50} = 81 nM) with no effect on β -CTF levels or Notch signalling. In contrast to the GSIs LY450139 and BMS-708,163, administration of GSM-2 for 8 days ameliorated the cognitive deficit in 5.5-month-old Tg2576 mice as measured in the Y-maze test. All three drugs reduced $A\beta_{42}$ levels, but only the two GSIs increased β -CTF levels and some have suggested that β -CTF elevation could partially explain the cognitive impairment associated with GSI treatment. In addition, 8-day administration of GSM-2 to 18-month-old Tg2576 mice reduced newly synthesized soluble $A\beta_{42}$ (measured using stable isotope-labeling) and significantly ameliorated cognitive deficits in the Y-maze test [63]. Brain soluble $A\beta_{42}$ was not reduced, suggesting that newly synthesized $A\beta_{42}$ contributes more to the cognitive deficits in Tg2576 mice than plaque-associated soluble $A\beta_{42}$. Merck has also disclosed a series of fluorinated piperidine acetic acid GSMs, including the isomeric 1,2,3-trisubstituted piperidine **13** [64].

Pfizer and Astellas have disclosed a variation of the piperidine acetic acid GSM series that involves transposing the piperidine nitrogen out of the ring providing an aminocyclohexane. This was done in part to alleviate the concern of metabolic activation of the piperidine ring system and subsequent hepatotoxicity that has been observed for some piperidine carboxylic acid derivatives [65]. The amino group was substituted with various alkyl and cycloalkyl groups to give potent GSMs [66]. For example, the bis (cyclopentylmethyl)amino analog **14** has an $A\beta_{42}$ IC_{50} of 229 nM. It was also possible to incorporate an oxygen in the ring to give the corresponding aminotetrahydropyrans. The 2,5,6-trisubstituted tetrahydropyran **15** and 2,4,5-trisubstituted tetrahydropyran **16** were reported to reduce $A\beta_{42}$ levels in cells with an IC_{50} of 357 and 777 nM, respectively. This modification is potentially attractive because it lowers the lipophilicity of the compounds and decreases the basicity of the nitrogen. Furthermore, Astellas extended the series to include 1,2,3-trisubstituted aminocyclohexanes with the analogous substitution pattern as piperidine **13** [67]. An example is AS2715348 (**17**), which has been reported to lower $A\beta_{42}$ levels in various cell types with an IC_{50} between 62 and 156 nM and did not affect β -CTF levels or Notch signalling. In addition, AS2715348 selectively reduced $A\beta_{42}$ in the brain of rats, Tg2576 mice, and non-human primates. It remains to be seen if an acceptable safety profile with adequate brain exposure at the target can be achieved with the class of acid GSMs.

2.2 Heterocyclic γ -Secretase Modulators

The second major class of GSMs is characterized by an A–B–C–D-type heterocyclic scaffold in which the A–B subunit often features an arylimidazole moiety. This series was first disclosed by NeuroGenetics and was further refined by Eisai [68, 69]. NeuroGenetics recently reported on the discovery of **18** [70], which has been identified as NGP-555 (Fig. 6). This series originated from a high-throughput screen of an 80,000 compound library that was assembled from commercial sources. An initial screening hit with an A β 42 IC₅₀ of 15 μ M was optimized through a series of targeted libraries and follow-up analogues to arrive at **18**. This lead compound was reported to have an A β 42 IC₅₀ of 10 nM in SH-SY5Y APP cells, and 29 nM in Tg2576 MBCs. Unlike acid GSMs, which selectively reduce A β 42, the A–B–C–D-type heteroaryl series reduces both A β 42 and A β 40 while increasing the levels of A β 38 and A β 37, as confirmed by immunoprecipitation mass spectrometry experiments. Four-month-old Tg2576 mice were dosed with **18** at 5–100 mg/kg p.o. for three consecutive days, and brain and plasma samples were analysed 6 h post-dose. Dose-dependent reductions of A β 42 and A β 40 were observed, and a 50 mg/kg dose afforded approximately a 30% reduction of brain A β 42. Next, **18** was tested in a 7-month plaque reduction study in Tg2576 mice in which the compound was administered via the food pellets to achieve a plasma concentration of 3.95 μ M [70]. Serial extraction of brain samples was performed to analyse the levels of soluble, detergent-soluble, and formic acid-soluble A β peptides. Not only was a significant reduction of A β 42 and A β 40 observed in all three fractions, but a robust reduction in A β 38 was observed as well. This result stands in sharp contrast to observations in the acute in vivo studies where the levels of A β 38 increase. The

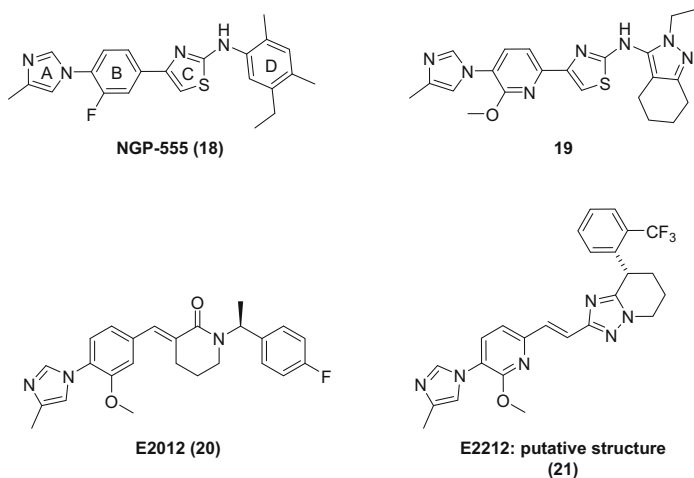


Fig. 6 NeuroGenetics and Eisai GSMs

reason for the reduction of A β 38 levels in the plaque reduction study is not understood at this point.

The physicochemical properties of **18** and related compounds required further optimization. In particular, the kinetic solubility of **18** was found to be very low ($<0.1\text{ }\mu\text{M}$), which may be attributed to high lipophilicity ($\text{clogP} = 7.7$) in combination with four aromatic rings [71]. A recent report described efforts to improve solubility by replacing two of the phenyl rings with heterocycles, and the exemplified compounds have clogP values ranging from 3.3 to 5.7 and kinetic solubility $<1.6\text{--}18.4\text{ }\mu\text{M}$ [72]. Compound **19** incorporating a methoxypyridine as the B-ring and a fused pyrazole in place of the D-ring was the most potent analogue with an A β 42 IC_{50} of 30 nM ($\text{clogP} = 5.5$, kinetic solubility = $2.7\text{ }\mu\text{M}$). Although **19** is an improvement relative to **18**, it highlights the significant challenge of balancing sufficient potency with adequate physicochemical properties within this chemotype, and this problem is evident throughout the GSM literature (*vide infra*). Nevertheless, in spite of the challenges encountered in this series, NGP-555 was recently reported to enter Phase I clinical development [73].

In 2005, Eisai published a patent application that claimed compounds such as E2012 (**20**) featuring a lactam as the C-ring hydrogen-bond acceptor and with reduced aromatic ring count relative to **18** and **19** ($\text{clogP} = 4.8$) [69]. E2012 has been extensively profiled *in vitro* and *in vivo*: A β 42-lowering activity was determined in several cell lines including HEK 293^{Swe-Lon} cells [74], rat cortical neuronal cultures [75], and CHO-APP cells [76], and the IC_{50} values were 64, 92, and 143 nM, respectively. *In vivo* reduction of A β 42 has been assessed in rodent and dog CSF biomarker studies, and robust and dose-dependent reduction of brain A β 42 has been reported [77, 78]. A Phase I trial with E2012 began in 2006, marking the first non-NSAID GSM to enter clinical development [79]. However, it was subsequently put on hold due to the observation of lenticular opacity (cataract) in a 13-week rat safety study. A recent paper linked the ocular toxicity to increased levels of lenticular desmosterol resulting from inhibition of DHCR24, which is the enzyme responsible for conversion of desmosterol to cholesterol [80]. Elevation of plasma desmosterol was demonstrated to be an effective toxicity biomarker. Furthermore, cataracts were not observed in a 13-week monkey study, and the clinical trial was therefore allowed to proceed. E2012 was tested at doses of 1–400 mg in a single ascending dose study, and plasma A β 42 was reduced by ~50% at the 400 mg dose [79].

Further development of E2012 was not pursued, and focus was rather shifted to the more potent clinical candidate E2212 (**21**; putative structure). This compound has an IC_{50} of 9 nM in rat cortical neuronal cultures ($\text{clogP} = 4.8$), and it was reported to have an improved safety profile [81]. Robust reductions of brain A β 42 have been demonstrated in multiple preclinical species. In a rat *in vivo* efficacy study, brain A β 42 was reduced by 47.7% and 70% following doses of 15 and 100 mg/kg *p.o.*, respectively [78]. This novel GSM retains the olefin linker of E2012, but the central lactam has been replaced with a fused triazole moiety. It is interesting to note that **21** incorporates conformational constraint in the C–D region, and this has become a reoccurring theme in the subsequent patent literature

(vide infra). E2212 was evaluated in a single ascending dose study in healthy human volunteers, and it was found to be well tolerated following single doses up to 250 mg. It has a terminal half-life ranging from 12.5 to 19 h, and a 53.6% reduction in plasma A β 42 was achieved following the 250 mg dose [81]. However, further clinical development of E2212 has not been reported.

The discovery and initial optimization of the A–B–C–D-type heteroaryl series by NeuroGenetics and Eisai generated substantial interest throughout the industry, and these chemotypes have served as starting points for numerous GSM programs (vide infra) [82–85]. Analysis of the literature reveals that the A–B region is highly conserved whereas much greater structural diversity has been achieved within the C- and D-ring sections of this class of GSMs. The frequently used A-ring imidazole appears to be making a key polar interaction with the target binding site. Recently, the imidazole has successfully been replaced with a variety of other heterocycles that can maintain the polar interaction including triazole, pyrazole, oxazole, and pyridyl [83]. The B-ring functions as a rigid spacer to link the A- and the C-rings together within a defined spacial configuration. The C-ring makes a critical polar interaction with the enzyme presumably serving as a hydrogen-bond acceptor; however, there is considerable flexibility as to what types of polar functionality are tolerated in this region. Examples include a wide variety of heterocycles as well as carbonyl-containing scaffolds. Finally, the D-ring appears to be directed into a lipophilic pocket, and the vast majority of GSMs incorporate a lipophilic aromatic ring that is substituted with halogens or other non-metabolizable lipophilic substituents. Only recently have GSMs with robust A β 42-lowering activity appeared in the literature where this aromatic ring has been replaced with aliphatic systems [83].

The GSM program at Schering-Plough (now Merck) has developed several alternative chemotypes using E2012 as the starting point. Many creative C-ring replacements have been examined including imino heterocycles [86], and 5,6-bicyclic heterocycles [87], the latter conceptually arising from cyclizing the carbonyl of E2012 onto the olefin linker. These efforts ultimately led to the discovery a pyrazolopyridine series as exemplified by **22** (A β 42 IC₅₀ = 107 nM, clogP = 5.4, Fig. 7) [88]. When dosed in rat at 30 mg/kg p.o., this compound achieved a 45% reduction of CSF A β 42 at the 3-h time point, and the corresponding brain and plasma exposures were 5.02 and 1.22 μ M, respectively. One of the challenges associated with this series was to reduce P-gp mediated efflux in order to obtain acceptable brain exposure. This problem could be mitigated by introducing a lipophilic and electron-withdrawing chloro-substituent onto the pyridopyrazine core.

An alternative strategy by Schering/Merck was to prepare amide bioisosteres such as an oxadiazoline by forming a ring between the C-ring hydrogen-bond acceptor of E2012 and the right-hand methylene-linker, e.g. **23** [74]. Initial, monocyclic versions of this chemotype were found to be unstable under acidic conditions whereas bicyclic structures such as **23** had significantly improved stability. Furthermore, chemical stability was also dependent on the nature of the substituent at the quaternary centre. GSM **23** displayed an A β 42 IC₅₀ value of 29 nM in HEK293^{Swe-Lon} cells

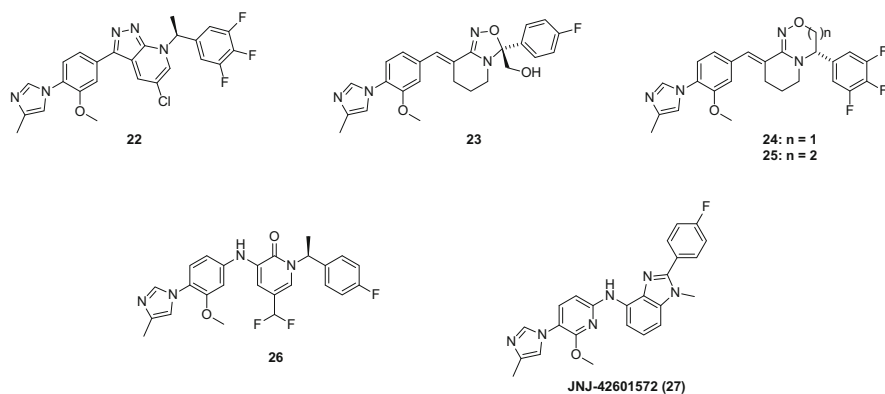


Fig. 7 Schering/Merck and Janssen GSMs

($clogP = 4.9$), and this compares favourably to E2012, which had an $A\beta_{42}$ IC_{50} of 58 nM under the same assay conditions. Given the excellent pharmacokinetic profile of compounds in the oxadiazoline series, this amide bioisostere was expanded further to afford the corresponding six-membered oxadiazine, e.g. **24** ($A\beta_{42}$ $IC_{50} = 33$ nM, $clogP = 6.6$) [74, 89]. Immunoprecipitation/mass spectrometry analysis revealed that **23** and **24** reduce formation of $A\beta_{42}$, $A\beta_{40}$, and $A\beta_{39}$ whereas production of the shorter peptides $A\beta_{38}$ and $A\beta_{37}$ is increased. Both lead compounds exhibited robust *in vivo* activity across multiple preclinical species. A 3 mg/kg oral dose of **24** in rat resulted in a 37% reduction of $A\beta_{42}$ in cortex and a 35% reduction in CSF. In cynomolgous monkeys treated with **24** at 30 mg/kg p.o., 30% and 64% reductions of cortical and CSF $A\beta_{42}$ were observed. This dose resulted in an exposure of ~ 6 μM of **24** in plasma and a brain/plasma ratio of about 1.4.

Inhibition of hERG remained a key challenge in the oxadiazoline and oxadiazine series. The two lead compounds **23** and **24** had hERG IC_{50} values of 3.3 and 1.0 μM , respectively, and additional heterocyclic cores were evaluated in an effort to address this problem. Ring expansion to generate fused oxadiazepines ultimately resulted in the discovery of **25** ($A\beta_{42}$ $IC_{50} = 39$ nM, $clogP = 7.1$) [90]. This structural modification not only retained comparable *in vitro* $A\beta_{42}$ -lowering activity, but it also reduced hERG activity with **25** only exhibiting 33% inhibition of hERG at 10 μM . When dosed in rat at 10 mg/kg p.o., **25** achieved a 61% reduction of CSF $A\beta_{42}$ at the 3-h time point. The corresponding plasma AUC exposure was 15,800 nM $\times$ h with a B/P of 3.2. It is also worth noting that **25** did not exhibit CYP inhibition, which has been a reoccurring problem in the A–B–C–D-type heteroaryl GSM series. GSM **25** was tested for inhibition of CYP3A4, 2D6, and 2C9 and was found to have IC_{50} values greater than 30 μM against these isoforms.

A characterizing feature of GSMs **20–25** is the rigid olefin linker that joins the B- and the C-rings (“olefin-linked heteroaryl series”). A key design objective for multiple organizations has been to identify a suitable replacement for the electron-rich olefin, and a common tactic has involved using an amine linker (“amine-linked heteroaryl series”) [83]. This approach was pioneered by Schering/Merck as

exemplified by **26** (A β 42 IC₅₀ = 44 nM, clogP = 4.6, Fig. 7). The team hypothesized that the NH-linker could form an intramolecular hydrogen bond with the C-ring pyridone carbonyl, thus maintaining the necessary rigidity [91]. The same design principles were utilized by researchers at Janssen, who further developed this concept to deliver GSMs with exquisite *in vitro* and *in vivo* A β 42-lowering activity [92]. Janssen evaluated several heteroaromatic C-rings such as **27** that incorporate an sp² nitrogen as a hydrogen-bond acceptor in the same location relative to the NH-linker as the pyridone carbonyl of **26**. Imidazopyridines were initially explored as a pyridone bioisostere, and this was followed by benzimidazoles, indazoles, and oxazoles. Introduction of a methyl substituent on the benzimidazole C-ring was found to have a big impact on potency. Presumably, this substituent serves as a conformational control element to increase the dihedral angle between the C- and the D-rings thus locking the molecule in the putative pharmacologically active conformation. Furthermore, calculations indicated that introduction of the methyl substituent results in an increase in the hydrogen-bond acceptor strength of the sp² nitrogen.

Ultimately, benzimidazole JNJ-42601572 (**27**) was selected for further profiling (A β 42 IC₅₀ = 16 nM, clogP = 6.1, Fig. 7) [92]. A 10 mg/kg oral dose in mouse afforded a 51% reduction of brain A β 42 at the 4-h time point. The corresponding brain and plasma exposures of **27** at this time point were 2.8 and 5.3 μ M, respectively. A 36% reduction of brain A β 42 was observed in rat at the same dose and time point with a concomitant increase in both A β 37 and A β 38 (402% and 49%, respectively) [92]. JNJ-42601572 was further evaluated in beagle dog at 20 mg/kg p.o., and this resulted in a 19% and 48% reduction of CSF A β 42 at the 4- and 8-h time points, respectively. As with a majority of GSMs described in the literature up to this point, **27** maintained high aromaticity and high lipophilicity (clogP = 6.1), and this may explain the signs of liver toxicity that were observed in dogs when dosed at 20 mg/kg p.o.

Connection of the B- and the C-rings via an amine linker has been explored by a number of additional companies including Hoffmann-La Roche, AstraZeneca, BMS, Boehringer Ingelheim, and Forum. A frequently used strategy has been to incorporate an aminopyrimidine C-ring, and often to introduce a second, aliphatic ring as a conformational control element (Fig. 8). Hoffmann-La Roche has reported the discovery of GSMs such as RO-02 (**28**), which has an A β 42 IC₅₀ of 15 nM in the HEK293/sw cells, and 13 nM in rat primary cortical neurons (clogP = 5.1). This compound served as a starting point for the development of a biotinylated photoaffinity probe to study the mechanism of action of this class of GSMs [46]. A similar series of GSMs has been reported by AstraZeneca as highlighted by AZ4800 (**29**) and AZ3303 (**30**) [93]. These compounds were tested in HEK APPsw cells and exhibited A β 42 IC₅₀ values of 26 and 74 nM, respectively. As in the case heteroaryl GSMs described previously, the series from AstraZeneca increased formation of A β 37 and A β 38. It is interesting to note that while **29** exhibited a stronger increase in A β 37 than A β 38, the opposite trend was observed for **30** where a more robust increase was seen for A β -38. This finding suggests that structural modification even within the same series can impact the A β profile. *In vivo* efficacy of **29** and **30** was evaluated in wild-type mouse. At a dose of

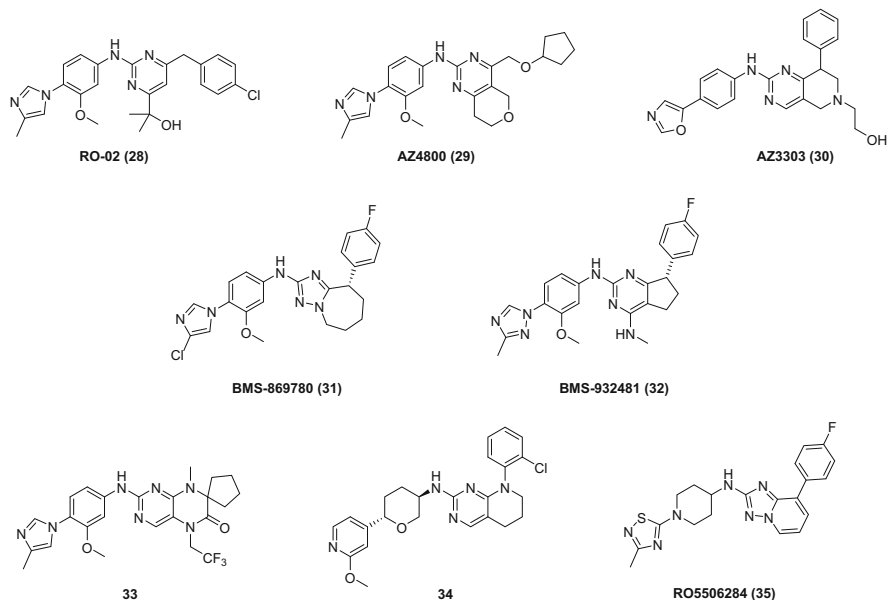


Fig. 8 Hoffmann-La Roche, AstraZeneca, BMS, Boehringer Ingelheim, and Forum GSMs

300 $\mu\text{mol/kg}$ p.o. (130 mg/kg for **29** and 124 mg/kg for **30**), GSM **29** and **30** achieved 46% and 25% reduction of brain A β 42, respectively. The corresponding free brain concentrations at these doses were 880 and 570 nM. GSM **29** is noteworthy in that it represents one of the first examples where robust A β 42-lowering activity has been maintained while using a fully saturated ring system as the lipophilic D-ring. This resulted in a significant improvement in physicochemical properties such as reduced aromaticity, increased sp^3/sp^2 ratio (fsp^3), and reduced lipophilicity. GSM **29** has a clogP value of 3.3, which has generally been challenging to achieve – both within the NSAID-derived acid series as well as the non-NSAID heteroaryl series.

Starting from lead matter identified in a high-throughput screen, the team at BMS has developed potent GSMs that contain an aminotriazole C-ring. These efforts resulted in preclinical candidate BMS-869780 (**31**), which has an A β 42 IC_{50} of 5.6 nM in H4-APPsw cells (clogP = 5.5) [94]. In vivo efficacy was extensively characterized in time-course studies in rats and triple transgenic mice to establish pharmacokinetic/pharmacodynamics (PK/PD) relationships and to enable human dose prediction. Through these studies and subsequent PK/PD modelling, in vivo IC_{50} values were determined for both species: The mouse and rat unbound plasma A β 42 IC_{50} values were 22 and 5.1 nM, respectively, which correlates well with the in vitro IC_{50} . The PK profile of BMS-869780 was assessed in rat, dog, and non-human primate from which a projected human clearance of 5.6 mL/min/kg, half-life of 13 h, and oral bioavailability of 28% were established. A 25% reduction of brain A β 42 human was projected to require a dose of 700 mg/day. Unfortunately,

hepatotoxicity (lipidosis) was observed in a 4-day rat safety study at doses of 10 mg/kg/day that afforded a mean plasma $C_{\max}$ concentration of 1.9 μM . The toxicity was attributed to agonism of the pregnane X receptor (PXR), which is a nuclear hormone receptor involved in induction of cytochrome P450 3A4 (CYP3A4). Because of the high projected human dose and inadequate therapeutic index, further development of BMS-869780 was halted.

Continued efforts led to the identification of an alternative candidate molecule, BMS-932481 (**32**), where the chloroimidazole has been replaced with a methyltriazole and the central aminotriazole C-ring has been replaced with an aminopyrimidine [95]. This compound has similar potency and lipophilicity as the previous candidate (A β 42 IC_{50} of 5.5 nM, $\text{clogP} = 5.3$). In vivo efficacy was established in rat at doses of 1, 5, and 10 mg/kg administered intravenously to minimize variability, and A β 42 was maximally reduced in brain by about 75% at the 10 mg/kg dose. When administered orally in dog at 30 mg/kg, A β 42 and A β 40 were both maximally reduced by about 65% in CSF at the 24-h time point. Robust increases in A β 37 and A β 38 were observed (5-fold and 1.8-fold for A β 37 and A β 38, respectively), which is consistent with the profile of A–B–C–D-type heteroaryl GSMs. Finally, in vivo efficacy was also established in non-human primate, where an intravenous dose of 15 mg/kg afforded approximately a 75% reduction of CSF A β 42 12–24 h post-dose.

In November 2012, BMS announced entering Phase I clinical trials with BMS-932481, and the clinical data has recently been published [95, 96]. BMS-932481 was initially evaluated in a single ascending dose study with oral doses ranging from 10 to 1,200 mg. The compound was generally well tolerated, and a 50% reduction of CSF A β 42 was achieved in healthy adults following a single dose of 900 mg that afforded a plasma $C_{\max}$ exposure of 1.68 μM . BMS-932481 was subsequently evaluated in a multiple ascending dose study with doses of 50 and 100 mg for 28 days and 200 mg for 14 days. The corresponding reductions in CSF A β 42 were 38%, 46%, and 69–77% for the three doses, respectively. A similar reduction of A β 40 was achieved along with robust and dose-dependent increases in A β 37 and A β 38. The PK/PD relationship in human closely matched that obtained in preclinical species. Significant drug accumulation was observed in the multiple ascending dose study, and at the last day of dosing, $C_{\max}$ exposures reached 0.66, 1.50, and 4.43 μM for the three doses, respectively. Administration of BMS-932481 at a dose of 200 mg/day was halted after 14 days due to changes in liver enzymes (ALT). Ultimately, the development of BMS-932481 was terminated because the reduced therapeutic index due to liver toxicity would only enable reductions of CSF A β 42 up to 25%.

Boehringer Ingelheim has also pursued *N*-linked heteroaryl GSMs and has published three patent applications describing compounds such as **33** that incorporate an aminopyrimidine C-ring [97–99]. It is interesting to note that this modulator achieved good in vitro activity (A β 42 $\text{IC}_{50} = 22$ nM, $\text{clogP} = 5.0$) without an aromatic D-ring. Forum has recently published three patent applications that also describe GSMs incorporating an aminopyrimidine moiety as the C-ring hydrogen-bond acceptor [100–102]. Although many of the exemplified compounds utilize the typical arylimidazole A–B unit, certain compounds such as **34** (A β 42 $\text{IC}_{50} = 41$ nM,

$clogP = 5.3$) achieve good in vitro activity with a saturated ring system such as a tetrahydropyran. This represents a significant departure from a majority of the GSMs described in the patent literature and offers an alternative to the commonly used (and potentially problematic) aniline B-ring.

Roche has combined a thiadiazole and a piperidine ring as the A–B moiety and introduced conformational constraint in the C-ring to afford GSMs with good A β -lowering activity [103]. GSM RO5506204 (**35**) has an A β 42 IC_{50} of 25.7 nM in H4 neuroglioma cells overexpressing human APP-Swe and reduced lipophilicity ($clogP = 3.4$). Reductions of brain A β 42 of 40%, 48%, and 73% were achieved at the 4-h time point when dosed in APP-Swe mouse at 10, 30, and 100 mg/kg p.o., respectively. The effect on plaque reduction was then evaluated in a chronic study in the TG2576 mouse model. GSM RO5506204 was dosed for 6 months at 30 mg/kg/day p.o., whereupon a 40% reduction of insoluble A β 42 was observed relative to the vehicle group. Plaque deposition was also examined using positron emission tomography (PET) imaging and was found to show good correlation with terminal analysis of A β levels. Analysis of fibrillar A β plaques using methoxy-X04 staining indicated a 56% reduction relative to the vehicle group. Interestingly, there were considerably fewer small plaques in the GSM-treated animal group relative to the vehicle group, indicating that GSM treatment may have a significant effect on de novo plaque formation. The authors propose that a combination of a GSM with an A β antibody, which targets existing plaques, may have the potential for increased therapeutic benefit relative to either treatment alone.

In addition to Roche, other companies have also explored significant modifications to the A–B section of the A–B–C–D-type heteroaryl series. An interesting example has been published by researchers at Merck, who identified a novel chemotype through a high-throughput screening campaign. This series contains a methoxyphenyl piperazine in the A–B region, and a pyrimidine unit as the central hydrogen-bond acceptor [104]. Further optimization by replacing the pyrimidine with a purine moiety afforded GSM leads such as **36** (Fig. 9) [105]. This structural modification resulted in a tenfold boost in potency (A β 42 $IC_{50} = 21$ nM in SH-SY5Y cells stably overexpressing the β -CTF SPA4CT, $clogP = 8.0$). In addition to the methoxyphenyl moiety, a number of additional groups were well tolerated including a 5-methyl-1,3-pyrimidine. Interestingly, **36** is about 16-fold selective for A β 42 over A β 40 in vitro, and this is borne out in vivo using the APP-YAC transgenic mouse model. When dosed at 100 mg/kg p.o., **36** selectively reduces brain A β 42 by 73% at

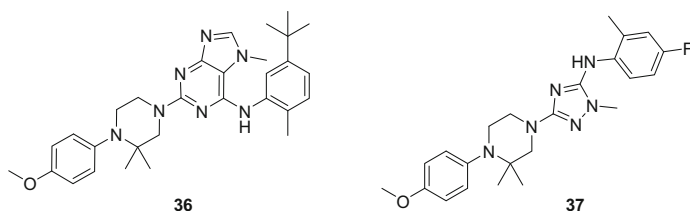


Fig. 9 Merck and Janssen GSMs

the 6-h time point. The corresponding plasma and brain exposure was 23 and 7.8 μ M, respectively. More recently, Janssen has described a similar chemotype, where the purine of **36** has been replaced with a triazole, e.g. **37** [106]. A β 42-lowering activity was measured in SKNBE2 cells (wtAPP 695), and **37** displayed good in vitro activity (A β 42 IC₅₀ = 50 nM, clogP = 5.5) [103]. This translated into a robust response in vivo with a 45% reduction of brain A β 42 in rodents at a dose of 30 mg/kg p.o.

The strategy of directly connecting the B- and the C-rings has also been explored within the original arylimidazole moiety “C-linked heteroaryl series” [83]. GSK has utilized a pyridine and a pyridazine as the C-ring hydrogen-bond acceptor and has directly attached it to the B-ring to afford GSMs such as **38** (Fig. 10) [107–109]. A- β -lowering activity was evaluated in SHSY5Y-APP_{swe} cells, and **38** displayed an IC₅₀ value of 63 nM (clogP = 5.2). Combined with a good pharmacokinetic profile, this translated into robust, dose-dependent A β -lowering in vivo. When dosed in rat at 10, 30, and 100 mg/kg p.o., brain A β 42 was reduced by ca 20%, 40%, and 75% for the three doses, respectively.

As discussed above, achieving robust A β -lowering activity (both in vitro and in vivo) while maintaining drug-like physicochemical properties has continued to be a significant challenge for the GSM field. In particular, good in vitro potency has often come at the expense of high lipophilicity, which in turn has reduced the odds of achieving adequate safety margins [110]. After encountering liver toxicity with **27**, the team at Janssen focused on reducing lipophilicity and aromaticity, resulting in compounds such as C-linked GSM **39** (Fig. 10) [111, 112]. This compound has an A β 42 IC₅₀ of 56 nM, but with dramatically reduced lipophilicity relative to **27** (clogP of 3.1 and 6.1 for **39** and **27**, respectively). When dosed orally at 20 mg/kg in dog, GSM **39** afforded a 30–40% maximal reduction of CSF A β 42 at the 4- and 8-h time points, and there were no signs of liver toxicity in spite of high plasma exposure (24 μ M). The safety profile of **39** was further evaluated in a 2-week safety study in rat at 200 mg/kg/day, and no liver toxicity was observed. Although this result represents a significant advancement for the program, the authors note that

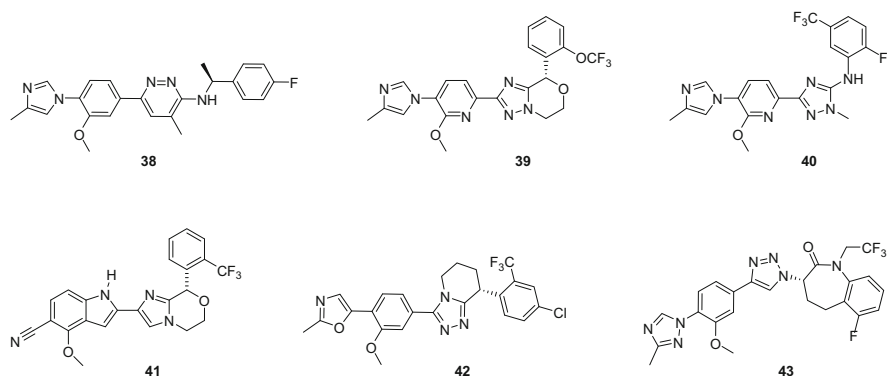


Fig. 10 GSK, Janssen, Takeda, and Merck GSMs

the high plasma exposure required for in vivo efficacy of **39** would not be suitable for chronic treatment, and additional gains in in vivo efficacy would be therefore be required.

Another approach used by Janssen to optimize the C-linked triazole GSMs involved using an aniline linker between the central triazole and the terminal phenyl ring along with an *N*-methyl substituent on the triazole as a conformational control element (e.g. **40**) [113]. Although **40** is more potent than **39** ($A\beta_{42}$ IC_{50} = 19 nM), it is also considerably more lipophilic ($clogP$ = 4.8). Aminotriazole **40** exhibited robust central efficacy in a time-course study in non-transgenic mouse. Brain $A\beta_{42}$ was maximally reduced by 61% at the 4-h time point following a 30 mg/kg oral dose. Efficacy was also assessed in dog, where a dose of 20 mg/kg p.o. resulted in 56% reduction of CSF $A\beta_{42}$ at the 8-h time point. No changes in liver enzymes (ALT and AST) were detected in a 1-week dog safety study at doses of 10 and 20 mg/kg/day, and only a small change in bilirubin was observed. Thus, **40** appears to have an improved safety profile relative to Janssen's early lead compound **27**.

Janssen has continued their search for new chemotypes with the goal of further improving alignment of potency and physicochemical properties. In early 2013, a patent application was published that disclosed compounds such as **41** that incorporate a novel A–B moiety [114]. In this series, the commonly used arylimidazole has been replaced with a cyanoindole unit. Compound **41** was reported to have an $A\beta_{42}$ IC_{50} value of 20 nM and $clogP$ of 3.9. Takeda has pursued chemical matter related to that described by Janssen [115]. Combination of an oxazolylphenyl A–B moiety with known C-ring heterocycles from the GSM literature afforded compounds such as **42** ($A\beta_{42}$ IC_{50} of 60 nM in rat primary neuronal cells, $clogP$ = 4.6, measured $logD$ = 3.5). This compound reduced brain $A\beta_{42}$ by 50% following a 10 mg/kg oral dose in mouse.

Scientists at Merck have designed GSMs that incorporate a 1,2,3-triazole linked to a benzoazepinone [116, 117]. Initial analogs in this series did not have adequate oral bioavailability in dog and monkey [116], but this problem could be addressed by modifications to the phenylimidazole A–B moiety leading to modulators such as **43** (Fig. 10) [117]. This compound has an $A\beta_{42}$ IC_{50} of 42 nM in SH-SY5Y cells overexpressing APP C-terminal fragment SPA4CT ($clogP$ = 3.8). Oral bioavailability in rat and dog was 25% and 84%, respectively, and brain $A\beta_{42}$ was reduced by 38% when dosed at 50 mg/kg p.o. in the APP-YAC transgenic mouse model. Finally, robust central efficacy was also achieved in non-transgenic rat with an ED_{50} value of 4.7 mg/kg.

Pfizer has also pursued non-NSAID-derived GSMs using E2012 as a starting point [76]. However, while a majority of the previously highlighted chemotypes relied on a heterocyclic C-ring as the central hydrogen-bond acceptor, Pfizer retained the amide carbonyl of E2012 while removing the cinnamide olefin and directly attached the carbonyl to the A–B arylimidazole moiety. A conformationally restricted benzofuran unit was introduced as the D-ring with the goal of improving potency while moving toward improved physicochemical space. These efforts ultimately led to GSM **44** (Fig. 11), which has a $clogP$ of 4.0 and moderate in vitro potency: $A\beta_{42}$ IC_{50} = 188 nM in CHO-APP cells (E2012 has an $A\beta_{42}$ IC_{50} value of

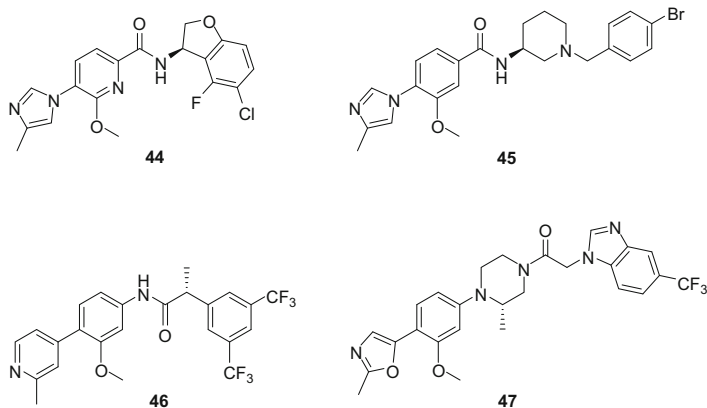


Fig. 11 Pfizer, Dainippon, Amgen, and Takeda GSMs

143 nM in the same assay). In vivo efficacy was assessed in guinea pig, and a 36% reduction of brain A β 42 was achieved at the 4-h time point when dosing **44** orally at 100 mg/kg.

Related examples of GSMs that rely on a carbonyl as the central hydrogen-bond acceptor have recently been reported in the literature. Dainippon has disclosed amide GSMs such as **45** that incorporates a 3-aminopiperidine linker between the amide carbonyl and the terminal phenyl ring [118]. This compound has an A β 42 IC₅₀ of 250 nM in rat primary neurons (*clogP* = 5.0), and a 55% reduction of A β 42 was observed in hippocampus when dosed in rat at a 100 mg/kg p.o. Amgen has reported GSMs with a reversed amide linker and a 2-methyl pyridine replacing the ubiquitous 4-methyl imidazole as exemplified by **46** (A β 42 IC₅₀ = 260 nM in HEK293 cell stably expressing the Swedish mutation, *clogP* = 5.3). This compound afforded a 33% reduction of A β 42 in brain at the 4-h time point when dosed orally in rat at 100 mg/kg [119]. Although in vitro potency could be improved tenfold relative to **46**, this was achieved at the expense of significantly increased lipophilicity resulting in reduced permeability and poor in vivo efficacy. Finally, Takeda has reported amide GSMs such as **47** that incorporate a piperazine linker between the A–B–ring system and the carbonyl and where the imidazole has been replaced with an oxazole [120]. In vitro potency was determined in primary rat neuronal cells, and potency is reported as inflection points (IPs) along with maximum percent reduction of A β 42, since many compounds did not reduce A β 42 by 100%. The lead GSM **47** has an IP of 21 nM with a 60% maximum reduction of A β 42 (*clogP* = 4.1). Interestingly, many compounds in this series did not reduce formation of A β 40. In fact, **47** was profiled in IMR-32 human neuroblastoma cells where a *slight increase* in A β 40 was observed along with robust reduction of A β 42. This stands in sharp contrast to the majority of A–B–C–D heteroaryl GSMs which typically reduce both A β 42 and A β 40. The in vitro selectivity of **47** was recapitulated in vivo where a 100 mg/kg oral dose in C5BL/6 mice resulted in a 59%

reduction of brain A β 42 at the 3-h time point while no statistically significant change in A β 40 was observed.

Further efforts to optimize the amide GSMs to improve A β 42-lowering activity while maintaining acceptable physicochemical properties and ADME profile have proven to be very challenging. To address this issue, the team at Pfizer shifted focus toward the A–B arylimidazole moiety with the goal of developing a novel, more polar heterocyclic core (Fig. 12). This was accomplished by introducing conformational constraint in the form of a two-carbon linker to connect amide NH to the pyridyl nitrogen and in the process converting the B-ring methoxy substituent to a pyridone carbonyl. The resultant pyridopyrazine-1,6-dione core (i.e. **48**) had significantly improved alignment of potency and ADME (A β 42 IC₅₀ = 103 nM in CHO-APP cells) as well as reduced lipophilicity (clogP = 3.3) [121, 122]. A dose-dependent reduction of brain A β 42 was observed with reductions of 19%, 44%, and 68% at the 4-h time point when dosed in guinea pig at 10, 30, and 90 mg/kg p.o., respectively. The corresponding brain exposures at this time point and doses were 2.9, 9.2, and 52 μ M indicating that further gains in vitro and in vivo efficacy would be needed. Through strategic use of fluorine to design lipophilic substituents with improved metabolic stability, this series was further optimized to deliver PF-06442609 (**49**), which exhibits excellent in vitro potency without a concomitant increase in lipophilicity (A β 42 IC₅₀ = 6 nM, clogP = 3.1) [123]. However, while excellent activity was achieved in vitro, this compound still required high drug exposure to achieve a robust reduction of brain A β 42 in a guinea pig time-course experiment: A 41% reduction in brain A β 42 was measured at the 3-h time point following a 60 mg/kg oral dose, and the corresponding total and unbound brain exposures were 17.3 μ M and 225 nM, respectively.

During this time, naphthyl GSM **50** (A β 42 IC₅₀ = 42 nM, clogP = 3.3) was identified as a promising starting point for further optimization in part due to its favourable ligand efficiency (LE = 0.35). A combination of parallel medicinal chemistry and design of individual analogues led to the discovery of indoles as a suitable replacement for the naphthyl unit [124]. The resulting GSM **51** once again demonstrated that the pyridopyrazine-1,6-dione series had the potential to deliver

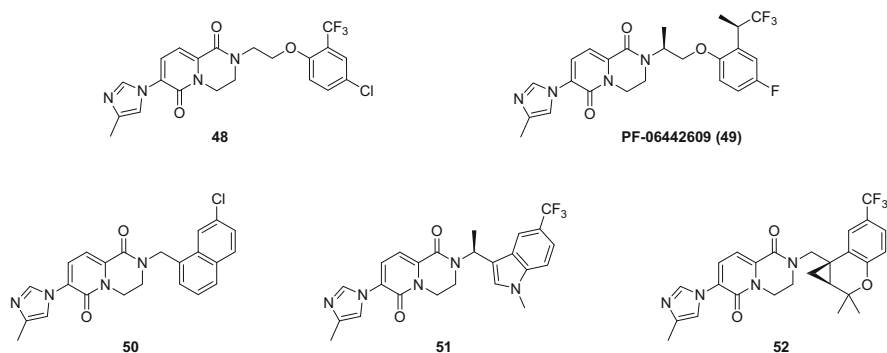


Fig. 12 Pfizer GSMs

GSMs with good alignment of in vitro potency, ADME, and acceptable lipophilicity: GSM **51** has an A β 42 IC₅₀ of 6 nM and clogP = 3.1. However, this compound was shown to be a substrate for P-glycoprotein (P-gp) efflux, which contributed to reduced central efficacy, and alternative heterocyclic replacements for the naphthyl unit were therefore required. Toward this end, the naphthyl moiety was replaced with a chromene, which was further refined into cyclopropyl chromane GSM **52** (A β 42 IC₅₀ = 4.9 nM, clogP = 3.2) [125]. This compound demonstrated dose-dependent and sustained reductions of brain A β 42 at low unbound brain concentrations, thus overcoming significant hurdles encountered with earlier compounds. Reductions of 40% and 62% were observed at the 4-h time point following a 10 and 40 mg/kg oral dose, respectively, and the corresponding unbound brain concentrations were 11 and 67 nM. The successful identification of **52** was accomplished by using design tactics such as molecular modelling, designing conformational control elements to lock the compound in the putative bioactive conformation, blocking metabolic soft spots to increase LipMetE [126], and using the unpaced isolated heart Langendorff model to triage compounds with respect to cardiovascular safety. Pfizer has disclosed additional, related GSMs in two recent patent applications [127, 128]. Likewise, Janssen has pursued a similar approach and also arrived at the pyridopyrazine-1,6-dione heterocyclic core as disclosed in the patent literature [129–131]. Discovery of the pyridopyrazine-1,6-dione based GSMs and subsequent optimization to provide highly potent compounds in good physicochemical property space represents a key milestone for the GSM field.

In July 2016, Pfizer reported the first clinical data from a Phase 1 study with GSM clinical candidate PF-06648671 (structure not disclosed) [132]. The compound was evaluated in a double-blind, placebo-controlled single ascending dose study with doses ranging from 2 to 360 mg. The terminal half-life was found to be between 13.9 and 23.1 h, and a 56.9% reduction of plasma A β 42 was reported following a single dose of 360 mg. GSM PF-06648671 was reported to be well tolerated at all doses, and therefore was advanced to a multiple ascending dose study to measure A β biomarkers in CSF at steady state.

2.3 Natural Product-Derived γ -Secretase Modulators

Satori Pharmaceuticals has reported a novel class of GSMs that originated from triterpene glycoside **53** (A β 42 IC₅₀ = 100 nM in H4 cells overexpressing human APP, Fig. 13) [133]. This compound was originally isolated from the black cohosh plant and was identified from a screen of a natural products collection. Interestingly; this class of GSMs reduces both A β 42 and A β 38 and increases A β 37 and A β 39 while A β 40 remains relatively unchanged. The initial lead had poor metabolic stability, and lead optimization involved reduction of the olefin, replacement of the labile acetate with an ethyl substituent, and removal of the glycoside to install a substituted morpholine [134, 135]. These efforts ultimately resulted in preclinical candidate SPI-1865 (**54**), which has an A β 42 IC₅₀ of 106 nM in CHO-2B7 cells

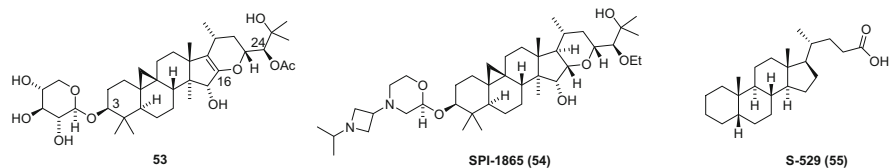


Fig. 13 Satori and University of Florida GSMs

expressing human wild-type APP [136, 137]. Dose-dependent reductions of brain A β 42 were demonstrated in an acute rat study with doses of 10, 30, and 100 mg/kg p.o. resulting in brain A β 42 reductions of 21%, 37%, and 50%, respectively, at 24 h post-dose. The corresponding brain exposures at this time point were 2.8, 11, and 33 μ M, respectively. Development of SPI-1865 was eventually halted due to adverse effects on adrenal function in monkeys [85].

Jung and co-workers have reported on acidic steroids as another new class of GSMs [138]. Among the compounds studied, S-529 (**55**) was the most potent analogue with an A β 42 IC₅₀ value of 5.68 μ M in CHO-2B7 cells. Unlike the steroidal GSMs from Satori, S-529 and related compounds display an A β modulatory profile that is similar to the NSAID-derived carboxylic acid class of GSMs, where A β 42 is selectively reduced over A β 40 with a concomitant increase in A β 38. Closely related non-acidic steroids such as the corresponding esters act as inverse GSMs (iGSMs) resulting in an increase of A β 42. The fact that these acidic steroids are derived from normal oxidative metabolism of cholesterol suggests that endogenous GSMs may exist.

3 Summary

GSMs reduce A β 42 levels without affecting total A β by increasing shorter A β species such as A β 38 and/or A β 37. This generally results in a reduction in the A β 42/A β 40 ratio and an even larger reduction in the ratio of A β 42 to shorter A β fragments including A β 38 and A β 37. This is particularly promising since A β 42 and oligomers thereof are thought to represent major neurotoxic species contributing to AD [139, 140] and FAD presenilin mutations generally result in an increase in the A β 42/A β 40 ratio. The early clinical results with aducanumab, a monoclonal antibody that targets aggregated A β , showing a reduction of brain A β plaques and a slowing of clinical decline in patients with prodromal or mild AD are encouraging and provide support for the amyloid hypothesis [141]. The recent cryo-EM studies on the γ -secretase complex, taken together with development of improved GSMs that are progressing in the clinic, represent another major step forward toward testing the amyloid hypothesis. Ongoing and future studies to understand the GSM binding sites on γ -secretase and mechanism of action could reveal new insights into how γ -secretase contributes to AD and inform future design of γ -secretase targeted therapies.

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Inhibitors of Tau-Phosphorylating Kinases

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and Maurizio Botta**

Abstract The phosphorylation of tau protein is finely regulated by a balance between phosphorylation and dephosphorylation processes carried out by kinases and phosphatases. It has been suggested that the disruption of this equilibrium and consequent abnormal tau phosphorylation contribute to the aggregation of tau. The understanding of this important mechanism is of high interest because of the implication of tau aggregates in the development of Alzheimer's disease (AD). In the last few years, among the possible strategies which could be used to reduce tau phosphorylation, the inhibition of certain tyrosine kinases has been suggested as a promising alternative to the common therapeutic approaches. In this chapter we will first give an overview of the tau protein kinases, their roles in cells, regulation and importance in AD. This will be followed by a more detailed description of the role of Fyn, a member of the Src family kinases, in the physiological development of CNS and the pathological progress of AD. How the inhibition of Fyn could be used as a new strategy in the fight against AD will be discussed.

Keywords Alzheimer's disease, CNS, Fyn, Inhibitors, Tyrosine kinases

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Abbreviations

5-HT6R	5-Hydroxytryptamine 6 receptor
AD	Alzheimer's disease
ADRA	Alzheimer's Disease and Related Disorders Association
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APP	Amyloid precursor protein
A β	Amyloid β
Blk	B lymphocyte kinase
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CDK5	Cyclin-dependent protein kinase-5
CHK1	Checkpoint kinase 1
ChIs	Cholinesterase inhibitors
CK	Casein kinase
c-KitR	c-Kit receptor
CNS	Central nervous system
CSF	Cerebrospinal fluid
Csk	C-terminal Src kinase
DYRK	Dual-specificity tyrosine phosphorylation-regulated kinase

ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
Erk	Signal-regulated kinase
FAD	Familial Alzheimer's disease
Fgr	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
Frk	Fyn-related kinase
GABA(A)R	γ -Aminobutyric acid A receptor
GAP	GTPase-activating protein
GSK3	Glycogen synthase kinase-3
HRI	Hydrophobic region I
HRII	Hydrophobic region II
IGF-1R	Insulin-like growth factor 1 receptor
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
KDR	Kinase insert domain receptor
Lck	Lymphocyte-specific tyrosine kinase
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinases
MARK	Microtubule affinity-regulating kinases
MBD	Microtubule-binding domain
MCI	Mild cognitive impairment
MCT	Mast cell tumours
MD	Molecular dynamics
MMGBSA	Molecular mechanics Generalised born surface area
mTOR	Mammalian Target of Rapamycin
NFT	Neurofibrillary tangles
NIA	National Institute of Ageing
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NMDA	<i>N</i> -methyl-D-aspartate
NMDAR	NMDA receptor
Non-PDPK	Non-proline-directed protein kinases
nRTK	Non-receptor tyrosine kinase
NSAIDs	Nonsteroidal anti-inflammatory drugs
PDGFR	Platelet-derived growth factor receptor
PDPK	Proline-directed protein kinases
PhK	Phosphorylase kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKN	Protein kinase N
PrP	Cellular prion protein
PSD	Postsynaptic density
PyrPyr	Pyrazolo[3,4-d]pyrimidine

RPTP	Receptor protein tyrosine phosphatase
SFK	Src family kinase
SP	Senile plaques
Syk	Spleen tyrosine kinase
TK	Tyrosine kinase
TPK	Tyrosine protein kinases
TTBK	Tau-tubulin kinase
WHO	World Health Organization

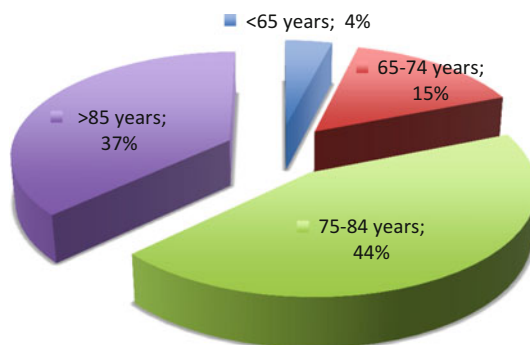
1 Alzheimer's Disease (AD)

As defined by the National Institute of Ageing (NIA), “Alzheimer’s disease (AD) is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills and, eventually, the ability to carry out the simplest tasks” [1]. AD was named in 1910 after the German neurologist Alois Alzheimer, who reported for the first time the case of a patient with symptoms that were unusual at the time: reduced memory and comprehension, disorientation, aphasia, paranoia, unpredictable behaviour, auditory hallucination and pronounced psychosocial impairment [2]. After the patient’s death, Dr. Alzheimer could observe brain atrophy and the presence of “dense bundles of fibrils” as well as of “minute miliary foci which are caused by the deposition of a special substance” distributed all over the cortex [3]. Indeed, it is now well known that AD is a neurodegenerative disease characterised by the presence of these two types of hallmarks. The “dense bundles of fibrils” are neurofibrillary tangles (NFT): intraneuronal aggregations, the main constituent of which is abnormally phosphorylated tau protein [4]. The “miliary foci”, also called senile plaques (SP), are extracellular deposits of amyloid β ($A\beta$) proteins. In addition to these, loss of connections between neurons in the brain, inflammation, severe tissue shrinkage and eventual death of brain cells are often considered features of AD [1, 5].

1.1 Epidemiology

In developed countries, birth control has led to a general decline in fertility, accompanied by an enhancement of the lifestyle that improves longevity. Developing countries are following the same trend. Fewer children are entering the world population, and people are living longer, making the proportion of old people increase from 7 to 12% in 2015 [6, 7]. According to the World Health Organization (WHO), the pace of population ageing is much faster than in the past, and it is estimated that in 2050 the proportion of the world’s population over 60 years will be approximately 22% [6]. Given that AD generally becomes apparent after the mid-60 years of age (see Fig. 1) [1, 8], it is not surprising that AD prevalence is

Fig. 1 Age distribution of people with AD in the USA in 2016. Data from [9]



significantly increasing, with a dramatic impact on public health and healthcare financing, mainly because of its requirement of persistent caregiving [1, 6, 7]. Indeed, in the USA alone, over five million people are currently living with AD, and experts say that this will cost over \$236 billion in 2016, making AD one of the potential most expensive chronic diseases [5, 8]. It is clear that the development of a cure for AD is compelling.

1.2 Symptoms and Disease Progression

Criteria for clinical diagnosis for AD were first established only in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRA) [10]. Due to brain plasticity, the symptoms of AD usually appear approximately a decade after the beginning of brain damage. Symptoms usually affect people in different ways, and the criteria for AD diagnosis are regularly updated [5, 8]. Since brain damage usually involves regions responsible of forming new memories, the first sign of AD is the gradual worsening of the ability to remember new information [1, 8]. With disease progression, other signs that are indicative of AD include [11–13]:

- Memory loss disrupting daily life
- Challenges in planning or solving problems
- Confusion with time or place
- Difficulty in completing familiar tasks
- Increasing problems with words in speaking or writing
- Trouble in understanding visual images and spatial relationships
- Decreased or poor judgement
- Loss of the ability to retrace steps
- Misplacing things
- Changes in mood and personality
- Withdrawal from work or societal activities

Symptoms range in severity according to the level of damage of the brain. Three stages of the disease have been identified to date: preclinical AD, mild cognitive impairment (MCI) and dementia [11–13]. In preclinical AD, patients have not yet developed visible symptoms, for example, memory loss, and the disease goes unnoticed. However, measurable changes in the brain and cerebrospinal fluid (CSF) and/or blood biomarkers can be detected [14]. As already mentioned, brain damage indeed starts approximately a decade before the appearance of signs [1, 14].

The symptomatic predementia phase of AD (MCI due to AD) is characterised by mild but measurable problems in performing complex functional tasks that were previously executed regularly. However, this does not impact on the independence in daily life, and only minimal aid or assistance is needed [13]. In the last phase of AD, dementia, there is a more important impairment in the patient's memory, thinking and behaviour, decreasing the ability to be independent in everyday life. In this case, continuous care is needed [12].

1.3 Causes of AD

The exact mechanism behind the brain lesions that cause AD is still not completely understood. It is plausible that for most patients there is a combination of genetic, environmental, lifestyle factors that sum to the age-related changes in the brain (e.g. atrophy, inflammation, production of free radicals or mitochondrial dysfunction) [1, 5].

Nevertheless, in 1–5% of AD cases, there is an early onset of the symptoms (before 60 years of age, Fig. 1), and the disease itself progresses at a faster rate. This condition, known as familial AD (FAD), is inherited in an autosomal dominant fashion of mutated genes (amyloid precursor protein, presenilin 1 and presenilin 2) that lead to A β aggregation [15, 16].

1.4 AD Aetiology

In AD, the dysfunction and cognitive impairment in patients are closely associated with synaptic loss, neurotransmitter depletion and brain lesions, in particular in regions associated with memory and cognition [17–20]. These lesions result in a multiplicity of irregular protein changes that occur in the brain. To date, the complete picture of the molecular and cellular mechanisms that are behind these cascades is still not clear and sometimes controversial. However, the increasing understanding of the complex brain changes involved in the onset and progression of AD allows the development of several and different intervention strategies focused on targeting the causes of neuronal cell death [1, 20, 21]. The main

hypotheses on the causes of AD are the cholinergic hypothesis, the glutamatergic hypothesis, the amyloid hypothesis and the tau hypothesis. An important aspect of the disease is the consistent finding of abnormal inflammatory responses in AD brains, with upregulation of pro-inflammatory cytokines in the CSF, as confirmed by the fact that the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been correlated to a decreased risk of developing AD [22–24].

1.4.1 Cholinergic Hypothesis

This theory was first proposed in 1982 and is based on the relation between dementia symptoms and impaired neurotransmission and degeneration of neuronal circuits, in particular of the cholinergic system [25]. The functional disturbances in cholinergic activity, for example, the progressive loss of cholinergic neurons or the reduced activity of choline acetyltransferase in neocortex and hippocampus, produce a decline in levels of acetylcholine in the brain and play a role in memory loss and related cognitive problems [26, 27].

1.4.2 Glutamatergic Hypothesis

N-Methyl-D-aspartate (NMDA) receptors are ionotropic glutamate channels that are inhibited by Mg^{2+} at resting potential and activated by glutamate, the main excitatory neurotransmitter in the central nervous system (CNS). AD patients present high levels of glutamate that over-activate the NMDA channels, leading to excessive Ca^{2+} influx and cell death [28].

1.4.3 Amyloid Hypothesis

Probably one of the most studied hypotheses for AD, the amyloid hypothesis, was first presented in 1991 [29, 30]. In this case, sequential proteolysis of the amyloid precursor protein (APP) produces the $A\beta$ peptides, which accumulate into toxic protein aggregates that cause the formation of the SP, with consequent synaptic failure and neurodegeneration [31]. APP is cleaved following two possible pathways (the non-amyloidogenic and the amyloidogenic), and predisposition for AD has been observed when the second one is prevalent over the first one. In the amyloidogenic pathway, the cleavage of APP is heterogeneous and the produced $A\beta$ peptides are different in length. Among these, the hydrophobic $A\beta_{42}$ is more aggregation prone and is considered more toxic [31–34].

1.4.4 Tau Hypothesis

One limitation of the amyloid hypothesis is that it does not explain the presence of the NFT in AD brains but only of SP. On the other hand, NFT can be explained by the tau hypothesis, and indeed AD is often accounted as a “tauopathy” [35]. Tau is abundantly expressed in the brain and belongs to the microtubule-associated protein (MAP) family. Its ability to bind to microtubules makes tau crucial in the stabilisation of neuronal cytoskeleton [35–37]. As further explained in Sect. 2, the hyperphosphorylation of this protein induces a decrease in tau-microtubule interaction, triggering a destabilisation of the neuronal cytoskeleton and tau accumulation [36–40].

1.5 *Current Treatment of AD*

Despite the increasing prevalence of AD, there is still no cure, but only symptomatic therapy [1, 5, 41, 42]. The available drugs (donepezil [43], galantamine [44], memantine [45], rivastigmine [46]) and a recently approved drug combination (donepezil with memantine [47]) aim to help patients to maintain mental function, to manage behavioural symptoms and, in general, to slow or delay the progression of the disease [41, 42]. These compounds are classified in two groups: cholinesterase inhibitors (CIs) and NMDA antagonist. Donepezil (Pfizer), galantamine (Janssen) and rivastigmine (Novartis) belong to the first group and are regarded as standard and first-line treatment for all stages of AD [41]. As explained in Sect. 1.4.1, the loss of cholinergic transmission has been correlated with AD symptoms. Thus, the inhibition of the degradation of acetylcholine improves the functional disturbances in cholinergic activity, with a consequent delay in the decline in cognitive function [43, 48]. Memantine (Lundbeck) belongs to the NMDA antagonists family. It is usually prescribed for moderate-to-severe AD and is believed to protect neurons from the excitotoxicity of NMDA channel over-activation (see Sect. 1.4.2). This compound improves cognition, and it may reduce behavioural and psychological symptoms [49]. The combination of a CI (donepezil) and a NMDA antagonist (memantine) produces a significant improvement in cognitive function, language, behaviours and global state in moderate-to-severe AD, but less in mild-to-moderate AD [5, 47]. Besides the FDA-approved therapies, current suggestions for prevention of AD include the use of antioxidants, omega fatty acid supplements, physical activity and cognitive engagement [42].

2 Implications of Tau Phosphorylation in AD

Tau is a 55-kDa protein expressed mainly in neurons and presents four regions: an acidic portion at the N-terminal, a proline-rich region, a microtubule-binding domain (MBD) and a C-terminal. The MBD contains repeat domains with a conserved KXGS motif and is consequently also called the repeat region [32, 36–38]. In the adult brain, tau exists in six isoforms that are formed through alternative mRNA splicing of the same gene on chromosome 17q. The six isoforms mainly differ in the absence/presence of one of the acidic inserts in the N-terminal acidic region and in the number (three or four) of repeat domains in the MBD [36, 50]. Since the repeat domains are responsible for the binding of tau to microtubules, isoforms containing four repeat domains bind with greater affinity to microtubules than do the ones containing only three repeat domains [36].

Up to 85 phosphorylation sites have been identified in tau. Among them, 28 are phosphorylated only in AD brains, 16 are phosphorylated both in control and AD brains and 31 are phosphorylated at physiological conditions (Table 1) [37]. Phosphorylation can include the serine (Ser) residues of the KXGS motif or threonine (Thr) residues in the repeat domains (residues in red in Table 1). Within these amino acids, the phosphorylation of specific sites (262, 293, 324, 356, highlighted in Table 1) produces a change in tau conformation with consequent decrease in tau binding to microtubules [36–38]. Thus, on one hand, hyperphosphorylation of tau produces a general cytoskeleton destabilisation with a disruption of neuronal functions that involve axonal growth, vesicle and organelle transport and nervous signal propagation. On the other hand, hyperphosphorylated tau proteins are less susceptible to protease processing and accumulate in cells as paired helical filaments (PHFs), the unit fibrils of NFT, and eventually produce cell death [36–39, 51]. The association of NFT with AD symptoms was confirmed by the positive correlation between the number of NFTs and the severity of dementia [52].

3 Kinases Involved in Tau Phosphorylation

The kinases responsible for tau phosphorylation are clustered in three groups (Fig. 2): proline-directed protein kinases (PDPK), non-proline-directed protein kinases (non-PDPK) and tyrosine protein kinases (TPK) [37].

3.1 Proline-Directed Protein Kinases, PDPKs

PDPKs phosphorylate a serine or threonine residue that precedes a proline amino acid. Three main groups of kinases phosphorylate tau by recognising this S/TP

Table 1 Tau phosphorylation sites

Tau phosphorylation sites found in:					
Only AD brain		AD and control brains	Physiologic conditions		Putative sites
Y18	S258	S46	T17	S241	T30
S68	S262	T181	Y29	T245	S61
T71	S289	S198	T39	T263	T63
S113	S356	S199	T50	S285	S64
T123	Y394	S202	T52	S293	T76
T153	T403	T205	S56	S305	S129
T175	S409	T212	T95	S316	S137
T184	S422	T217	T101	S320	Y310
S185	T427	T231	T102	S324	T319
S191	S433	S235	T111	S431	T377
Y197	S435	S396	S131	S352	
S208		S400	T135	T361	
S210		S404	T149	T373	
S214		S412	T169	T386	
S237		S413	S195	T386	
S238		S416	T220		

Adapted from [36, 37]. See text for details

motif: glycogen synthase kinase-3 (GSK3), cyclin-dependent protein kinase-5 (CDK5) and mitogen-activated protein kinases (MAPK) [37].

3.1.1 GSK3

GSK3 was initially discovered for its involvement in glycogen metabolism and is one of the most exploited kinase targets in AD. It plays roles in general neuronal functions, apoptosis, cell proliferation, oncogenesis, embryonic development and immune response [53–57]. There are two main GSK3 isoforms (GSK3α and GSK3β) sharing 85% sequence homology, but the isoform associated to tau

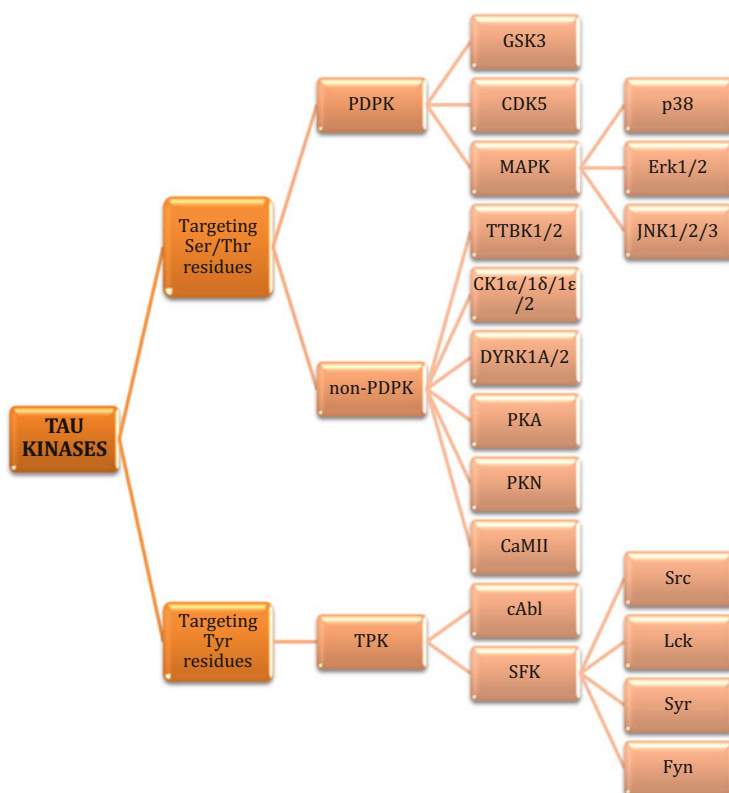


Fig. 2 Summary of tau-phosphorylating kinases

hyperphosphorylation is GSK3 β [58, 59]. GSK3 β can phosphorylate tau at 42 sites, of which 29 are phosphorylated in AD brains [60]. Overexpression or increased activity of GSK3 β in mouse models induces tau hyperphosphorylation and other tau-mediated effects, for example, neurodegeneration, inflammatory response, increased production of A β and reduced acetylcholine synthesis [61–63]. GSK3 β co-localises with NFT, and its increased activity is observed in AD brains, making this a promising therapeutic target [55, 64–66].

3.1.2 CDK5

CDK5 is pivotal in the development of the CNS as it is involved in several key processes, like the regulation of synaptic functions, neuronal cytoskeleton dynamics and neuritic development [67–70]. The monomeric form of CDK5 is enzymatically inactive and needs to interact with a co-activator (namely, p39/p35 and p29/p25) to phosphorylate substrates. Thus, the restriction of CDK5 activity to

neurons is related to the specific CNS distribution of the activators [71, 72]. Tau phosphorylation is performed by the most active complex (CDK5/p25) on 11 sites of the protein. This kinase has been also correlated with NFT formation and GSK3 β activation [73–75]. Furthermore, CDK5 stimulates neuronal apoptosis, in particular A β -induced, and is consequently considered a potential target for AD therapy [76, 77].

3.1.3 MAPK

Three kinases belonging to the MAPK family have been associated with abnormal tau phosphorylation in AD patients: p38, the extracellular signal-regulated kinase (Erk) and the c-Jun N-terminal kinase (JNK) [78, 79].

P38

P38 is involved in cell apoptosis and is generally activated in response to cytokine exposure or extracellular stress [80]. It phosphorylates 21 sites of tau, 15 of which are phosphorylated in AD brains, as well as other proteins like p53 and GSK3 β . In AD, p38 co-localises with NFT and in this context is activated by cytoskeleton destabilisation, as well as by environmental, oxidative and chemical stresses [81–85]. Indeed, this kinase could be involved in the inflammatory response associated with AD, and p38 inhibitors could be used as neuroprotective agents [86].

Erk

Two Erk isoforms (Erk1 and Erk2), sharing 83% sequence homology, have been correlated to AD. Both of them regulate cell growth, proliferation and division, as well as cell death [87, 88]. Erk1/2 can be activated by cytoskeleton abnormalities, and their increased activity produces tau hyperphosphorylation (at 16 possible sites) and apoptosis, becoming a putative drug target [81, 87, 89].

JNK

All three isoforms of JNK (JNK1, JNK2 and JNK3) are expressed in the brain and phosphorylate tau in 12 sites [81]. Similarly to p38, JNK can be activated by cytoskeleton destabilisation and induces apoptosis in response to stress. JNK-activating stresses include anticancer drugs (e.g. etoposide), ultraviolet or ionising radiation, nerve growth factor deprivation or cytokines [90, 91]. As well as co-localising with NFT, JNK is involved in SP formation by inducing γ -secretase activity and consequent A β aggregation, making JNK inhibition a possible therapeutic strategy [92].

3.2 *Non-PDPK*

Several kinases that belong to the non-PDPK family are able to phosphorylate tau: tau-tubulin kinase (TTBK), casein kinase (CK), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK), microtubule affinity-regulating kinases (MARK), phosphorylase kinase (PhK), protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), protein kinase N (PKN) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). Some of them do not represent interesting potential therapeutic targets and will not be further discussed here.

3.2.1 TTBK

Two tau-tubulin kinases (TTBK1 and TTBK2) are encoded by different genes and are expressed in nonoverlapping regions of the brain [93]. In addition to ten possible sites in tau, TTBK1/2 also phosphorylate tubulin. TTBK1, in particular, was found to phosphorylate tau and neurofilaments as well as increase CDK5/p25 complex formation, activate GSK3 β and consequently induce NFT formation and neuronal loss [81, 94].

3.2.2 CK

Both CK1 and CK2 are promising targets for the development of new drugs because they phosphorylate tau in 46 sites and, thus, regulate microtubule dynamics, apoptosis and cell survival pathways [95–97]. Of the many different isoforms of these kinases, only some specific ones (CK1 α , CK1 δ , CK1 ϵ and CK2) are involved in AD and are activated by A β . Notably, CK1 isoforms are increased in AD and co-localise with NFT [98–102].

3.2.3 DYRK

Only two of the six DYRK isoforms (DYRK1A and DYRK2) are involved in AD. They both phosphorylate two proteins involved in learning and memory processes: tau and the transcription factor cAMP response element binding [103, 104]. DYRK1A is encoded by a gene on the 21st chromosome and contributes to the positive correlation between Down syndrome and AD, along with the APP gene located in the same chromosome. Moreover, its increased levels in AD brains make this isoform particularly interesting for the development of AD and Down syndrome therapies [105–107].

3.2.4 PKA

PKA is a pro-apoptotic kinase activated by cAMP and responsible for the phosphorylation of several proteins, for example, PKB, PKC, GSK3 and tau [108, 109]. Of the 25 tau phosphorylation sites targeted by PKA, 17 are phosphorylated in AD brains. Indeed, it has been associated with AD tau hyperphosphorylation and co-localises with NFT. PKA is activated in AD, causing memory loss and making it a potential target for AD therapy [110, 111].

3.2.5 PKN

The inhibition of PKN is another possible therapeutic strategy. This protein kinase is highly expressed in the brain and has been co-localised with NFT. It phosphorylates GSK3 β as well as tau in four sites, two of which are phosphorylated in AD brains [112–115].

3.2.6 CaMKII

CaMKII kinase is involved in apoptosis induced by retinoic acid and phosphorylates tau in several sites, five of which are phosphorylated in AD brains. When phosphorylated at Thr287, CaMKII binds to the NMDA receptor. This kinase is expressed in neurons with NFT, and the inhibition of this protein could be a promising therapy for AD [116–118].

3.3 Tyrosine Protein Kinases, TPKs

The majority of tau phosphorylated amino acids are serine and threonine residues. However, five sites are represented by tyrosines 18, 29, 197, 310 and 394 (in green in Table 1). Four of these (18, 29, 197 and 394) are phosphorylated only in AD brains, and tyrosine phosphorylation might promote additional phosphorylation at other sites. Consequently, TPK that can phosphorylate tau are of particular therapeutic interest [119–124]. The main non-receptor tyrosine kinases (nRTKs) identified to phosphorylate tau are non-receptor Src family kinases (SFKs) members and cAbl kinase [37].

3.3.1 cAbl

This nRTK regulates cell proliferation and apoptosis through several pathways [123]. It is involved in the first steps of NFT formation, by phosphorylating tau at

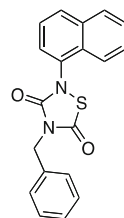
Tyr18 and Tyr394 [122]. The activation of cAbl occurs through phosphorylation of Tyr245 that can be mediated by SFKs. In neuronal cells, cAbl activation allows it to bind to p73 and trigger apoptotic signalling. Importantly, in AD both cAbl and p73 are highly expressed and stimulated by A β itself [124, 125].

3.3.2 SFK

SFK members involved in tau phosphorylation are mainly Src, lymphocyte-specific tyrosine kinase (Lck) and Fyn [37]. Among these, Fyn has been strongly related to AD symptoms, and its involvement in AD as well as its potential as a novel cure for AD will be further explored later in this manuscript.

4 The Development of Inhibitors of Tau-Phosphorylating Kinases Is a Promising Strategy in AD Drug Development

As already mentioned, there is no available AD disease-modifying therapy, which means that drugs only ameliorate symptoms and do not stop neuronal degeneration. Thus, there is no halt in the progressive deterioration of the cognitive faculties, and only patients presenting mild-to-moderate AD have benefits [41, 126, 127]. The need of novel efficacious therapy has encouraged research on understanding AD aetiology and progression. The increased knowledge has pushed the development of novel disease-modifying strategies, for example, immunotherapy or small molecules involved in amyloid deposition modulation, metal interference, secretase modulation, sirtuins and caspase inhibition, tau deposition modulation, oxidative damage reduction and cholesterol and vascular-related risk factor modulation [37, 41, 126–129]. These novel strategies have already been reviewed elsewhere and will not be further explored, as it is beyond the scope of this manuscript. The success of these approaches would improve the duration and quality of life of AD patients and allow disease intervention at the predementia stage, expanding considerably the market for AD drug development, which was already around \$4.1 billion in 2014 [130]. Indeed, today more than 100 clinical trials that have AD as therapeutic indication are ongoing, and a high proportion of them, for example, immunotherapy and secretase modulation, follow the historically most studied amyloid hypothesis (see Sect. 1.4.3) [127, 130]. Unfortunately, despite all the efforts, clinical trials are producing minimal results, and AD drug development in clinical trials has proved to be challenging [131]. On the other hand, the tau hypothesis (see Sect. 1.4.4) explains more aspects of AD-related events. Several encouraging molecules have been developed following this hypothesis, acting at different levels: microtubule stabilisation, tau oligomerisation, tau phosphorylation, tau dephosphorylation, tau degradation and tau vaccination. Most of these agents

Fig. 3 Tideglusib

are still in preclinical tests, and the hope is that they will perform better than the current molecules in clinical trials [129].

The strong evidence supporting the contribution of tau-phosphorylating kinases suggests that these enzymes are very promising targets for the development of novel anti-AD agents [126, 132]. The development of protein kinase inhibitors is a long-established strategy in cancer therapy, where inhibitors reduce the hyperactivity of kinases that causes uncontrolled cell division, migration and invasion. These compounds often display limited side effects and are well tolerated in patients, allowing the use of protein kinase inhibitors in AD drug development as well [132–134]. Inhibitors are being developed against almost all the tau-phosphorylating protein kinases mentioned above: GSK3 β , CDK5, p38, Erk1/2, JNK, TTBK1/2, CK1/2, DYRK1A, PKA, CaMKII and TPKs [37]. Compounds in preclinical and clinical development have been extensively reviewed elsewhere [37, 126–128, 132]. Among tau-phosphorylating kinases, GSK3 β is one of the most studied with respect to AD, and some of its inhibitors have reached clinical development. Of interest, lithium (as Li⁺), the classic mood stabiliser used in bipolar depression, was discovered to compete with Mg²⁺ and to inhibit GSK3 β selectively. Phase I and II clinical trials are ongoing to investigate the efficacy of this ion in AD (NCT02129348, NCT02204969, NCT02601859) [37, 128]. Furthermore, it has been demonstrated that intranasal insulin has inhibitory effects on GSK3 β , and two of these products have reached phase II clinical development: Humulin R for mild AD (NCT02503501) and glulisine for mild-to-moderate AD (NCT01436045) [127]. On the other hand, another very attractive GSK3 β inhibitor, tideglusib (Fig. 3), has failed a safety phase I/II clinical trial (NCT00948259) and an efficacy, safety and tolerability phase II study (NCT01350362), and its development was halted [127, 128].

This suggests that there is room for improvement also within the development of tau-kinase inhibitors. In this respect, Fyn represents a very promising drug development target, as it is involved in several aspects of AD pathogenesis, as further analysed later in the manuscript. As a proof of concept, two Fyn inhibitors, masitinib (NCT00976118 and NCT018725998) and saracatinib (NCT01864655 and NCT02167256), have reached clinical development (see Sect. 8) [135].

5 Fyn Kinase: Structure and Functions

Human Fyn is a tyrosine kinase (TK) belonging to the SFKs (Src family kinases), of which c-Src is the prototypical and best-studied family member. This family is constituted by nine members grouped into subclasses: the SrcA subclass includes Src, Yes, Fyn and Fgr and the SrcB subclass includes Lck, Hck, Blk and Lyn, while Frk is in its own subfamily. SFKs have been implicated in most cancer types [136], where they play a critical role in the regulation of cellular processes like proliferation, cell-cell and cell-matrix (ECM) adhesion and angiogenesis [137, 138]. In the CNS Src, Fyn and Yes are widely expressed, while Lck is commonly found in T cells. Blk, Hck, Fgr and Lyn are each expressed primarily in haematopoietic cells, while Fyn-related kinase (Frk) is a nuclear TK, and its functions mostly concern the regulation of G1 and S phases of the cell cycle [139]. Fyn is a 59-kDa protein comprising 537 amino acids encoded by the FYN gene located on chromosome 6q21 [140]. There are three different isoforms of Fyn: isoform 1 (FynB), the first identified; isoform 2 (FynT), which is expressed in T cells and differs from FynB in the linker sequence between SH2 and SH1 domains; and isoform 3, which has been found in blood cells and differs from isoform 1 by deletion of residues 233–287 [141]. Even if the first two forms are expressed as a mixture in different tissues, FynB seems to be highly expressed in the brain, while FynT tends to be expressed in T cells.

Like the other members of SFKs and many other TKs involved in signal transduction, Fyn consists of domains which mediate protein-protein interactions. Typically, five regions have been recognised as important for protein functions (Fig. 4a). These regions are the SH1 domain, the SH2 and SH3 domains, the unique domain and the SH4 domain. SH1 is also known as the catalytic domain and was the first region of homology identified among different Src family members [142]. It shares the characteristic bilobal structure with all tyrosine and ser/thr kinases. The N-terminal (small) lobe of SH1 is composed of five β -strands and one α -helix, termed the C-helix, which is a crucial element in the regulation of the enzyme activity. The C-terminal (large) lobe is constituted by α -helices and contains the activation loop with its DFG (Asp-Phe-Gly) motif. The hinge region connects the two lobes and represents an important anchor point for ATP. The SH2 domain is about 100 amino acids long and is important because it binds the tyrosine-phosphorylated residues in a specific sequence [143, 144].

The SH3 domain is approximately 75 amino acids in length and usually keeps contacts with proline-rich sequences on target substrates. SH3 is involved in the regulation of the autoinhibition mechanism of SFK activity by binding the region which links the SH2 domain to the SH1 domain [145–147]. The unique domain is located at the N-terminus of the kinase and it is subjected to a high variability among the SFKs. The role of the Fyn unique domain has not been completely clarified, but it has been proposed to be required for subcellular localisation [148]. Finally, the N-terminal seven residues of Fyn constitute the SH4 domain, which contains potential sites for post-translational modifications, like fatty

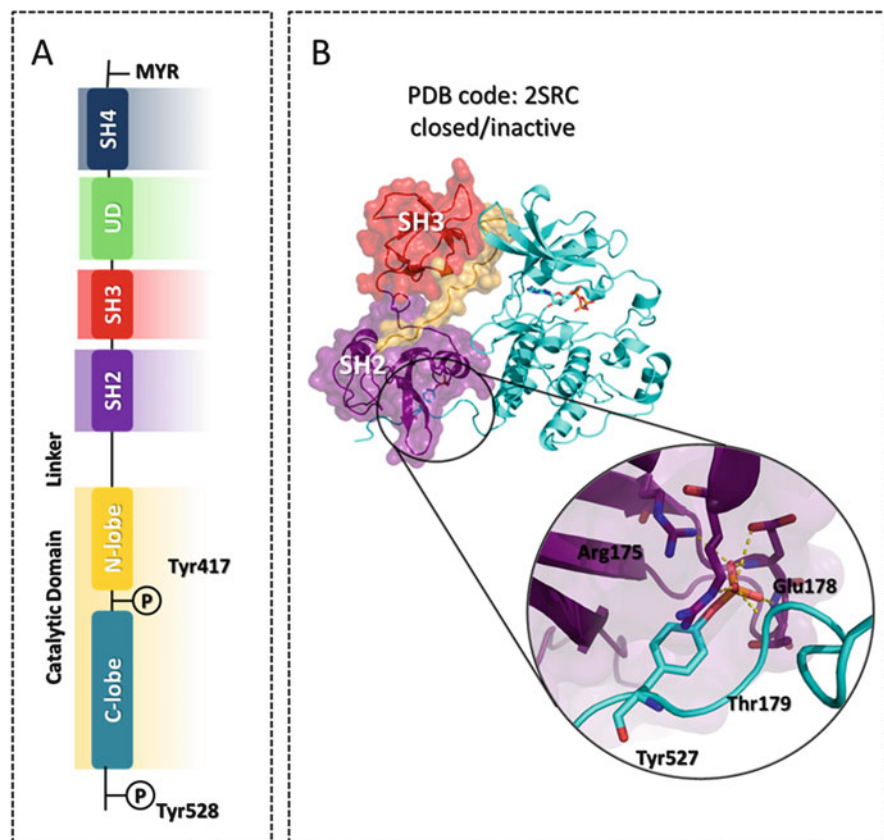


Fig. 4 Panel A, schematic representation of Fyn kinase structure. Phosphorylation sites have been highlighted. Panel B, X-ray structure of the closed/inactive conformation of c-Src. The binding of Tyr527 to the SH2 domain which stabilises the closed conformation is highlighted in the magnification

acylation, important for association with the plasma membrane [140, 149]. The presence of a glycine residue in position 2 (common in many TK such as Abl) serves, after the removal of the leading methionine, as a site of modification by a myristate group, while cysteine 3, not present in Src and Blk, can be modified with palmitate [150]. These acylations allow the anchorage of the kinase to the inner surface of the cell membrane.

The activation of Fyn depends on the binding of the SH2 and SH3 domains to their ligands and the phosphorylation/dephosphorylation of specific tyrosine residues. In particular, the phosphorylation of Tyr528 on isoform 2 or FynT (residue 531 on FynB) by the C-terminal Src kinase (Csk) inhibits the enzyme, promoting the formation of a closed conformation in which the catalytic site is not accessible to ATP and protein substrates (Fig. 4b). On the other hand, the phosphorylation on

Tyr417 on Fyn T (residue 420 on FynB) promotes Fyn activation and leads to an open/active conformation of the protein structure [151].

6 Functions of Fyn in the Central Nervous System (CNS)

Fyn is considered to be involved both in the development of the CNS and in normal adult brain physiology. The biological importance of Fyn in the brain has been extensively investigated using transgenic animal models. These studies indicated that Fyn is a key element for the development of CNS. Mice deficient in Fyn display various neural defects, including disruption of hippocampal architecture, impaired spatial learning and increased sensitivity to ethanol [152], suggesting the importance of Fyn in various neural signalling pathways. Moreover Fyn plays important roles in CNS myelination, and it has been found in many brain areas [153]. In the last decade, many efforts have been devoted to the study of the role of Fyn in CNS. Fyn has been found in glial cells in white matter and cultured oligodendrocytes [153], where it is important for the formation of neuritis. Indeed, Fyn interacts with γ -tubulin, a member of the tubulin family involved in the nucleation and polar orientation of microtubules. Consistently, the pretreatment of P19 embryonic carcinoma cells with SFK inhibitors blocked the nucleation activity of γ -tubulin [154].

Fyn and other members of SFKs are involved in synaptic transmission and plasticity, and indeed it is localised to the postsynaptic density (PSD), the primary cytoskeleton specialisation at neuronal excitatory synapses, constituted by many proteins such as PSD95, NMDAR and AMPAR. In particular, NMDAR forms a multiprotein complex, with PSD95 directly binding to the NR2 subunit of NMDAR. PSD95 interaction with the SH2 domain of Fyn has been proposed to induce the phosphorylation of NMDAR subunit NR2A [155, 156]. In this way Fyn regulates the NMDA phosphorylation and the NMDAR-dependent synaptic potentiation [157]. It was demonstrated that Fyn phosphorylates PSD93, another protein usually associated with NMDAR. PSD93 serves as an anchor substrate for Fyn, placing Fyn very close to NMDAR, regulating the receptor activity [158].

Fyn has many different roles in the development and regulation of physiological functions of the CNS, and every year new associations with target proteins are discovered.

Jud and colleagues reported that Fyn phosphorylates the GABA(A)R gamma2 subunit on Tyr365 and Tyr367. In particular, phosphorylation on Tyr367 seems to be important for the modulation of GABA(A)R and consequently the inhibition of synaptic functions in mammalian brain [159].

Interactions of Fyn with other proteins in CNS have recently been reported, such as p250GAP, a RhoGAP protein expressed predominantly in the brain [160, 161], and RPTPrho, exclusively expressed in CNS [162, 163].

The description of the role of Fyn in phosphorylating tau and other specific proteins in CNS has increased the interest on this TK as a potential therapeutic target for neurodegenerative diseases.

7 Fyn in AD

Fyn hyperactivation/deregulation is involved in neurodegenerative disorders (tauopathies) characterised by alterations of tau, a microtubule-associated protein that is the main component of NFTs in AD. As previously described, the most important hallmarks of AD are the presence of SP and NFT. Plaques result from the self-aggregation of A β into insoluble fibrils, which represent the endpoint of an aggregation process generating a broad range of complexes of different molecular weights and solubility [164, 165]. NFTs are located inside the neurons, and their formation is a consequence of pathological post-translational modifications of tau [166, 167]. Indeed, NFTs are composed of highly phosphorylated forms of tau. The mechanisms leading to AD are not completely understood, but recent evidence suggests that alterations in Fyn kinase contribute to AD pathogenesis.

The initial evidence that Fyn may be implicated in some ways in the development of AD was reported in 1993 [168]. Shirazi and Wood found that a subset of neurons from AD brains exhibited a strong Fyn immunoreactivity compared to controls. The possible connection between Fyn and AD led to numerous studies in the past decades, focusing mainly on the effects of A β on TKs in cell culture. An early study suggested that treatment of cells with A β_{25-35} and A β_{1-40} increased tyrosine phosphorylation in a dose-dependent manner, although the exact A β preparation of the mixtures was not well characterised [169]. Even though the majority of early studies in AD used fibrillary or mixed preparations of A β , it is now widely believed that soluble intermediates of this peptide, termed A β oligomers (A β o), are the most relevant assemblies in AD pathogenesis [170]. A critical early step in AD pathophysiology is the process by which A β o interacts with the neuronal surface, and studies of this interaction have further implicated Fyn in AD pathophysiology. In a unique unbiased screen for cell surface proteins, A β was found to bind to the cellular prion protein (PrP^C) with high affinity and in a specific manner for the oligomeric form, with little or no affinity for fibrillary or monomeric A β peptide [171–174].

Once A β o forms and binds to PrP^C at the cell surface, changes in neuronal biochemistry occur. Both PrP^C and Fyn were found to be enriched in the PSD fraction upon exposure of cultured mouse hippocampal neurons to A β o, activating Fyn by phosphorylation of its Tyr420 residue [175]. Brain extracts from patients with AD also stimulate Fyn activation in mouse cortical cultures, while control brain extracts do not, further suggesting that the A β o-PrP^C-Fyn pathway is relevant in human disease [175]. The connection between A β o-PrP^C complexes at the cell surface and intracellular Fyn has recently been shown to require the metabotropic glutamate receptor mGluR5 [176]. This transmembrane protein has a physiological role in linking extracellular glutamate levels to calcium mobilisation, to protein

translation in dendrites and to TK signalling. In neurons and in mice, mGluR5 is required for downstream neuronal dysfunction caused by A β . Extracellular A β oligomers thus trigger neuronal signal transduction from PrP^C to mGluR5 to Fyn kinase.

7.1 Implications of Fyn-Tau Interaction in AD Development

In addition to its role in A β signalling, Fyn is also involved in tau phosphorylation. Tau may play a critical role in mediating downstream neurodegeneration in AD, and a report showed that a 50% reduction in tau expression reversed cognitive impairments in a transgenic AD model [177]. Similarly to A β , studies on tau have mechanistically implicated Fyn in AD. Fyn physically associates with tau, via its SH3 domain, and phosphorylates tyrosine residues, including Tyr18, near the amino terminus [119, 178–180]. The phosphorylation of Tyr18 has been found in neurofibrillary tangles in human AD brain, suggesting a possible clinical relevance. Activation of Fyn by the A β -PrP^C complex was also demonstrated to lead to downstream tau phosphorylation [181].

Under normal conditions in neurons, tau is primarily located in axons but can also interact with Fyn in dendrites. The phosphorylation of NMDAR by Fyn was reported to stabilise the interaction between NMDAR and PSD95, a protein recruited into the NMDA receptor cluster [182]. This stabilisation enhances signalling by the neurotransmitter glutamate which strengthens A β toxicity. During AD development, the redistribution of hyperphosphorylated forms of tau from axons to dendrites may increase the tau-dependent storing of Fyn, leading to an abnormal NMDAR phosphorylation and consequently to toxic effects on neurons [183].

Fyn is not only involved in the development of AD by its interactions with NMDAR, tau and A β , but Fyn also binds 5-HT6R, widely distributed in the limbic region. 5-HT6R binds the Fyn SH3 domain via its C-terminal region, and activation of ERK1/2, a downstream target of 5-HT6R, is regulated in a Fyn-dependent manner [184].

8 Fyn Inhibitors for AD Treatment

Taken together, the preclinical evidence strongly supports that Fyn overexpression or hyperactivation is involved in brain pathologies, in particular AD. For this reason, the search for new molecular scaffolds able to inhibit Fyn activity represents an expanding field of study. Most Fyn inhibitors reported so far are ATP-competitive inhibitors, targeting the catalytic site of the enzyme. Unfortunately, because of the high sequence homology among SFKs, no specific Fyn inhibitor has been identified to date: all the reported compounds are also active against the other members of the family and sometimes against other TKs.

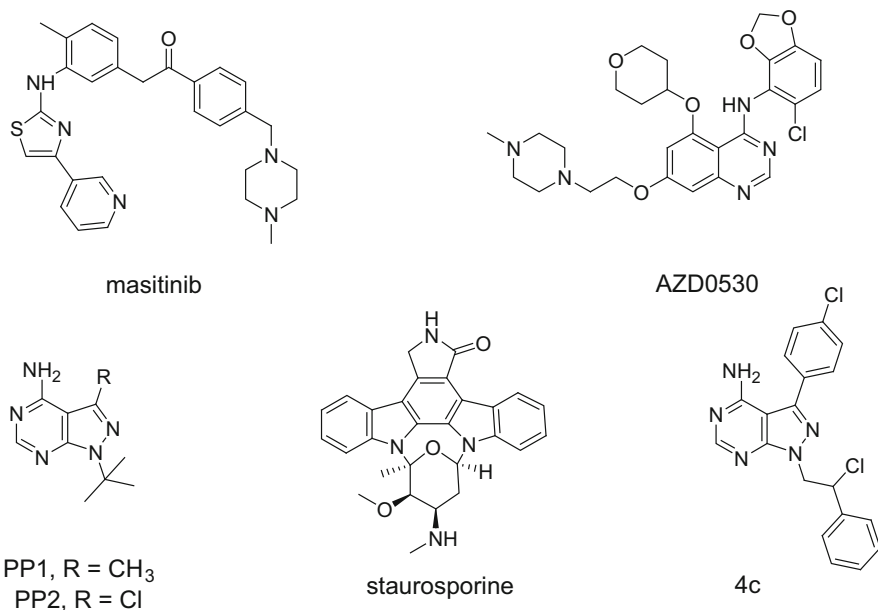


Fig. 5 Molecular structures of Fyn kinase inhibitors

8.1 Masitinib

Masitinib mesylate, also known as AB1010 (Fig. 5), is a phenylaminothiazole-type selective inhibitor targeting the ATP binding site of c-Kit receptor (c-KitR). Masitinib was originally approved for the treatment of mast cell tumours (MCT) in dogs [185, 186] and is currently under evaluation for the treatment of human cancers [187, 188]. In human medicine, masitinib is also considered a potential treatment for inflammatory diseases, including rheumatoid arthritis (RA), inflammatory bowel diseases and mastocytosis [119, 168, 189, 190].

In recent years, a new potential application of masitinib in neurodegenerative disease has emerged.

Even though masitinib is known as a selective inhibitor of c-Kit, it is also capable of blocking the SFK Fyn with a potency in the nanomolar range. As Fyn is involved in tau phosphorylation, it is possible that masitinib could prevent NFT formation. A 24-week randomised, placebo-controlled, phase II study was performed in patients with mild-to-moderate AD, receiving masitinib as an adjunct to a cholinesterase inhibitor and/or memantine [191]. Patients were randomly assigned to receive masitinib or placebo, administered twice per day. A lower rate of clinically relevant cognitive decline was observed after 12 and 24 weeks

with masitinib adjunct treatment compared with placebo. The trial provided evidence that masitinib could represent an innovative treatment for AD.

8.2 *Saracatinib (AZD0530)*

In 2006, AstraZeneca reported the discovery of AZD0530 (Fig. 5), also known as saracatinib, a small-molecule inhibitor of SFKs (Src, Fyn, Lyn and Yes) with a 2–10 nM potency [192]. At 10- to 100-fold higher concentrations, saracatinib was also active against Abl, but no detectable activity was found for other kinases, including c-Kit, Csk and PDGFR. Because of its selectivity against Fyn and SFKs, saracatinib was developed at first as therapy for solid tumours [192]. Clinical trials demonstrated that saracatinib is well tolerated and has a good oral bioavailability, but unfortunately a phase II study showed that it could not be used as a single agent in specific oncological indications because of its limited benefits [193–197].

In 2013, researchers from the Yale University School of Medicine launched a phase Ib study of saracatinib in patients with AD. The goal was to establish the safety, tolerability and CNS penetration of the drug. The study represented one of the prime success stories from the *Discovering New Therapeutics Uses for Existing Molecules* programme, established by the US National Institutes of Health in 2012. Typically in the drug development process, the passage from the discovery of a lead compound to the entry in a phase IIa trial takes at least one decade, but in the case of saracatinib, the research team completed all preclinical and clinical safety studies in 18 months. The multicentre phase IIa study is still recruiting participants, and the estimated primary completion date is December 2016 [ClinicalTrial.gov Identifier: NCT02167256].

9 Rational Design of Pyrazolo[3,4-D]pyrimidines as Fyn Kinase Inhibitors Active on AD Models

The pyrazolo[3,4-d]pyrimidines PP1 and PP2 (Fig. 5) were reported by Pfizer in 1996 as the first inhibitors of Lck and Fyn, with IC₅₀ values ranging from 4 to 6 nM. Further studies indicated that PP1 is also active against Src and Hck kinase [198]. PP1 and PP2 are ATP-competitive inhibitors that bind the catalytic site of the enzyme, with the pyrazolopyrimidine core taking the place of the adenine ring of ATP. A large amount of biological data has been reported so far for PP1 and PP2, which are commonly used as reference compounds for newly synthesised derivatives or as pharmacological tools in the investigations on SFKs [199]. Even if biopharmaceutical properties of PP1 and PP2 appeared to limit their use as

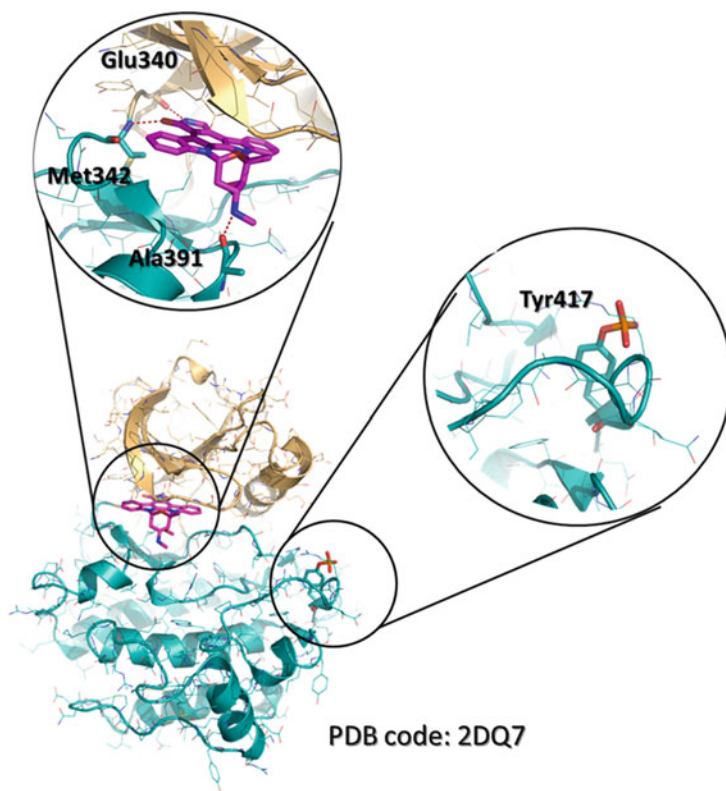


Fig. 6 X-ray crystal structure of the catalytic domain of Fyn (PDB code: 2DQ7). The N-terminal (small) lobe (*golden*) and the C-terminal (large) lobe (*cyano*) of the catalytic domain are highlighted. The C-lobe contains the activation loop with its Tyr417. Between the two lobes, the ATP-binding site is occupied by the staurosporine (*magenta*)

therapeutics, their discovery represents a significant advance in the use of TK inhibitors in the study of different cell functions.

Recently we reported the design, synthesis and biological evaluation of new pyrazolo[3,4-d]pyrimidine (PyrPyr) derivatives as selective inhibitors of Fyn kinase [200]. Selected compounds have been evaluated as anticancer agents, but surprisingly some of them were also found to be active in AD models. In particular, one molecule **4c** (Fig. 5) showed good potency in SH-SY5Y cell lines differentiated into neurons and treated with A β_{42} oligomers/protofibrils in order to induce AD-like neurotoxicity [201, 202]. At first, **4c** was identified as a possible inhibitor of Fyn by applying a combination of molecular docking and molecular dynamics (MD) simulation protocols to an in-house library of pyrazolo[3,4-d]pyrimidines (consisting of ~300 structurally characterised compounds with >98% purity). The 2DQ7 [203] X-ray structure of the enzyme bound by staurosporine (Fig. 6) was retrieved from the Protein Data Bank (PDB) and used for computational studies.

Table 2 Molecular structures and enzymatic K_i values against Fyn of compounds **3a–p** and **4a**

Cpd	R	R1	R2	R3	Fyn (K_i) (μ M)
3a	NHCH ₂ CH ₂ C ₆ H ₅	SC ₄ H ₉	CH ₂ –CHCl–C ₆ H ₅	H	NA
3b	NHC ₆ H ₄ - <i>m</i> Cl	SC ₄ H ₉	CH ₂ –CHCl–C ₆ H ₅	H	1.4
3c	NHCH ₂ CH ₂ C ₆ H ₅	S-ciclopentyl	CH ₂ –CHCl–C ₆ H ₅	H	NA
3d	NHC ₆ H ₄ - <i>m</i> F	S-ciclopentyl	CH ₂ –CHCl–C ₆ H ₅	H	NA
3e	NHC ₆ H ₄ - <i>m</i> Cl	S-ciclopentyl	CH ₂ –CHCl–C ₆ H ₅	H	NA
3f	NHC ₃ H ₇	SCH ₂ CH ₂ -4morpholino	CH ₂ –CHCl–C ₆ H ₅	H	8.15
3g	NHCH ₂ C ₆ H ₄ - <i>p</i> F	SCH ₂ CH ₂ -4morpholino	CH ₂ –CHCl–C ₆ H ₅	H	15.5
3h	NHCH ₂ CH ₂ C ₆ H ₅	SCH ₂ CH ₂ -4morpholino	CH ₂ –CHCl–C ₆ H ₅	H	NA
3i	NHC ₆ H ₅	SCH ₂ CH ₂ -4morpholino	CH ₂ –CHCl–C ₆ H ₅	H	7.5
3j	NHCH ₂ C ₆ H ₅	S-CH(CH ₃)C ₂ H ₅	CH ₂ –CHCl–C ₆ H ₅	H	NA
3k	NHCH ₂ CH ₂ C ₆ H ₅	S-CH(CH ₃)C ₂ H ₅	CH ₂ –CHCl–C ₆ H ₅	H	NA
3l	NHC ₆ H ₄ - <i>m</i> Cl	S-CH(CH ₃)C ₂ H ₅	CH ₂ –CHCl–C ₆ H ₅	H	12.5
3m	NHCH ₂ CH ₂ C ₆ H ₅ - <i>m</i> Cl	SCH ₃	CH=CH–C ₆ H ₅	H	16
3o	NHCH ₂ C ₆ H ₅	CH ₂ C ₆ H ₅	CH ₂ –CHCl–C ₆ H ₅	H	13
3p	NHCH ₂ C ₆ H ₅	H	CH ₂ –CHCl–C ₆ H ₅	H	3.5
4a	NH ₂	H	CH ₂ –CHCl–C ₆ H ₅	C ₆ H ₅	0.9

From a structural point of view, PyrPyr compounds are related to the Src inhibitors PP1 and PP2 but bear a different substitution pattern on the pyrazolo [3,4-*d*]pyrimidine nucleus. According to the predicted score values and the binding modes observed, 17 compounds were selected to be tested as Fyn inhibitors (Table 2). Among them, some derivatives (**3a–i**, **3p**) had already been reported by us as Bcr-Abl/Src inhibitors [204–207].

Three different binding modes emerged from docking studies. Compounds **3a–3o**, C3 unsubstituted and functionalised at positions 1, 4 and 6, showed an interaction network similar to that previously found within the ATP-binding site of c-Src (BM-A, Fig. 7a) [208]. The pyrazolo[3,4-*d*]pyrimidine core occupies the ATP adenine region, and the C4 substituent establishes hydrophobic interactions with Leu329, Phe409, Ile340, Phe311, Met318 and Leu411 (hydrophobic region I, HRI), while the N1 side chain is involved in van der Waals contacts with Tyr344, Met345 and Leu277 (hydrophobic region II, HRII). The C6 substituent is directed towards the external region. Two hydrogen bond interactions were also found, one involving the C4 amino group and the side chain of Thr342 and the other between the N2 of the pyrazolo[3,4-*d*]pyrimidine nucleus and the backbone NH group of Met345.

Conversely, the binding mode B (BM-B, Fig. 7b) was identified for compound **4a**, which contains a substituent at the C3 position while being unsubstituted at C6.

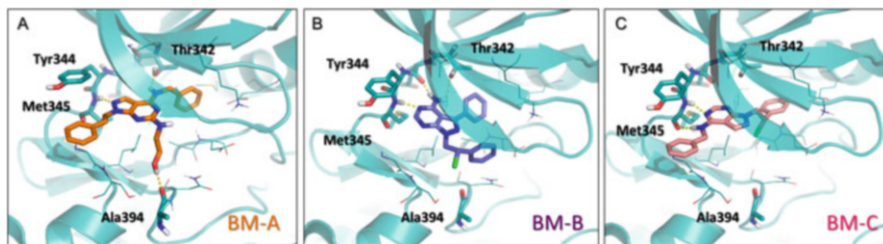


Fig. 7 3D graphical representation of predicted binding modes of pyrazolo[3,4-*d*]pyrimidines **3a–p** and **4a**. Three binding modes have been predicted, namely, BM-A (A), BM-B (7B) and BM-C (C). Compounds **3n** (orange), **4a** (violet) and **3p** (pink) are visualised

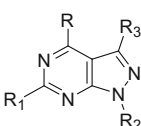
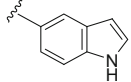
4a is a close analogue of PP1 and PP2, the first reported Fyn inhibitors [198]. Accordingly, its binding mode is consistent with that previously identified for PP1 and PP2 by crystallographic experiments [209, 210]. In detail, the exocyclic amine makes an H-bond with the carbonyl backbone of Glu343, while the nitrogen at N5 position acts as an H-bond acceptor in the interaction with Met345. The C3 phenyl group binds to the residues of hydrophobic region I through van der Waals interactions, while the N1 group is solvent exposed. Finally, compound **3p**, which is unsubstituted at both C3 and C6 positions, showed the binding mode C visualised in Fig. 7c. According to our prediction, the ligand binds the Met345 of the hinge region through two hydrogen bonds by means of the atom N5 and the exocyclic amino group at C4. Moreover, the N1 side chain is located within the hydrophobic pocket I formed by residues Leu329, Phe409, Ile340, Phe311, Met318 and Leu411, while the substituent at C4 interacts with Tyr344 and Leu277 of hydrophobic region II. Finally, biological investigation on isolated Fyn kinase (Table 2) revealed that **3b**, **3n**, **3p** and **4a** were the most active compounds, being endowed with K_i values of 1.4 μ M, 1.15 μ M, 3.5 μ M and 0.9 μ M, respectively. Derivatives **3f**, **3g**, **3i**, **3l**, **3m** and **3o** exhibited moderate activity with K_i ranging from 7.5 to 16 μ M. No activity was detected for the remaining compounds.

Based on this finding, a hit-to-lead optimisation campaign was then initiated on compound **4a** to improve its potency. Nine analogues were synthesised (**4b–e**, **5a–e**) (Table 3).

The C3 moiety was differently functionalised, while minor modifications were carried out on the 2-chloro-2-phenylethyl N1 side chain by replacing or removing the chlorine atom. Furthermore, with the aim of exploring the effect caused by the introduction of a *S*-methyl moiety at the C6 position of the C3-phenyl-substituted pyrazolo[3,4-*d*]pyrimidine core, derivatives **6a** and **6b**, previously synthesised by our group [211], were added to this set of compounds. The 11 compounds were tested in a cell-free assay to evaluate their affinity towards isolated Fyn kinase.

As expected, derivatives **4c** and **4d** showed potent *in vitro* inhibitory effect against Fyn, with K_i values in the nanomolar range (70 nM and 95 nM, respectively). These potencies were most likely evoked due to the contribution of a chlorine or methyl substituent in *para* position of the C3 phenyl ring (compare **4c**

Table 3 Structure and activity against Fyn of compounds **4b–e**, **5a–e** and **6a–b**

					
Cpd	R	R1	R2	R3	Fyn (K_i) (μ M)
4b	NH ₂	H	CH ₂ –CHCl–C ₆ H ₅	C ₆ H ₄ –pF	0.36
4c	NH ₂	H	CH ₂ –CHCl–C ₆ H ₅	C ₆ H ₄ –pCl	0.07
4d	NH ₂	H	CH ₂ –CHCl–C ₆ H ₅	C ₆ H ₄ –pCH ₃	0.095
4e	NH ₂	H	CH ₂ –CHCl–C ₆ H ₅	C ₆ H ₄ –pOCH ₃	1.485
5a	NH ₂	H	CH ₂ –CHCH ₃ –C ₆ H ₅	C ₆ H ₅	3.35
5b	NH ₂	H	CH ₂ –CHCH ₃ –C ₆ H ₅	C ₆ H ₄ –pCl	0.78
5c	NH ₂	H	CH ₂ –CHCH ₃ –C ₆ H ₅	C ₆ H ₄ –pCH ₃	0.995
5d	NH ₂	H	CH ₂ –CHCH ₃ –C ₆ H ₅	C ₆ H ₄ –pCOCH ₃	> 10
5e	NH ₂	H	CH ₂ –CHCH ₃ –C ₆ H ₅		1.625
6a	NHCH ₃	SCH ₃	CH ₂ CH ₂ C ₆ H ₅	C ₆ H ₅	> 10
6b	NH ₂	SCH ₃	CH ₂ CH(OCH ₃)–C ₆ H ₅	C ₆ H ₅	> 10

and **4d** with **4a**). Interesting activities were also found for compounds **4b**, **5b** and **5c** which exhibited submicromolar affinities (0.36 μ M, 0.78 μ M and 0.995 μ M, respectively). As a general trend, the substitution of chlorine by methyl in the N1 side chain led to a considerable reduction of the affinity with about tenfold decreased activities (compare **5a** with **4a**, **5b** with **4c** and **5c** with **4d**). All studied compounds adopted a binding mode in line with that of **4a** with the exception of the inactive derivatives **5d** and **6a,b**.

In order to assess the stability of the complexes and to gain further insight into the ligand-protein interactions, molecular dynamics (MD) simulations were carried out starting from the geometry obtained by docking. The complexes between Fyn and derivatives **4a**, **4b**, **4c** and **4d** were selected as the training set. Each simulation was carried out for a total of 12 ns. Molecular mechanics generalised born surface area (MM-GBSA) approaches were then applied to estimate the free energy of binding between protein and ligands. The computed values are reported in Table 4. As expected, **4c**, the most active compound against Fyn kinase, showed the lowest favourable binding energy (-40.51 ± 2.63 kcal/mol).

Next, to determine the details of the interactions which play a pivotal role in the ligand-protein recognition process, a post-processing analysis was conducted for the four complexes. H-bonding interactions between ligands and enzyme as well as between the main residues of the active site were determined and are listed in Table 5 together with their occupancy in the investigated time period.

The analysis reveals that the H-bond which occurs between the nitrogen at position 5 (N5) of each ligand and the NH backbone of Met345 is weaker

Table 4 Calculated binding free energies of complexes between Fyn and compounds **4a–d**

Cpd	ΔG_{bind} MMGBSA (kcal/mol)
4a	-35.07 ± 2.25
4b	-38.21 ± 2.75
4c	-40.51 ± 2.63
4d	-38.36 ± 3.26

Table 5 Hydrogen bonds occupancy calculated from MD for **4a–d** ligands in complex with Fyn

Cpd	Glu343(O) N4	Met345(NH) N5	Thr342(OGH) Ala297(O)	Thr342(OGH) Glu343(O)	Thr342(NH) Ala297(O)
4a	67.4 %	33.3 %	54 %	5.7 %	88.3 %
4b	62 %	32.9 %	20 %	24.18 %	85.5 %
4c	76 %	32.8 %	53.2 %	–	85.7 %
4d	77.1 %	30.6 %	51.5 %	–	89.4 %

(occupancy of about 30%) than the one between the carbonyl backbone of Glu343 and the exocyclic amine (N4) (occupancy higher than 60%). As a consequence of this H-bond, the intermolecular interaction between the Thr342 side chain and the carbonyl backbone of Glu343 is weakened. This effect is particularly pronounced for the most active inhibitors **4c** and **4d**, for which the Thr342-Glu343 polar contact is totally lost. Furthermore, no interaction was detected between N4 and the Thr342 side chain, and this could be due to the fact that the “gatekeeper” residue is stabilised in its position by favourable interactions with Ala297 (Table 5). Such a network of interactions contributes to the stabilisation of the binding of our PyrPyr together with the enzyme. The global effect of stabilisation was more pronounced for the most active inhibitors, in line with the trend observed for the activity against Fyn.

9.1 Selectivity Against a Human Kinase Panel

To assess its specificity against Fyn, compound **4c** was tested in a panel of kinases including other Src family members (Hck, Blk, Fgr, Fyn, Src, Lck, Lyn and Yes), tyrosine kinases (Abl, EGFR, IGF-1R, JAK2, PDGFR, KDR) as well as some serine-threonine kinases. The percentage of enzymatic activity was measured for each kinase by using **4c** at 10 μM (Millipore); results are listed in Table 6.

Remarkably, **4c** proved to be more efficient against SFKs than towards the other investigated kinases, confirming such a compound as a useful probe to study Src family function. Furthermore, high activity against Abl was also detected, as expected for the high structural similarity between Abl and the SFKs. Notably, **4c** was not able to significantly inhibit any of the serine-threonine kinases tested (Pim-1, mTOR, JNK, CDK5, CHK1) and also was not active towards the serine-threonine kinases DYRK1a and GSK3- β that were reported as implicated in AD [212].

Table 6 Kinase profiling of compounds **4c** by Millipore

	Residual activity (%)
Fyn (h)	1
Abl(h)	9
Abl (H396P) (h)	5
Abl (M351T)(h)	6
Abl (Q252H) (h)	3
Abl(T315I)(h)	97
Abl(Y253F)(h)	2
Blk(h)	19
CDK5/p25(h)	86
CDK5/p35(h)	99
CHK1(h)	97
cKit(h)	36
cSRC(h)	17
EGFR(h)	47
Fgr(h)	4
GSK3 β (h)	66
Hck(h)	7
Hck(h) activated	2
IGF-1R(h)	71
JAK2(h)	118
JNK3(h)	53
KDR(h)	31
Lck(h)	2
Lck(h) activated	9
Lyn(h)	15
mTOR(h)	47
PDGFR β (h)	51
Pim-1(h)	91
Src(1–530)(h)	2
Src(T341M)(h)	98
Yes(h)	5
DYRK1a	85

9.2 Biological Evaluation in Alzheimer's Disease Model

Since Fyn mediates the phosphorylation of tau on the Tyr18 residue and is therefore considered a promising therapeutic target for the treatment of AD, the most interesting compounds identified during in vitro inhibition assays, **4c** and **4d**, were evaluated for their ability to inhibit the Fyn-mediated phosphorylation of residue Tyr18 on tau in an AD model cell line. To this aim, neuroblastoma SH-SY5Y cells were differentiated to mature neurons with the administration of retinoic acid, followed by brain-derived neurotrophic factor, neuregulin β 1, nerve growth factor and vitamin D3 treatment. Once differentiated, SH-SY5Y cells were

treated with A β ₄₂ oligomer/protofibril in order to induce AD-like neurotoxicity [201, 213]. Both compounds significantly affected A β ₄₂-induced Tyr18-tau phosphorylation to a similar degree and in a dose-dependent manner [200]. Moreover, the inhibitory activity of **4c** and **4d** was constant over time, being effective up to 6 h after compound administration [200].

10 Conclusions

The accelerating pace of population ageing is producing an increased prevalence of those neurodegenerative diseases that appear in the late stages of life and that require persistent caregiving, for example, AD. Despite the important impact on public health costs that this provokes, current therapy does not stop neuronal degeneration and only ameliorates symptoms. Thus, there is a compelling need for the development of AD disease-modifying agents. Some of these molecules are in clinical trial studies, but discouraging results indicate that more efforts are needed. The increasing understanding of AD aetiology indicates that tau-phosphorylating kinases are strongly involved in the disease and thus represent promising drug targets. However, the difficulties in the development of GSK3 β inhibitors suggest that research should move towards other tau-phosphorylating kinases.

Fyn is responsible of the development and regulation of the physiological functions of the CNS, and its overexpression or hyperactivation is strongly correlated with tauopathies. Interestingly, in addition to tau hyperphosphorylation, several other cascades link Fyn to AD aetiology, making this kinase a very promising anti-AD drug target. Indeed, Fyn ATP-competitive inhibitors are in preclinical and clinical development with encouraging results. This might indeed be one of the drug development strategies to follow for the development of efficacious disease-modifying drugs for AD.

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Microtubule-Stabilizing Agents for Alzheimer's and Other Tauopathies

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Abstract In neurodegenerative tauopathies, of which the most prevalent example is Alzheimer's disease (AD), the aggregation of the microtubule (MT)-stabilizing protein tau is believed to have neuropathological consequences. Multiple studies indicate that deficits in axonal MTs and axonal transport may contribute to the neurodegenerative processes of these diseases. MT-stabilizing molecules have shown promise in restoring axonal MTs and transport, as well as cognitive performance, in animal models of human tauopathies. As a result, such compounds may be considered as potential candidates for the treatment of AD and related tauopathies. Many examples of MT-stabilizing natural products and derivatives thereof have been approved for cancer treatment; however, the use of these compounds for central nervous system (CNS) diseases may be challenging due to limited brain penetration and oral bioavailability, as well as potential systemic side effects. In this chapter, we review the progress made toward the identification and development of CNS-active MT-stabilizing candidate compounds, with an emphasis on nonnaturally occurring small molecules that exhibit favorable drug-like properties.

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1 Introduction

Neurodegenerative tauopathies [1] are a group of >20 sporadic and familial diseases (e.g., see Table 1) characterized by progressive dysfunction of nerve cells that correlates with the appearance of insoluble intracellular aggregates comprised primarily of tau proteins. Importantly, a causal relationship between the formation of tau inclusions and the process of neurodegeneration has been established via a series of genetic studies [2–4]. Among neurodegenerative tauopathies, the most prevalent example is Alzheimer's disease (AD), a complex multifactorial disease which features, in addition to tau inclusions, senile plaque pathology caused by the aggregation of amyloid β (A β) peptides.

Tau is a microtubule (MT)-associated protein that is highly expressed in the axons of neurons. The primary function of tau appears to be the promotion of MT assembly and stabilization [5]. As highlighted in Fig. 1, tau is comprised of: (1) either three or four alternatively spliced MT-binding amino acid repeats that interact with the β -tubulin subunit in a binding site that is believed to overlap the taxane-binding site [6]; (2) a proline-rich region, which at physiological pH is mostly positively charged and that is thought to interact electrostatically with the negatively charged surface of MTs; and (3) an N-terminal domain containing either zero, one, or two 29 amino acid-long alternatively spliced inserts, known as the projection domain region, which at physiological pH is mostly negatively charged and thus extends outward, away from the surface of the MT due to electrostatic repulsion. In the adult brain, there are six tau isoforms that result from the aforementioned alternative splicing of the MT-binding repeats (i.e., 3R or 4R) and the N-terminal inserts (i.e., 0N, 1N, or 2N). In the healthy adult brain, the ratio of 3R and 4R tau is close to 1:1, and a deviation from this ratio is associated with certain forms of frontotemporal lobar degeneration (FTLD).

Tau is known to undergo a number of posttranslational modifications [7] that are believed to play an important role in modulating binding to MTs, as well as the propensity of the protein to undergo misfolding and aggregation. In neurodegenerative diseases, tau is typically hyperphosphorylated and aggregated in neurons, forming either paired helical filaments (PHF) or straight filaments that comprise

Table 1 Selected examples of neurodegenerative amyloidoses featuring tau inclusions

Alzheimer’s disease
Chronic traumatic encephalopathy
Corticobasal degeneration
Frontotemporal dementia and parkinsonism linked to chromosome 17
Frontotemporal lobar degeneration
Pick’s disease
Progressive supranuclear palsy

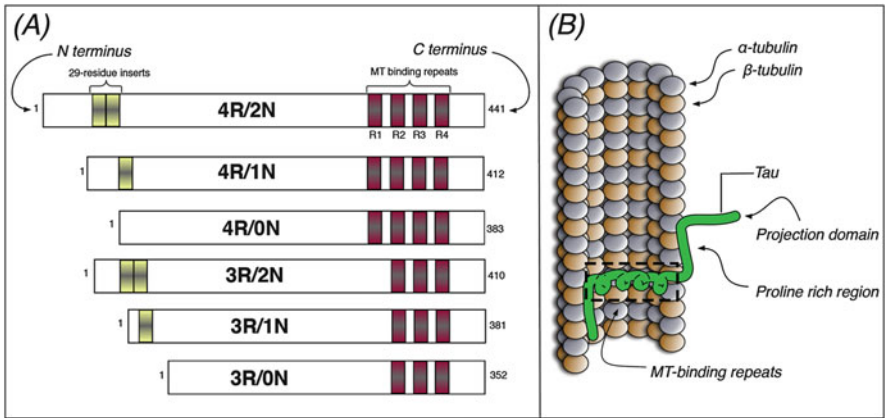


Fig. 1 (a) The six isoforms of tau; (b) A schematic model of the tau MT complex revealing key interactions (adapted from Amos [6])

neurofibrillary tangles (NFTs) in the cell soma and neuritic threads in neuronal processes [1, 5]. The degree of cognitive impairment in AD appears to correlate closely with the density of NFTs, although a small number NFTs can also form as part of normal aging [8–10]. Furthermore, in the diseased state, aggregated tau is comparatively hyperphosphorylated relative to aggregated tau in non-diseased adult brains [11].

A growing body of evidence indicates that a common feature of neurodegenerative tauopathies is compromised axonal transport that appears to be associated with a faulty axonal MT network [12]. Axonal transport is an essential process in neurons that is responsible for the movement of various types of cargoes to and from the cell body and the synapses [13]. MTs are essential constituents of the intracellular infrastructure, and they form polarized linear arrays in the axons of neurons that provide both structural support and directionality to motor proteins, such as kinesins and dyneins (see Fig. 2). Thus, an appropriate structure and organization of axonal MTs is believed to be required for efficient axonal transport. In addition, there is evidence that alterations in MT dynamics in other neuronal compartments may play a role in the neurodegenerative process. For example,

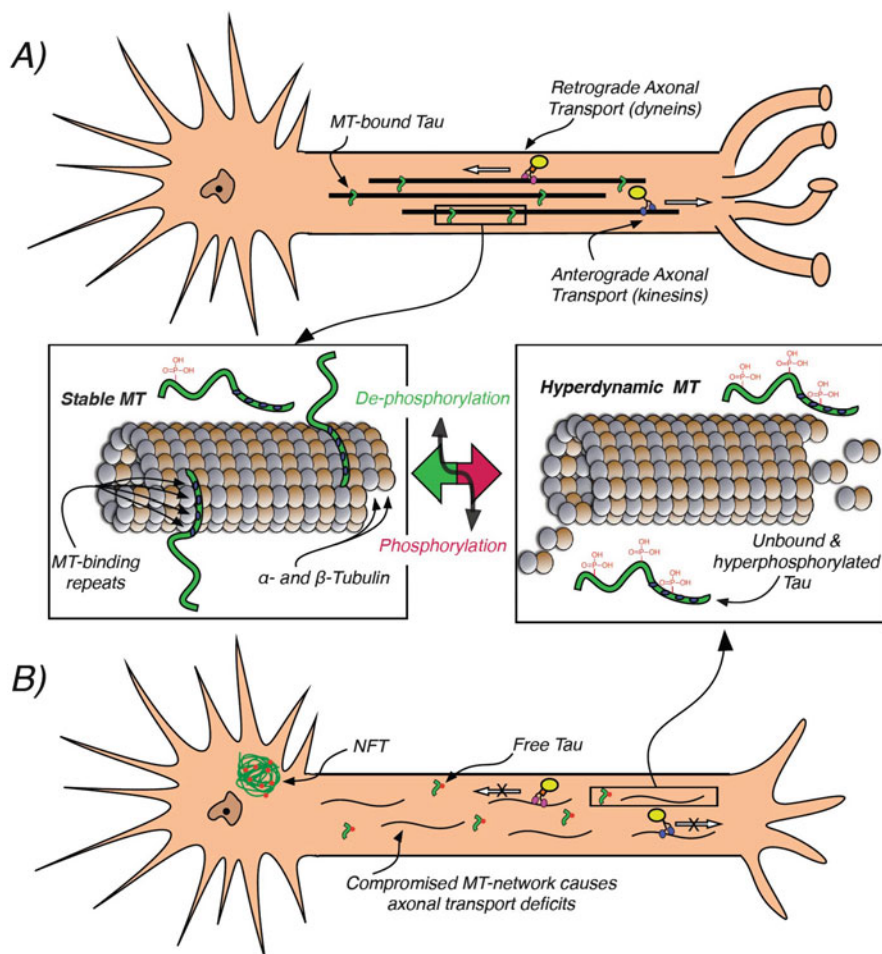


Fig. 2 Schematic representation of the role of tau, axonal MTs, and axonal transport under physiological conditions (a). The hyperphosphorylation and disengagement of tau from MTs result in compromised axonal transport, leading to a pathological condition (b)

abnormal MT dynamics in the dendrites may be involved in the loss of dendritic spines that constitute another distinctive neuropathological feature of AD [14–17]. Interestingly, a decreased level of biomarkers of stable MTs, such as acetylated and de-tyrosinated α -tubulin [18, 19], as well as a reduction in the total number and lengths of neuronal MTs, have been observed in the brains of AD patients [20–22], suggesting that hyperdynamic MTs, axonal transport deficits, and neurodegeneration may be causally related.

Mechanistically, the alteration of axonal MTs in tauopathy neurons may derive at least in part from the fact that tau, when hyperphosphorylated and sequestered into aggregate structures, can no longer bind to and stabilize MTs [23]. Evidence of

a causative relationship between the hyperphosphorylated/aggregated tau and disrupted MTs has been found in cell culture [24, 25], as well as in animal models [26–28]. The binding affinity of tau for MTs is influenced by the phosphorylation state of the protein [29], as increased phosphorylation is believed to reduce the binding affinity of tau for MTs [30]. Under normal conditions, tau exhibits a relatively low phosphorylation status (i.e., an average of ~1.9 mol of phosphate per mole of tau [31]) and is mostly bound to MTs [32]. In the AD brain, however, the tau phosphorylation increases to ~2.6 mol of phosphate per mole of tau, with PHF tau exhibiting 6–8 mol of phosphate per mole of tau [31]. Thus, the hyperphosphorylation and aggregation of tau are likely to cause an abnormal disengagement of tau from MTs that may lead to cytoskeletal alterations [33] and axonal transport deficits in affected neurons. In addition, as there is evidence that MT-bound tau may also be involved in regulating the attachment of motor proteins, an aberrant detachment of tau from MTs could also affect axonal transport by interfering with normal motor protein function [34]. Finally, it is possible that tau hyperphosphorylation and aggregation may contribute to neuronal dysfunction by additional mechanisms that are not directly related to a loss of MT-stabilizing function but rather are due to a gain-of-function toxicity of misfolded tau monomers, oligomers, or fibrils.

2 MT-Stabilizing Agents as Candidate Therapeutics

Naturally Occurring Compounds and Derivatives Thereof Stabilization of axonal MTs by means of MT-stabilizing agents has been proposed as a possible strategy for therapeutic intervention in neurodegenerative tauopathies [35]. A series of proof-of-concept studies [26–28, 36] in animal models have provided validation for this strategy. The first important demonstration that MT-stabilizing drugs could be used to ameliorate MT and axonal transport deficits was obtained in 2005 [28], where weekly doses of a formulated paclitaxel (Taxol®, **1**, Fig. 3) preparation were found to restore axonal transport and reduce the neurodegenerative phenotype of T44 tau transgenic mice.

Although paclitaxel is known to have limited brain uptake, efficacy was presumably obtained in the T44 mice because they exhibit tau pathology in motor neurons that reside in the periphery. As a result, blood–brain barrier (BBB) penetration was not a requirement in these particular studies. Subsequent studies from our group [27, 36] and others [26] that utilized the brain-penetrant natural product, epothilone D (**2**, Fig. 4), in transgenic mouse models in which tau pathology is confined predominantly to the brain further validated the potential of MT-stabilizing agents for the treatment of tauopathies.

Several reports, including competition binding [37], X-ray [38], NMR [39], and photoaffinity-labeling [40] studies demonstrated that the epothilones, like other classes of MT-stabilizing natural products, interact with MTs at the taxane-binding

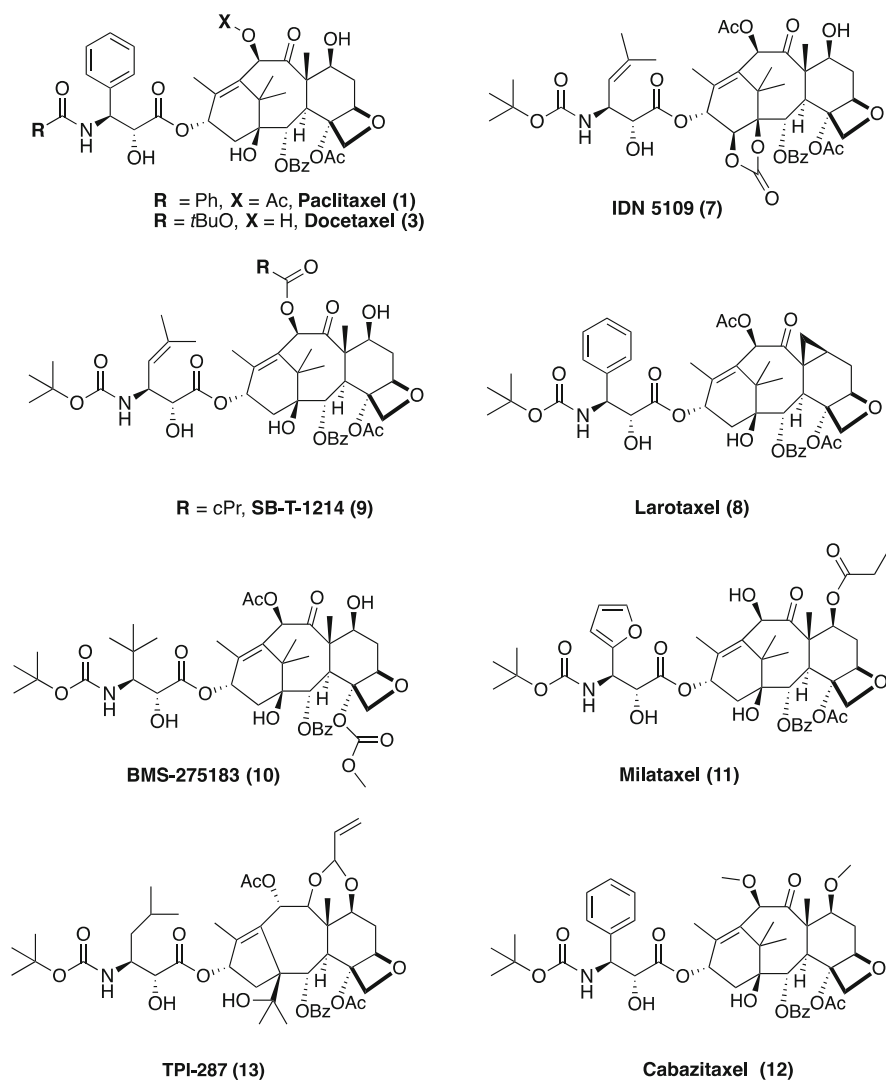


Fig. 3 The structure of paclitaxel and related taxanes

site located in the lumen of the MT in the β -tubulin subunit. The different taxane site MT-stabilizing agents are believed to promote MT stabilization by inducing a similar structuring of the M-loop of β -tubulin [38], which results in stronger lateral interactions between adjacent protofilaments. However, at the molecular level, the mechanism of action of these compounds is not identical due to differing binding modes. Indeed, even relatively small structural differences within the same class of compounds, such as those between paclitaxel and docetaxel (3, Fig. 3), are known to be responsible for noticeable changes in the structure of MTs [41].

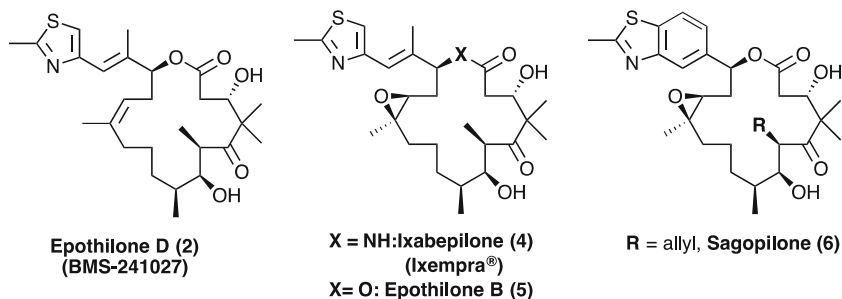


Fig. 4 The structures of selected epothilones

Paclitaxel exhibits limited brain penetration in part because of the active transporter, P-glycoprotein (P-gp) [42], which is highly expressed in the endothelial cells of the BBB. Epothilone D, unlike **1**, is not a good substrate for P-gp, but it is rather a potent inhibitor of the active transporter [43]. The ability of epothilone D to overcome P-gp-mediated efflux, combined with the fact that this molecule is comparatively smaller and less functionalized than paclitaxel and related taxanes, provides a possible explanation for the superior brain penetration of this natural product [43]. Treatment of tau transgenic mice, such as the PS19, 3xTg, and rTg4510 models that progressively develop tau pathology in the CNS with age, with relatively low weekly doses of epothilone D led to a reduction in the number of dystrophic axons and a reduction in tau pathology that coincided with a normalization of MT mass and dynamics, as well as an improvement in axonal transport [26, 27, 36]. Importantly, epothilone D treatment led to an improvement in cognitive performance and a reduction in neuronal loss in the tau transgenic mice. Interestingly, the effective doses of epothilone D in these studies (i.e., 0.3 and 1 mg/kg) were approximately equivalent to 1/30th and 1/100th of the chemotherapeutic doses when adjusted by allometric scaling for species differences. Treatment of tau transgenic animals with these relatively low weekly doses of epothilone D did not result in detectable side effects, such as neutropenia or peripheral neuropathy [44] that are typically encountered with chemotherapeutic regimens based on MT-stabilizing drugs.

Given the very promising results from these preclinical studies, the development of CNS-directed MT-stabilizing therapies holds promise as a possible strategy to treat tau-mediated neurodegeneration. Although epothilone D may be considered as a potentially promising clinical candidate, as this compound progressed to Phase 1b clinical testing in a short 9-week study with AD patients, **2** is only one of very few examples of brain-penetrant MT-stabilizing agents to be evaluated in animal models. Moreover, this compound may have potential deficiencies as a drug candidate to be used for a prolonged treatment period, including an IV route of administration and inhibition of P-gp, which may lead to undesired drug-drug interactions [45] and/or to CNS toxicities [46].

Over the past several years, a growing number of MT-stabilizing compounds have been identified among natural products and nonnaturally occurring small

molecules [47, 48]. The screening and evaluation of additional examples of brain-penetrant MT-stabilizing agents may lead to alternative and potentially improved clinical candidates. In this context, ixabepilone (**4**, Fig. 4), which is the first member of the epothilones to have reached FDA approval for cancer chemotherapy [49], was evaluated in our laboratories. Ixabepilone is a close derivative of epothilone B (**5**, Fig. 4), in which the lactone moiety of the latter is replaced by a more stable lactam. Like epothilone D, ixabepilone was found to reach significant brain concentrations in mice after peripheral administration [43]. However, in stark contrast with the former compound, treatment of PS19 tau transgenic mice with **4** over several weeks was associated with adverse side effects at doses required to elicit an increase in a marker of stable MTs in the brain (data not published), suggesting that the repurposing of this drug for neurodegenerative diseases may not be possible. Interestingly, although both **2** and **4** exhibit prolonged half-lives in the brain (i.e., >16 h), epothilone D was found to be considerably less stable toward metabolism than ixabepilone, as suggested by a relatively short plasma half-life in mice (i.e., <2 h) [43]. A possible explanation for the noticeable difference in tolerability between these two compounds may be related in part to the fact that epothilone D is more rapidly metabolized outside of the brain and, therefore, has a lesser peripheral exposure than ixabepilone. This possibility may imply that a differential between brain and plasma half-life could be an important aspect to consider in the prioritization of candidate compounds for tauopathy treatment. A similar differential has been observed for other members of the epothilones [43], including sagopilone [50] (**6**, Fig. 4) and epothilone B [51]. Interestingly, epothilone B showed promise as a CNS-active agent in animal models of Parkinson's disease [52] and traumatic brain injury [53], with no signs of systemic toxicity.

Among the taxanes, different examples of paclitaxel derivatives have been identified that unlike **1** are capable of overcoming P-gp-mediated efflux and as a result may exhibit improved brain penetration and/or oral bioavailability (see compounds **7–12**, Fig. 3). These derivatives include IDN-5109 (**7**) [54], lorataxel (**8**) [55], SB-T-1214 (**9**) [56], BMS-275183 (**10**) [57, 58], and milataxel (**11**) [59]. Cabazitaxel (Jevtana®, **12**, Fig. 3) [60, 61], an FDA-approved anticancer drug, was also reported to exhibit increased brain penetration due to reduced transport by P-gp [62]; however, recent pharmacokinetic studies in P-gp-expressing and knockout mice clearly suggest that the brain accumulation of **12** may still be limited by the action of P-gp [63]. Another compound that was found to be particularly promising as a CNS-directed MT-stabilizing compound is TPI-287 (**13**, Fig. 3) [64]. This compound is a taxoid derivative that features a 11(15→1) abeotaxane scaffold, which is found also in several other naturally occurring taxoids from *Taxus chinensis*, *T. yunnanensis*, and *T. wallichiana* [65, 66]. This particular abeotaxane skeleton is characterized by a five-membered A ring of the baccatin core, compared to the larger six-membered ring found in all other taxanes. As TPI-287 was found to be brain penetrant, this compound is under evaluation as potential treatment for brain cancer and primary tumors that have metastasized to the brain [67–69]. In addition, although the activity of this compound in tau transgenic animal models has not been fully described, preliminary studies suggest

that low weekly doses of TPI-287 may improve behavioral impairment and tau pathology in PS19 mice [70]. Importantly, this compound is presently undergoing clinical trials in patients with tauopathies, such as corticobasal degeneration and progressive supranuclear palsy (see clinicaltrials.gov identifier NCT02133846).

In addition to taxanes and epothilones, other classes of MT-stabilizing natural products are being considered for evaluation. For example, dictyostatin [71] (**14**, Fig. 5), another compound that is known to interact with MTs at the taxane site within β -tubulin [72] and which demonstrated promising pharmacokinetic and pharmacodynamic properties that resembled those of epothilone D [73], has recently undergone evaluation in PS19 tau transgenic mice in our laboratories [74]. Moreover, peloruside A [75] (**15**, Fig. 5) has also been described as a potentially promising compound, as in vitro studies demonstrated that this MT-stabilizing agent is able to compensate for the loss of MT-stabilizing function of tau caused by okadaic acid-induced hyperphosphorylation of tau [76]. These results with peloruside A are especially interesting in consideration of the fact that this compound stabilizes MTs in a substantially different way than molecules that bind the taxane site on β -tubulin, interacting instead with a distinct site on the surface of MTs that does not overlap with the taxane site [77, 78]. The brain pharmacokinetic properties of this compound, however, have not yet been determined.

Nonnaturally Occurring Small Molecules Most of the MT-stabilizing compounds discovered to date comprise a large ensemble of structurally complex natural products and derivatives thereof [47, 48]. With the possible exception of the selected examples illustrated above, the majority of these compounds share similar potential limitations as therapeutic candidates for CNS indications, including suboptimal brain exposure and, in some instances, synthetic challenges. For these reasons, the development of MT-stabilizing small molecules that exhibit more favorable drug-like properties would be desirable to identify candidates that could combine synthetic accessibility with good pharmaceutical properties, including oral bioavailability and brain penetration. It is known that nonnaturally occurring small molecules having MT-stabilizing activity are relatively rare, especially compared

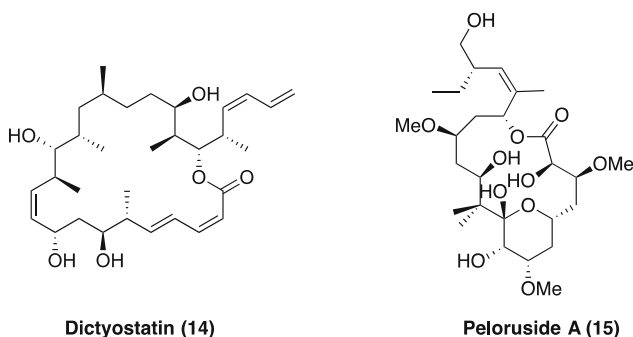


Fig. 5 Other MT-stabilizing natural products being investigated in the context of tau-mediated neurodegeneration

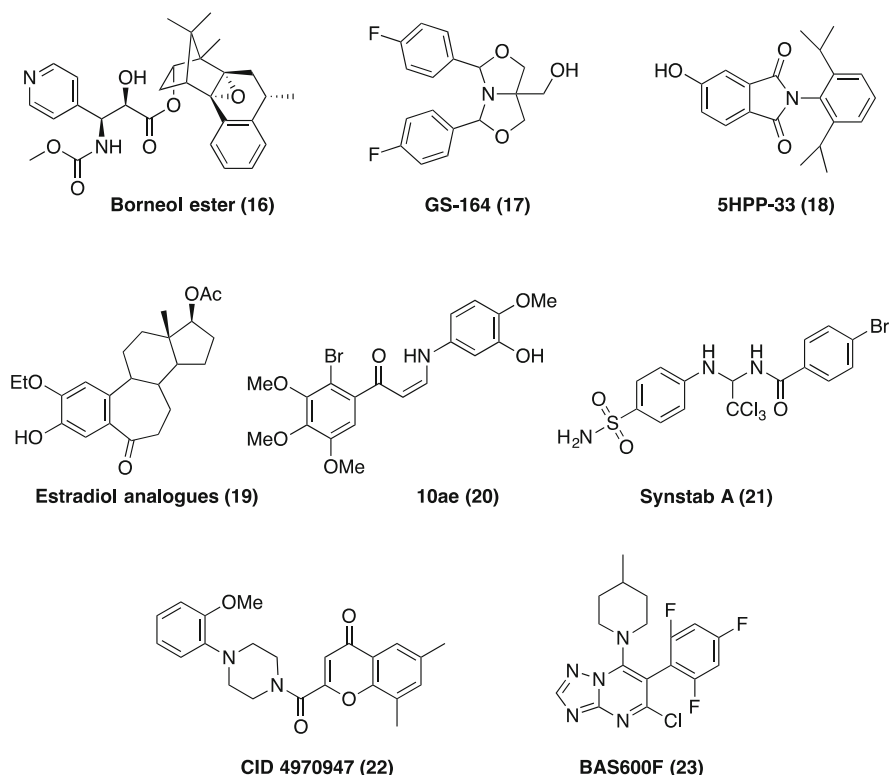


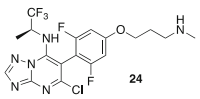
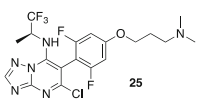
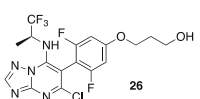
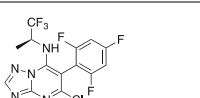
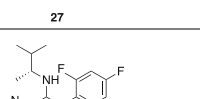
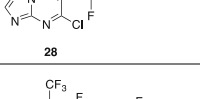
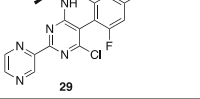
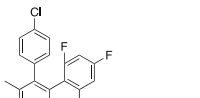
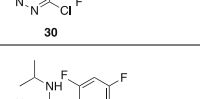
Fig. 6 Representative structures of nonnaturally occurring MT-stabilizing compounds

to compounds having the opposite effect of de-polymerizing MTs [79]. An overview of different classes of nonnaturally occurring compounds with reported MT-stabilizing activity is shown in Fig. 6 and Table 2.

Attempts to simplify the taxane structure and generate smaller and less complex analogs of **1** with paclitaxel-like MT-stabilizing properties resulted in the identification of borneol ester derivatives [94] (e.g., **16**, Fig. 6) and GS-164 [95] (**17**, Fig. 6). Interestingly, although these compounds have been found to promote MT polymerization in vitro in a manner similar to paclitaxel, compared to the natural product, these structurally simpler analogs appear to be considerably less potent as antimetabolic agents. This observation is somewhat paradoxical, as it is thought that the antimetabolic activity of MT-stabilizing agents directly results from alterations of MTs in the mitotic spindle during cell division. If the apparent separation between MT-stabilizing and antimetabolic activities is further substantiated for these compounds, they may serve as potentially attractive starting points for the treatment of neurodegenerative diseases [94].

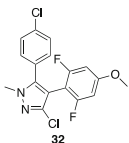
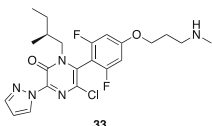
Relatively weak antimetabolic and MT-stabilizing properties have also been described for a group of thalidomide derivatives [96]. The most studied of these

Table 2 Representative examples of triazolopyrimidines and related heterocyclic compounds

Compound class/name	Representative structure	References
Triazolopyrimidines	 24	[80–83]
	 25	[83–85]
	 26	[83–86]
	 27	[83–85]
	 28	[84]
Phenylpyrimidines	 29	[84, 87, 88]
Pyridazines	 30	[89]
Pyridopyrazines	 31	[90]
Imidazoles	 31	[91]

(continued)

Table 2 (continued)

Compound class/name	Representative structure	References
Pyrazoles	 32	[92]
Pyrazinones	 33	[93]

derivatives, 5HPP-33 [97] (**18**, Fig. 6), is a compound that was initially identified for its anti-angiogenic properties [98]. The activity of **18**, however, is somewhat unclear as previous studies found that this compound exhibits inhibitory activity on tubulin polymerization [99]. In addition, recent studies [100] revealed that although 5HPP-33 suppresses the dynamics of individual MTs in MCF-7 cells, this compound causes an inhibition of MT assembly both in cell-free assays and in cultured MCF-7 cells.

Selected estradiol derivatives have also been identified that appear to promote MT polymerization [101]. Interestingly, the metabolite of estradiol, 2-methoxyestradiol, and other related synthetic congeners are known to act as inhibitors of MT polymerization and are thought to interact with tubulin at the binding site for colchicine [102], an inhibitor of MT polymerization. However, selected congeners (e.g., **19**, Fig. 6) demonstrated paclitaxel-like enhancement of MT polymerization [103]. Structure-activity relationship (SAR) studies demonstrated that the presence in these compounds of an acetyl group at C17, but not at C3, is essential to impart MT-stabilizing properties [101, 103].

In an apparently similar fashion to the estradiol derivatives, a series of enaminones, such as 10ae (**20**, Fig. 6), have also been identified that appear to be functionally related to paclitaxel with respect to MT stabilization and antimitotic activity [104]. However, several structurally related compounds have been found to compete with colchicine binding to tubulin and inhibit tubulin polymerization [105]. The tubulin polymerization promoter, 10ae, was found to be more potent than paclitaxel in a cell-free, light-scattering MT polymerization assay; however, in cells, this compound was not as potent. Furthermore, significant differences in the organization of cytoplasmic MTs have been noted upon treatment with paclitaxel or **20**, as cells treated with the latter compound did not show the characteristic bundling of MTs that is typically seen upon treatment with taxanes [104]. These results with enaminone derivatives and those mentioned above with estradiol derivatives demonstrate that appropriate structural modifications of certain colchicine-site tubulin polymerization inhibitors can lead to analogs with opposite

MT-stabilizing activities. It is not clear, however, whether these MT-stabilizing analogs interact with tubulin at the colchicine site or at a different binding site.

Additional compounds of interest have been identified via phenotypic screening for novel small molecules having paclitaxel-like properties. These compounds include synstab A [79] (**21**, Fig. 6) and CID 4970947 [106] (**22**, Fig. 6), which demonstrated MT-stabilizing activity both in cell-based and cell-free assays. The SAR of these compounds, however, has not yet been investigated. Finally, a promising class of tubulin polymerization promoters are the triazolopyrimidines (e.g., BAS600F [107], **23**, Fig. 6) and related heterocycles (Table 2). This class of small heterocyclic compounds was originally identified via screening for novel antifungal agents. Considerable efforts, led largely by the agrochemical industry, resulted in the identification of a growing number of mono- and bi-heterocyclic compounds that are structurally related to the triazolopyrimidines and which exhibit comparable activities as tubulin polymerization promoters and antifungal agents (e.g., see compounds **25–34**, Table 2). Interestingly, Wyeth developed a series of triazolopyrimidine congeners as potential anticancer agents, and this effort ultimately led to the identification of TTI-237 (cevipabulin, **24**, Table 2) as a preferred clinical candidate with a promising combination of antimitotic activity and drug-like properties, including metabolic stability and oral bioavailability [83]. Importantly, although cevipabulin was found to exhibit limited brain uptake, several examples of MT-stabilizing congeners (e.g., **25–29**, Table 2) have been identified that are brain penetrant and that can cause an elevation in biomarkers of stable MTs in the brain of wild-type (WT) mice [84, 85].

Early studies to elucidate the mode of action and the SAR of triazolopyrimidines indicated that these compounds alter MT dynamics in a manner potentially unique among all MT-active compounds [82, 83]. Competition binding studies revealed that cevipabulin does not compete with colchicine or paclitaxel for binding to tubulin. Instead, **24** appeared to inhibit vinblastine binding, suggesting that the triazolopyrimidine class of compounds may be competing directly or allosterically with the binding site of vinca alkaloids, which inhibit MT polymerization. This observation, combined with the fact that cells treated with cevipabulin manifested changes in the level of different phosphoproteins that are typically seen after vincristine treatment, led to the hypothesis that the mode of action of triazolopyrimidines may be both taxane- and vinca-like [82].

Further in vitro characterization of the pharmacological properties of cevipabulin, as well as the brain-penetrant derivatives **25** and **26** and phenylpyrimidine **29**, revealed that compound concentrations that are needed to elicit an increase in markers of stable MTs (i.e., acetylated- and de-tyrosinated- α -tubulin) in cells (QBI-293 or COS-7 cells) also produce a decrease in total α - and β -tubulin levels [84]. Notably, this compound-induced reduction of total tubulin, which occurs via the proteasomal degradation pathway, was not observed upon treatment with epothilone D or vinblastine. Furthermore, side-by-side comparison of cellular response after treatment with epothilone D, **25**, or **29** demonstrated that while all compounds produced a marked increase in acetylated α -tubulin, treatment with **25** or **29**, unlike epothilone D, resulted in altered distribution and intensity of β -tubulin labeling.

These results clearly suggest that while triazolopyrimidine **25** and phenylpyrimidine **29** can promote tubulin assembly, as determined by an increase in acetylated α -tubulin, the MT structures generated in the presence of these compounds may be abnormal and subject to degradation, an effect that would be undesirable for tauopathy treatment. However, an evaluation of a series of closely related congeners, such as triazolopyrimidine **27** and **28**, revealed that these compounds promote MT assembly without affecting total tubulin levels or disrupting MT architecture. These studies highlight that, depending on the particular scaffold and the nature of the substituents decorating the heterocycle, compound treatment can elicit substantially different cellular phenotypes [84]. Importantly, most of the preferred subset of MT-stabilizing triazolopyrimidines were found to be both brain penetrant and orally bioavailable and did not disrupt P-gp function. A representative example (**28**, Table 2) was also found to produce an elevation of markers of stable MTs in the brain of WT mice. Moreover, in neuronal culture, **28** could compensate for the loss of tau MT-stabilizing function caused by okadaic acid-induced tau hyperphosphorylation [84]. Thus, collectively, these results suggest that selected triazolopyrimidines and possibly other related heterocycles may provide a source of viable candidates for tauopathy treatment.

3 Concluding Remarks

CNS-active MT-stabilizing compounds hold promise as potential treatments for a variety of devastating conditions, including brain tumors and several neurodegenerative diseases [108]. Among natural products and related derivatives, selected compounds, such as epothilone D and TPI-287, have reached the early stages of clinical evaluation for neurodegenerative tauopathies. In addition, in spite of the fact that, historically, the identification of drug-like small molecules endowed with MT-stabilizing activity has proven to be challenging, over the past several years, there has been considerable progress as evidenced by the number of compounds that have been reported, most of which are related to the triazolopyrimidine class. Although the mode of action of these nonnaturally occurring compounds has not yet been completely defined, the data generated thus far clearly suggest that a select subset of these molecules may be viable candidates for neurodegenerative tauopathies and perhaps CNS oncological indications. Further evaluation of efficacy and tolerability in disease models will help determining the potential of these compounds as therapeutic agents.

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PET Imaging Agents for Alzheimer's Disease

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Abstract In the past four decades, PET (positron emission tomography) imaging has been developed into a common diagnostic tool. After an i.v. injection of a small amount of radioactive probe, PET images are taken based on the decay of short-lived positron-emitting isotopes. The emitted positron collides with a neighboring electron to produce two 511 KeV gamma rays roughly 180 apart. It is based on this physical principal and the positron coincident signals that the point source of the gamma rays can be deduced by using a coincident circuit for detectors 180 apart. Currently, there are three FDA-approved imaging agents (Amyvid, NeuraCeq, and Vizamy) available for mapping A β in the brain of patients suspected of having Alzheimer's disease. There is a strong interest in measuring tau deposition by PET imaging in a quantitative manner. Several useful Tau imaging agents have been tested in humans; however, they have not been fully validated as those of A β imaging agents.

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1 Introduction

1.1 In Vivo Positron Emission Tomography Imaging

In the past 4 decades, PET (positron emission tomography) imaging has been developed into a common diagnostic tool. After an i.v. injection of a small amount of radioactive probe, PET images are taken based on the decay of short-lived positron-emitting isotopes. The emitted positron collides with a neighboring electron to produce two 511 KeV gamma rays roughly 180° apart. It is based on this physical principal and the positron coincident signals that the point source of the gamma rays can be deduced, by using a coincident circuit for detectors 180° apart [1].

Two most commonly used positron-emitting isotopes are ¹¹C (*T*_{1/2} = 20 min) and ¹⁸F (*T*_{1/2} = 110 min) (Table 1) [1]. A metabolic tracer for brain imaging, ¹¹C glucose, has been reported for studying glucose utilization in the brain

Table 1 Radionuclides for PET imaging agents

Radionuclides	<i>T</i> _{1/2}	Production methods ^a
¹¹ C	20 min	N-14 (p, α) C-11, cyclotron
¹⁸ F	110 min	O-18 (p, n) F-18, cyclotron
¹³ N	10 min	O-16 (p, α) N-13, cyclotron
⁶⁸ Ga	68 min	⁶⁸ Ge/ ⁶⁸ Ga Generator

^aIn producing isotopes in this list, a proton, p, is accelerated in a cyclotron and when the high energy beam hits the target containing the parent element it knocks off either an alpha particle, α, or a neutron, n, from the parent isotope to produce the daughter isotope listed above

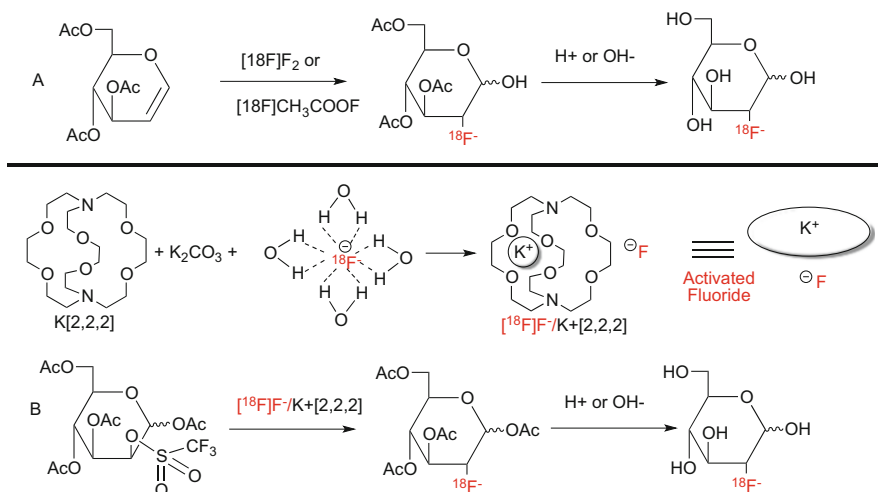


Fig. 1 The schemes show the production of $[^{18}\text{F}]\text{FDG}$ (2-fluoro-2-deoxy-D-glucose). **(a)** The original method for preparation of $[^{18}\text{F}]\text{FDG}$ employed fluorine gas ($[^{18}\text{F}]\text{F}_2$) [4, 5]. **(b)** The improved synthesis of $[^{18}\text{F}]\text{FDG}$ uses $[^{18}\text{F}]\text{fluoride}$ ion, potassium carbonate, and $\text{K}[\text{2.2.2}]$, a cryptand, to activate the fluoride ion [6, 7]. This nucleophilic reaction improved production of $[^{18}\text{F}]\text{FDG}$

[2, 3]. However, due to its limited physical half-life, it is challenging to utilize ^{11}C radiopharmaceuticals. Only a few major medical centers and clinics with technical skills can make ^{11}C radiolabeled probes for patient studies. The half-life of ^{18}F (110 min) is 5.5 times longer than that of ^{11}C ($T_{1/2} = 20$ min). Thus, it is generally accepted that ^{18}F tracers are more practical. An off-site cyclotron can manufacture them commercially. The distribution can be centralized by a radiopharmacy. Originally, $[^{18}\text{F}]\text{FDG}$ (2-fluoro-2-deoxyglucose) was prepared with fluorine gas ($[^{18}\text{F}]\text{F}_2$; Fig. 1a). The process is rather complicated, and the labeling yield was less than 30%. The reaction has been improved significantly by a direct nucleophilic reaction using a fluoride ion, potassium carbonate, and $\text{K}[\text{2.2.2}]$ Kryptofix[®], a cryptand, to activate the fluoride ion (see Fig. 1a, b) [4, 6]. This procedure can produce >370 GBq (>10 Ci) of $[^{18}\text{F}]\text{FDG}$ per batch (sufficient for hundreds of patient doses).

Currently, $[^{18}\text{F}]\text{FDG}$, a PET imaging agent with widespread clinical application, is transported across cell membranes via a glucose transporter and then phosphorylated by hexokinase in the cytosol. The process is closely linked with cellular metabolism, including glucose usage. FDG as an imaging agent is commonly employed for studying glucose metabolism in the brain as well as in detecting tumors, which show a higher rate of metabolism [8]. FDG-PET provides a diagram of cancer cell activity within the body. PET-CT (computer tomography) also offers a fusion image combining regional glucose metabolism and anatomical structure in one imaging session. This combined imaging method is now the standard of care in oncology for diagnosis, staging, and monitoring therapy [9].

PET imaging in conjunction with FDG has been approved by the FDA for diagnosis of Alzheimer's disease [10, 11], but the FDG regional distribution in the brain is a reflection of glucose metabolism, which may not be directly associated with AD symptoms or disease progression.

2 Alzheimer's Disease (AD): Pathogenesis and Amyloid Imaging

Alzheimer's disease (AD) is a neurodegenerative disorder of the brain, characterized by a loss of mental function and progressive dementia. This disease is important because it affects millions of older patients. Clinical symptoms of AD include cognitive decline, irreversible memory loss, disorientation, and language impairment. Major neuropathological observations of postmortem AD brain include the presence of senile plaques containing β -amyloid ($A\beta$) aggregates and neurofibrillary tangles (NFTs) containing highly phosphorylated tau proteins (Fig. 2a). Significant evidence suggests that accumulation and aggregation of $A\beta$ peptides may play a major role in AD pathogenesis [13, 16, 17]. The aggregated $A\beta$, produced by proteolytic processing of the β -amyloid precursor protein (Fig. 2b), may start a series of events (Fig. 2a).

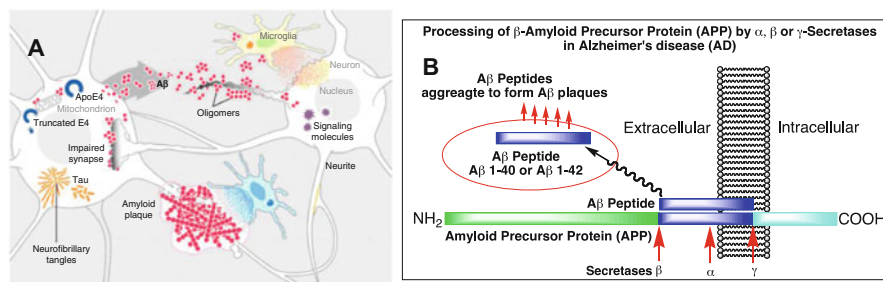


Fig. 2 (a) Processes implicated in AD pathogenesis. $A\beta$ peptides produced by neurons aggregate into a variety of assemblies (e.g., oligomers, plaques), some of which may impair synapses and neuronal dendrites. Build-up of pathogenic $A\beta$ aggregates could result from increased production or from deficient clearance mechanisms. This multifactorial scenario leads to progressive disintegration of neural circuits, isolation and loss of neurons, network failure, and neurological decline (reproduced with permission from Science, TBD) [12]. (b) A simplified model of the excessive $A\beta$ production leading to Alzheimer's disease (AD). Normally, the β -amyloid precursor protein (APP) is metabolized by at least three proteases (α -, β -, and γ -secretases). $A\beta$ peptides are produced by β -secretase, forming the N-terminus, and by γ -secretase, forming the C-terminus of $A\beta$. The fibrillar aggregates of amyloid peptides, $A\beta$ 40 and $A\beta$ 42, are the major constituent of senile plaques in AD patients. The $A\beta$ aggregates are believed to be responsible for initiating a cascade of events leading to neurotoxicity and AD [13–15]

2.1 Amyloid (A β) Plaque Imaging Agents

Development of PET imaging agents targeting A β aggregates in the living human brain has been a research topic for many studies and reviews [18–22]. An essential prerequisite for a successful imaging agent for A β plaques in the brain is the ability to rapidly penetrate the intact blood–brain barrier. A small and moderately lipophilic PET agent exhibiting an excellent initial brain uptake is an imperative requirement. Not only should the agent have a high initial brain uptake, but also have fast washout kinetics from the normal brain. The specific to nonspecific binding of the PET signal will be critical for quantitative determination of A β plaques in the brain [23–26]. These are highly desirable properties for a useful brain-imaging agent targeting A β plaques. The development of diagnostic imaging agents targeting A β aggregates may lead to improved selection and monitoring of patients undergoing drug treatment designed to reverse the build-up of A β plaque in the brain [16].

Thousands of compounds have been prepared and tested for imaging A β aggregates. However, the clinically validated and FDA-approved A β plaque-targeting PET imaging agents can generally be classified into two main groups: stilbenes and benzothiazoles (Fig. 3a, b, respectively). Chemical structures of benzothiazoles (PIB analogs [19]) and stilbenes (SB-13 analogs [21]) are similar; both are highly conjugated aromatic and planar molecules – an important attribute for insertion between the β -sheets of A β aggregates. Both types of molecules contain a common electron donating group (*N*-methylamine) at the end of the benzene ring [27], and they compete for similar binding sites on the A β aggregates [19–22]. It is important to note that these PET imaging agents for A β aggregates not only have fulfilled the

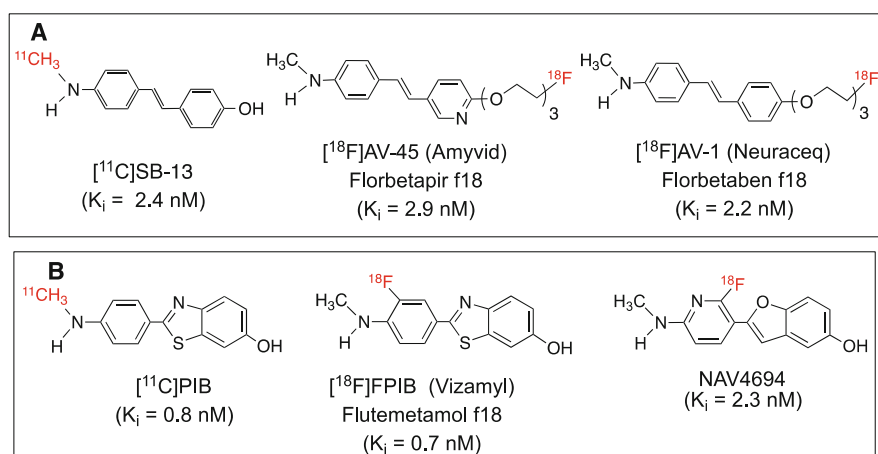


Fig. 3 Chemical structures of ^{11}C and ^{18}F labeled amyloid imaging agents. All of these agents have been successfully tested in humans. The FDA approved the commercial use of florbetapir f18 (AmyvidTM) in 2012, flutemetamol f18 (VizamylTM) in 2013, and florbetaben f18 (NeuraCeqTM) in 2014; the FDA approval of NAV4694 is still pending

basic requirements as listed above, they also have been extensively tested in living AD human brain. Postmortem studies have confirmed that the extent of A β aggregate accumulation in the brain (measured by pathological examination – plaque counting) correlated with the in vivo PET images. This is a very high criterion. Because PET imaging prior to death and the subsequent postmortem AD human brain studies involving hundreds of patients is very expensive, such studies could only be carried out by commercial companies with the intention to submit a new drug application (NDA).

In 2001, early reports on radiolabeled stilbenes (Fig. 3a) showed high binding affinity and selectivity towards A β aggregates, and the autoradiography of postmortem human AD brain sections confirmed the binding [27]. From these stilbenes, [^{11}C]SB-13 was selected and successfully tested in normal controls and AD patients [28]. Two successive F-18 labeled agents, NeuraCeqTM (florbetaben f18, AV-1, developed by Schering/Piramal Health) [29] and AmyvidTM (florbetapir f18, AV-45, developed by Avid/Eli Lilly) [30], were approved by the FDA for commercial use for detecting amyloid accumulation in the brain. The structure–activity relationship for this series of agents has been reviewed [20, 21]. Among the benzothiazole-based agents (Fig. 3b), [^{11}C]PIB is now the agent of choice for C-11 PET imaging, and a wealth of information on its use in AD patients has been reported [31–33]. The benzothiazoles as PET imaging agents for detecting the presence of amyloid accumulations in the brain have been reviewed [19, 22, 23]. FDA has approved the use of VizamyTM (flutemetamol f18, FPIB, developed by GE Health) in detecting A β plaques in the brain. Flutemetamol PET imaging of brain beta-amyloid plaque density improves diagnostic accuracy in cognitively impaired patients [34]. Another benzothiazole-related agent, NAV4694 (originally as AZ4694, developed by AstraZeneca/Navidea), was also comprehensively tested in humans [35, 36], and it is likely that NDA submission to the FDA is pending.

In summary, four F-18 PET imaging agents targeting A β aggregates have been studied in humans: florbetapir f18 (AmyvidTM), florbetaben f18 (NeuraCeqTM), flutemetamol f18 (VizamyTM), and NAV4694 (Fig. 3). Results of phase 3 clinical studies of these agents demonstrated a close correlation of in vivo amyloid PET imaging with postmortem histopathological findings. The FDA has approved three agents for routine clinical uses, and the approval of the fourth agent, NAV4694, is still pending.

2.2 Potential Applications of Imaging Plaques in the Brain

Clinically, β -amyloid imaging will be important for the differential diagnosis of dementia. A negative scan will indicate that dementia is not related to AD. A positive scan will imply a higher risk for AD. It is also important to detect preclinical AD and predict the progression from mild cognitive impairment (MCI) to AD, thereby establishing a temporal correlation between the β -amyloid imaging and neurological status. Early monitoring of patients with genetic or

family risk factors will also be highly desirable and reports of these type of early monitoring are coming out [37]. One of the significant considerations is for ApoE4-positive subjects. It has been demonstrated that ApoE4 carriers will show early development of positive scans and progression of plaque-build-up. A β plaque-positive patients even without memory deficit are likely at risk for AD [38]. PET imaging studies in patients with risk factors suggest that brain amyloid deposition is bad news, though it can take years until cognitive consequences emerge, and the initial cognitive decrements may be subtle.

Many treatments for AD have been aimed at stopping the formation of, or reversing, the A β aggregates. The two major approaches of the antiplaque treatments have been the inhibition of β - or γ -secretase (reducing A β production) and immunotherapy (removing A β from the brain). The past few years have witnessed a series of failures in AD drug development [39–41]. Antiplaque treatments based on the inhibition of γ - and β -secretase (also known as BACE) by small molecules have had difficulty meeting clinical endpoints. However, active efforts in this area of drug development are ongoing [42–46]. Negative clinical results have also been reported for the anti-amyloid antibodies solanezumab (Eli Lilly) and bapineuzumab (Pfizer), two leading immunotherapy drugs designed to remove A β in the brain. Additional trials are currently ongoing to further explore the potential benefits. The NIH has recently announced the selection of crenezumab, an antibody by Genentech, as the first therapeutic drug to be evaluated in a preventative clinical trial. Some recently developed amyloid plaque imaging agents, particularly Amyvid™, may be useful to select patients, at the early phase of amyloid plaque development.

Human amyloid imaging will be useful for evaluation of risk factors associated with Alzheimer's disease. Although this imaging procedure on determining the presence or the absence of A β plaques (or Tau, see Sect. 3 in this chapter) in the brain may prove to be tremendously valuable once it is incorporated into the routine clinical work-up of dementia patients, A β plaque imaging is not without its critics. The debate on the utility of human amyloid imaging may be just a part of the validation process for this new technology. The amyloid-specific imaging will provide a new tool in selecting and monitoring patients enrolled in clinical trials for AD drugs specifically aiming to reduce A β -amyloid in the brain. Correlation of plaque load vs PET imaging has been established via phase III clinical studies of three FDA-approved imaging agents, florbetapir f18 (Amyvid™), flutemetamol f18 (Vizamyl™), and florbetaben f18 (NeuraCeq™) [30, 34, 47]. Plaque load was not directly correlated with AD symptom, only a reflection of a risk factor. Using PET A β plaque imaging for selection of patients for drug trial evaluation of the effectiveness of anti-A β plaque treatment starts with selecting suitable AD patients with A β plaques.

3 Tau Imaging Agents

3.1 *Tau Aggregates as a Risk Factor for Developing Alzheimer's Disease*

The presence of tau aggregates in the brain is a major risk factor for the development of AD [48–50]. Normally, tau proteins serve several essential functions inside neurons, including promoting the assembly of tubulin into microtubules and the stabilization of their structure. Tau is mainly found in the axons of the CNS and is comprised of six isoforms generated by alternative splicing; the longest of them has 441 amino acids. There are three (3R tau) or four (4R tau) microtubule-binding repeats. The hyperphosphorylation of the tau protein at several serine, threonine, and tyrosine sites usually precedes both conformational changes and cleavage of the protein. Tau and mutated tau contain many potential cleavage positions vulnerable to multiple proteases, leading to breakdown products that are believed to be toxic and more susceptible to aggregation. Other biochemical modifications of tau proteins are oxidation, ubiquitination, O-GlcNAc glycosylation, and acetylation, the last of which impairs microtubule binding of tau, promotes aggregation, and potentially interferes with ubiquitination that leads to the degradation of tau. Recent reports suggest that the intracellular transport of the tau protein is consistent with tau protein diffusion along the microtubule lattice [17, 51, 52]. In addition to Alzheimer's disease, the aggregation of tau is also a presumed cause of several diseases known as tauopathies [53–55]. These tauopathies are heterogeneous, with spatiotemporal differences in affected brain areas and also differences in the spliced variants of aggregated tau as “paired helical filaments” (PHFs) [56]. The presence of tau aggregates in the absence of deposits of A β peptides (“tau only” brain) is seen in several tauopathies, such as Pick's disease, progressive supranuclear palsy (PSP), and corticobasal degeneration. Frontotemporal lobar degeneration (FTD) is the most common form of tauopathies.

Various approaches to the development of tau therapeutics have also been reported [57, 58]. However, similar to anti-amyloid treatment, it will be essential to select patients specifically for tau aggregates in the brain. This approach is further complicated by the presence of various forms of modified or mutated tau aggregates, which may contain different binding sites. Heterogeneous binding sites would create a challenging problem for the development of PET imaging agents for tau aggregates.

3.2 *Imaging Agents Tested for Tau Aggregates in Alzheimer's Disease*

Because the accumulation of tau NFTs occurs in AD, and the deposition of Tau aggregates correlates well with the progression of symptoms (cognitive

impairment) in AD patients [49, 50, 59], there is an urgent need for tau-specific probes for detecting and monitoring the progression of AD. There have been many attempts to develop tau-specific imaging agents, mainly for PET imaging.

3.2.1 FDDNP

The first notable agent was [^{18}F]FDDNP (Fig. 4) [60–62]. FDDNP is a PET imaging probe with binding affinity for “amyloid and amyloid-like structures.” It was produced for in vivo brain imaging of patients with AD and tauopathies (see discussion below) [60, 63, 64]. It was reported that this agent is useful for binding to both A β plaques and tau. The signal-to-noise ratios in humans were reported to be distinctively related to A β plaques and tau. The multiple binding sites for this probe make the interpretation of PET imaging highly puzzling – binding to both A β plaques and tau in the brain regions may not be easily and simultaneously validated (Fig. 4).

3.2.2 5H-pyrido[4,3-b]indoles (T807/AV1451 and T808) as Tau Imaging Agents

The Siemens molecular imaging group, which has reported a series of tau imaging probes (such as T807 and T808, Fig. 5), appears to have made the most progress in this field [65]. In the past 10 years, multiple patent applications have appeared in the literature. T807 and T808, probes for tau imaging, have been sold to Avid/Lilly, and T807 is now designated as AV1451 [65–69]. The leading candidate for tau imaging is ^{18}F -AV1451/T807 with a core structure of 5H-pyrido[4,3-b]indoles (Fig. 5). In in vitro binding assays, using tau-enriched AD brain sections with autoradiography, the K_d for AV1451/T807 was 14.6 nM and A β aggregate binding was $>2,000$ nM [71]; therefore, the selectivity for tau binding is excellent. Human imaging studies of this leading tau imaging candidate have been reported [66, 68, 71]. ^{18}F -AV1451/T807 PET imaging could be valuable as a biomarker that reflects both the progression of AD tauopathy and the emergence of clinical impairment [68]. Preliminary

Fig. 4 FDDNP as a tau imaging agent

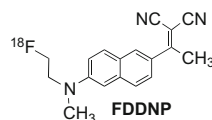
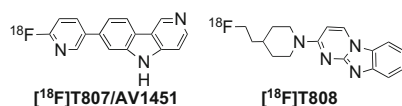


Fig. 5 T807/AV1451 and T808 as tau imaging agents

Siemens'/Avid/Lilly compounds



results of regional and substrate-specific autoradiographic and in vitro binding patterns of ^{18}F -AV1451/T807 in postmortem human brain sections suggested that it might be a useful surrogate marker for detection of tau in living human brain [72].

3.2.3 PBB3 as Tau Imaging Agents

Previously, a series of 2-ethenyl-benzoxazole derivatives (BF compounds; Fig. 6) was reported as A β and tau imaging agents. However, the binding selectivity of the series of probes was not optimal, as the affinity for tau fibrils was similar to that of A β fibrils [73–75]. Recent publications of using ^{11}C -PBB3 for imaging tau suggest that it may serve as a biomarker for detecting tau in the brain [76, 77]. Despite its brilliant fluorescent signal, the drawbacks of PBB3 are related to its low brain uptake after an i.v. injection. Although new PET image analysis methods have been developed for this probe, the low uptake in the brain makes quantification much harder to accomplish [70, 76, 78].

3.2.4 Quinolines (THK Series) as Tau Imaging Agents

More recently, quinoline-based probes (Fig. 7) for imaging tau pathology have been reported (from the research group of Tohoku University) [82–84]. It was reported that [^{18}F]THK-5105 appeared to show a higher affinity for tau fibrils than for A β aggregates (K_d for tau fibrils 1.99 nM vs 30.3 nM for A β aggregates). In vitro autoradiography studies of postmortem brain samples suggested different

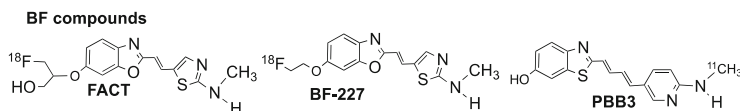


Fig. 6 BF series of compounds as tau imaging agents. Among these agents, [^{11}C]PBB3 has been evaluated in humans [70]

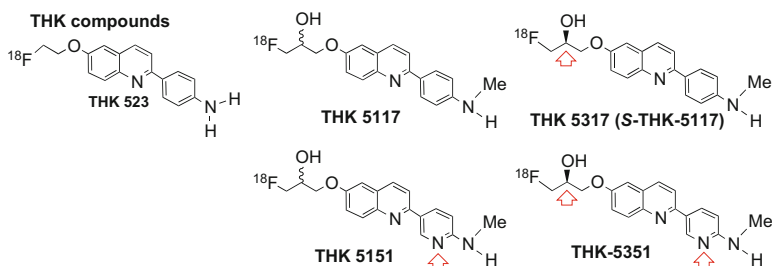


Fig. 7 THK compounds as tau imaging agents. It is important to note that THK5117 (phenyl) and THK5151 (pyridinyl) are two core similar structures for tau binding; their *S*-isomers, THK5317 [79] and THK5351 [80], both displayed high binding affinity and selectivity [81]

distribution patterns between THK-523 and PIB, showing different binding between A β and tau. These results suggest that the THK ligands are reasonable candidates for testing in humans [85]. The *S*-isomers of THK-5117, called THK-5317 (Fig. 7), were prepared and displayed better target-to-nontarget ratios [80, 82]. Human PET imaging displayed high selective binding to tau [80]. In a related effort, a stereo-selective quinolone-pyridyl derivative, ^{18}F -THK5351 (the *S*-isomer of THK5151), was prepared and tested in humans [79, 81]. The main difference in uptake and kinetics between THK-5317 and THK-5351 is likely due to the additional nitrogen atom (pyridyl), which makes the agent less lipophilic. Therefore, the in vivo kinetics in the brain were faster and provided a good selective tau signal [81].

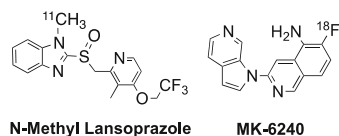
3.2.5 Other Tau Imaging Agents

As mentioned above, there are likely multiple mutated variants of tau aggregates and different binding sites. It is likely that many other tau imaging agents targeting different sites could be developed.

It was reported that lansoprazole and astemizole (Fig. 8) have nanomolar affinity for certain forms of tau and selectivity for tau over A β plaques [86]. These agents are normally for the treatment of allergies and gastrointestinal disorders, respectively. Recently, ^{11}C and ^{18}F labeled *N*-methyl lansoprazole have been reported as tau imaging agents [87, 88]. In vivo studies suggested a distinctive regional brain distribution different from that of A β plaques, but the binding did not overlap with the other tau agents listed above. Additional tau targeting probe, MK-6240, was recently reported from Merck [89], displaying very high affinity ($K_i = 0.36 \pm 0.8$ nM) and selectivity in NFT-rich AD brain homogenates. In vitro autoradiography also showed regional AD brain distribution consistent with known tau (NFT) burden.

A recent publication evaluating binding sites for tau imaging agents [90] suggested that there are two distinctive binding pockets for [^{18}F]THK-523 and [^{18}F]T807 (AV1451) using AD human brain homogenates. It is possible that we are entering a new phase of developing PET imaging agent specifically targeting tau aggregates. Many different binding sites may be available within the β sheet structure of tau aggregates, and the binding of each agent may show different preferences to different pockets.

Fig. 8 Other tau imaging agents: *N*-methyl-lansoprazole and MK-6240



3.3 *Summary for Tau Imaging Agents*

It is generally believed that tau pathology is closely linked to symptoms and progression of AD, and there is a strong interest in measuring tau deposition by PET imaging in a quantitative manner. Several useful Tau imaging agents have been tested in humans; however, they have not been fully validated as those of A β imaging agents. Correlation of tau deposition vs. PET images may require additional systematic studies, perhaps by a series of brain autopsies of a full range of AD patients, to fully demonstrate the effectiveness of monitoring tau accumulation in patients by PET imaging.

4 **Conclusions and Perspective**

Currently, there are three FDA-approved imaging agents (Amyvid, NeuraCeq, and Vizamyl) available for mapping A β in the brain of patients suspected of having Alzheimer's disease. Although the A β imaging in the brain is available, it has not yet achieved widespread use due to a limited Medicare reimbursement. When successful treatments of Alzheimer's disease are developed, its use may increase in the future. More and more tau targeting agents for PET imaging have been developed and clinical studies have generated exciting data suggesting the importance of tau in the disease process. However, there is still uncertainty with the correlation of PET imaging signal vs the presence of tau (or more precisely which version of mutated tau aggregates). Off-target signals also make the interpretation of the brain images difficult. It is likely that clinical studies correlating the tau signal with postmortem human brain sections will be part of the answers. The build-up of A β and tau and their relationship with disease process will likely be elucidated by PET imaging. The information will also play an important role in development of drug treatments.

In addition to the imaging agents based on A β and tau, many other PET imaging agents have been proposed for studying the brain function of AD patients, such as agents for neuroinflammation, nicotinic receptors, serotonin receptors, etc. So far none has gone through validation steps which are necessary to correlate the *in vivo* binding and the level of binding sites in connection with the disease status. Further work will be needed in order to improve tau binding and selectivity at various binding pocket sites.

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