

Giuliano Ciarimboli · Sophie Gautron
Eberhard Schlatter *Editors*

Organic Cation Transporters

Integration of Physiology, Pathology,
and Pharmacology

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This book is dedicated to the memory of Karl Julius Ullrich (1925–2010), former head of the Department of Physiology at the Max Planck Institute of Biophysics in Frankfurt am Main, Germany. Professor Ullrich was one of the founders of the renal transport physiology, especially with regard to the transport of organic anions and cations. Ullrich's department stood out as a beacon of transport physiology. Many of his scholars were able to further increase knowledge of transport physiology. In this way, we would like to express our admiration and gratitude for his outstanding work.

Preface

Transport across the cell membrane is essential for vital processes like entry of nutrients into the intracellular compartment, delivery of cellular products to extracellular and intracellular destinations, and handling of metabolism waste products and toxic substances and is necessary to keep the intracellular milieu constant. Transport across cell membranes is mediated by a variety of different transport proteins. This book focusses on transporters for organic cations, which are not directly energy-dependent, such as organic cation transporters (OCTs), organic zwitterions/cation transporters (OCTNs), and multidrug extrusion proteins (MATEs). Because these transporters are polyspecific, they accept many different substrates of endogenous (e.g. choline, acetylcholine, histamine, and monoamine neurotransmitters) as well as of exogenous (e.g. drugs like metformin, quinine, cimetidine, and cisplatin) origin.

Since the cloning of the first transporter for organic cations (rOCT1) in 1994, profound understanding of their structure, transport properties, and regulation has been obtained. In organs expressing these transporters at high levels, such as the intestine, liver, and kidney, transporters for organic cations play a pivotal role not only in absorption and in excretion of xenobiotics but also in their accumulation and toxicity. However, their expression is not restricted to organs typically involved in the transport of xenobiotics, but is found also in other tissues, such as the brain and reproductive organs. Recent studies with genetically modified animals have helped to unveil novel physiological, pathophysiological, and pharmacological roles of transporters for organic cations. While there is no doubt about the pharmacological and toxicological implications of transporters for organic cations for the organism, their physiological functions had remained largely elusive. Moreover, gender- and species-specific differences in the expression and properties of these transporters as well as the role of single nucleotide polymorphisms on their function have become a focus of attention in physiology, pathophysiology, and medical care.

This book presents current knowledge on the expression, physiological functions (see Chap. 1 by G. Ciarimboli), and regulation (see Chap. 5 by E. Schlatter and Chap. 6 by L.M. Aleksunes) of transporters for organic cations in various organs, on

their gender and species dependencies (see Chap. 9 by I. Sabolić, D. Breljak, and T. Smital), and on their role in pathophysiological situations. This overview should be of high interest for researchers and students in various areas of integrative, organ, cell, and molecular physiology and will contribute to delineate an integrative physiological interpretation of transporter function.

Another important aspect of the book is that it conjugates integrative transporter physiology with structural and molecular biology (see Chap. 2 by H. Koepsell and T. Keller), genetics (see Chap. 4 by M.V. Tzvetkov, N. Dalila, and F. Faltraco), pharmacology, and pathophysiology (see Chap. 3 by K. Inui and H. Motohashi and Chap. 8 by K. Tieu), offering an integration of the knowledge in these fields. The different chapters of the book present the state of the art of the research in these different fields. For this reason, the book addresses both expert readers and readers with a more general interest in understanding transporter function in physiology and pathophysiology. Hence, the book should also attract people interested in adaptive mechanisms of the organism to conditions, such as salt intake, anxiety, and stress (see Chap. 7 by A. Orrico and S. Gautron).

Since up to 40 % of the prescribed drugs are organic cations, this book will provide important information on the involvement of transporters for organic cations in determining specific effects but also side effects induced by particular drugs, offering new approaches for a successful translation from physiology to clinical therapy. Finally, because of the expression of transporters for organic cations in plants, the role of these transporters for the environmental cycling of pharmaceutical residues is also presented (see Chap. 10 by T. Eggen and C. Lillo).

In conclusion, we think that a book concentrating on the latest developments of integrative, organ, cell, and molecular aspects of function of transporters for organic cations will furnish an optimal platform to integrate the knowledge on these transporters and obtain a more comprehensive physiological understanding of their function.

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GC would like to thank Frank Thevenod for the support in organizing the symposium on organic cation transporters, whose idea came up when visiting together with him.

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Eberhard Schlatter is Full Professor at the faculty of medicine of the University of Münster, Germany. He studied Biology at the University of Hannover, Germany. In 1981, he received a Ph.D. in Biology at the University of Hannover, Germany. In 1989, he habilitated in Physiology at the University of Freiburg, Germany. In 1993, Eberhard Schlatter was appointed Full Professor for Experimental Nephrology at the Department of Nephrology and Hypertension of the University Clinic Münster, Germany.

His main research interests since then are focused on renal effects of cGMP activating peptide hormones, rejection processes in experimental kidney transplantation, and, most recently, characteristics of organic cation transporters, their interaction with drugs, and their role in drug toxicities.

Dr. Sophie Gautron is a permanent researcher at the Inserm (Institut National de la Santé et de la Recherche Médicale). She holds a PhD degree in Molecular and Cellular Genetics from Pierre et Marie Curie University and an HDR degree from Paris-Est Créteil University. She is appointed at the Neuroscience Paris Seine laboratory (CNRS UMR8246/Inserm U1130/UPMC UMCR18) in Paris where she co-leads of the “Physiopathology of Psychiatric Diseases” Team. Her recent work focuses on the function of low-affinity monoamine transporters in the central nervous system, with a particular interest in psychiatric disorders such as depression and addiction in preclinical models.

Chapter 1

Introduction to the Cellular Transport of Organic Cations

Giuliano Ciarimboli

Abstract Organic cations (OCs) are substances of endogenous and exogenous origin to which belong important neurotransmitters such as histamine and serotonin and also drugs such as metformin. Because OCs are positively charged they need membrane transporters to permeate the plasma membrane. Membrane transporters which translocate OCs according to their electrochemical gradient belong to the Solute Carrier (SLC) families 22 (organic cation transporters (OCT) 1–3, and organic cation transporters novel (OCTN) 1–2) and 47 (multidrug and toxin extrusion (MATE) 1–2). This chapter collects the information on expression and function of these transporters present in the literature, comparing the characteristics of human and rodent transporters. These data show that OCTs play an important physiological role for neurotransmitter balance in the body. Moreover, they are also important uptake routes for intracellular drug delivery and, considering their high expression in excretory organs, together with MATEs are responsible for drug excretion. For this reason, OCTs and MATEs can be important determinants of drug efficacies and also toxicities. OCTNs are transporters involved in the cellular uptake of substances, which are important in cell metabolism and in signal transmission, such as ergothioneine, carnitine and acetylcholine. Even though the expression and function of orthologs of transporters for OCs is generally similar, still there are important differences that have to be considered for a proper interpretation of translational studies. Paralogs of transporters for organic cations often display similar characteristics, however they show also important differences e.g. with regard to interaction with substrates and to regulation. Other important functional aspects of transporters for organic cations, such as the molecular correlates of polyspecificity, regulation, interaction with drugs, genetic variations, role in the central nervous system, and distribution in the plants are discussed in the other sections of this book.

Keywords Organic cations • Transporters • Neurotransmitters • Drugs • Plasma membrane

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Introduction

The development of a plasma membrane was a fundamental step in the evolution of the cell, because it allowed the separation of an internal milieu from the external environment, which is of special importance to protect the genetic material. However, this important evolutionary progress created new challenges, because now the cell had to find solutions able to guarantee the entry of all essential nutrients into the cytoplasmatic compartment, the distribution of cellular products such as proteins, complex carbohydrates and lipids into and beyond the plasma membrane, and the handling of waste products and toxic substances, processes aimed at keeping the intracellular milieu constant [1]. The solution of these problems was the development of specialized transport systems of proteinic nature (transporters) embedded in the plasma membrane. Thus, it is evident that transporters are essential to sustain life and adaptation to changes in the environment. Their malfunction can result in diseases and, therefore, they are target of therapeutic intervention. Some transporters are also responsible for efficacy and also dangerous side-effects of chemotherapy [2, 3].

A total of 40,678 transport proteins classified into 134 families were predicted by whole-genome transporter analysis of 141 species, including 115 Eubacteria, 17 Archaea and 9 Eukaryota [4]. Eukaryotic cells, especially those of multicellular eukaryotic organisms, express the largest total number of transporters, which display a high number of paralogs generated by gene duplication or expansion within certain transporter families. The formation of paralogs is a sign of specialization, since closely related paralog transporters become expressed in specific tissues or at specific subcellular localisation and developmental time points [4].

Based on mode of transport and energy-coupling source, molecular phylogeny, and substrate specificity, there are five main recognised classes of transporters: pores and channels, electrochemical-potential-driven transporters, primary active transporters, group translocators, and transmembrane electron carriers ([1], <http://www.tcdb.org>). Each transporter category is further classified into individual families and subfamilies (Table 1.1).

This book focuses on transporters for organic cations, which are not directly ATP dependent and mediate the substrate movement through the plasma membrane according to the electrochemical gradient. According to the “*Transporter Classification Database*” (<http://www.tcdb.org>), these transporters belong to the family 2, subfamily 2.A (Table 1.1). Here a special attention will be paid at organic cation transporters (OCTs), novel organic cation transporters (OCTNs), and multi-drug and toxin extrusion transporters (MATEs).

Basing on the amino acid sequences, the Human Genome Organisation (HUGO), classified human transporters in 54 Solute Carrier (SLC) families (a transporter has been assigned to a specific family if it has at least 20–25 % amino acid sequence identity to other members of that family [5]). These SLC families comprise 386 different SLC human transporters [6], additional new members being identified constantly [5].

Table 1.1 Transporter classification (classes and subclasses) according to the International Union of Biochemistry and Molecular Biology (<http://www.tcd.org>)

1. Pores and channels	1.A α -Helical channels
	1.B β -Strand porins
	1.C Pore-forming toxins
	1.D Non-ribosomally synthesized channels
	1.E Holins
These proteins catalyze facilitated diffusion by passage through a transmembrane aqueous pore or channel. They do not exhibit stereospecificity but may be specific for a particular molecular species or class of molecules	
2. Electrochemical-potential-driven transporters	2.A Transporters or carriers (uniporters, symporters and antiporters)
	2.B Non-ribosomally synthesized transporters
These transporters utilize a carrier-mediated process not directly linked to a form of energy other than chemiosmotic energy to catalyze uniport (a single species is transported by facilitated diffusion), antiport (two or more species are transported in opposite directions) and/or symport (two or more species are transported together in the same direction)	
3. Primary active transporters	3.A P-P-bond-hydrolysis-driven transporters
	3.B Decarboxylation-driven transporters
	3.C Methyltransfer-driven transporters
	3.D Oxidoreduction-driven transporters
	3.E Light-driven transporters
These transporters use a primary source of energy (chemical, electrical and solar) to drive active transport of a solute against a concentration gradient	
4. Group translocators	4.A Phosphotransferases
Transport systems of the bacterial phosphoenolpyruvate: sugar phosphotransferase system. The product of the reaction, derived from extracellular sugar, is a cytoplasmic sugar-phosphate. The enzymatic constituents, catalyzing sugar phosphorylation, are superimposed on the transport process in a tightly coupled process	
5. Transmembrane electron carriers	5.A Two-Electron Carriers
	5.B One-Electron Carriers
Systems that catalyze electron flow across a biological membrane, from donors localized to one side of the membrane to acceptors localized on the other side. These systems contribute to or subtract from the membrane potential, depending on the direction of electron flow. They are therefore important to cellular energetics	

According to this classification, OCTs and OCTNs belong to the SLC22 and MATEs to the SLC47 family (Table 1.2). The HUGO nomenclature system is also informally used with lowercase letters for rodents and this notation has been also extended to the spelling of protein (e.g., *Slc22a1* and Oct1 denote the rodent orthologs of the human *SLC22A1* gene and hOCT1 protein, respectively).

Many of the SLC families present in *H. sapiens* (among these also the SLC22 family) are highly evolutionary conserved in Bilaterian species [7]; moreover, the high representation of the SLC22 family in the plant *Arabidopsis thaliana*, suggests that it has an ancient origin [7]. More information about transporters for organic cations in plants will be presented in the Chap. 10 by T. Eggen and C. Lillo in this book.

Table 1.2 The SLC22A and SLC47A families

Gene name	Gene locus	Protein name	Function
<i>SLC22A1</i>	6q25.3	hOCT1	Electrogenic cation transport
<i>SLC22A2</i>	6q25.3	hOCT2	
<i>SLC22A3</i>	6q25.3	hOCT3	
<i>SLC22A4</i>	5q23.3	hOCTN1	Carnitine and cation transport
<i>SLC22A5</i>	5q23.3	hOCTN2/CT1	
<i>SLC22A16</i>	6q21	hCT2/hFLIPT2/hOCT6	
<i>SLC22A6</i>	11q12.3	hOAT1	Anion transport
<i>SLC22A7</i>	6q21.1	hOAT2	
<i>SLC22A8</i>	11q12.3	hOAT3	
<i>SLC22A9</i>	11q12.3	hOAT7	
<i>SLC22A11</i>	11q13.1	hOAT4	
<i>SLC22A12</i>	11q13.1	hURAT1	
<i>SLC22A13</i>	3p22.2	hOAT10	
<i>SLC22A20</i>	11q13.1	hOAT6	
<i>SLC22A10</i>	11q12.3	hOAT5	
<i>SLC22A14</i>	3p22.2	OCTL2/hORCTL4	
<i>SLC22A15</i>	1p13.1	FLIPT1	
<i>SLC22A17</i>	14q11.2	BOIT/BOCT	
<i>SLC22A18</i>	11p15.5	TSSC5/hORCTL2	Predominant substrates not yet determined
<i>SLC22A23</i>	6p25.2		
<i>SLC22A24</i>	11q12.3		
<i>SLC22A25</i>	11q12.3	UST6	
<i>SLC22A31</i>	16q24.3		
<i>SLC47A1</i>	17p11.2	hMATE1	H ⁺ -coupled electroneutral exchange of organic cations
<i>SLC47A2</i>	17p11.2	hMATE2	

The transporters presented in this book are in bold characters

Substrates of Transporters for Organic Cations

The substrates of the three types of transporters for organic cations discussed in this book (OCTs, OCTNs, MATEs) are mainly organic cations, even though also inorganic substances such as Cd²⁺ [8] and cisplatin [9, 10] have been demonstrated to be accepted as substrate by some of these transporters. Moreover, some of these proteins can transport also zwitterions such as L-carnitine [11, 12] (OCTNs) and cephalexin and cephradine [13] (human MATE1, hMATE1) and anionic substances such as estrone sulphate (hMATE1, [13]), acyclovir, and ganciclovir (hOCT1 and hMATE, [13, 14]).

Organic cations (OCs) can derive from endogenous and also exogenous sources. Endogenous OCs are important neurotransmitters such as histamine, serotonin and dopamine [15] and polyamines such as putrescine and spermidine [16], which have an important function in many cellular processes such as DNA stabilization,

regulation of ion channel activity, gene expression, and cell proliferation [17]. In general, neurotransmitters and polyamines seem to be low affinity substrates of transporters for OCs, underlying the importance of these transport systems in places, where the concentration of such substances is high. Exogenous OCs are drugs (up to 40 % of the prescribed drugs are OCs [18]), xenobiotics such as the herbicide paraquat and the DNA intercalating agent ethidium bromide [19, 20], and also several natural contents of fungi, fruits and vegetables. Of practical experimental interest are fluorescent OCs such as 4(4-dimethylaminostyryl)-*N*-methylpyridinium (ASP⁺), and rhodamine 123, which are substrates for several transporters for OCs and are therefore useful for investigating transporter activity [21–24].

OCs are also classified as type I and type II OCs depending on their chemical structure. Type I OCs are small (below 500 Da), strongly hydrophilic cations, such as tetraethylammonium (TEA⁺) and 1-methyl-4-phenylpyridinium (MPP⁺), while Type II OCs are large, more hydrophobic and mostly polyvalent substances, such as D-tubocurarine and quinine [25].

Even though many substrates are common between OCTs, OCTNs, and MATEs, every single transporter has a specific interaction spectrum with the substrates and inhibitors. For example, TEA⁺ is a substrate for OCT1 and OCT2 [26], but not for OCT3 [15]. Some substances are known to bind to, but not to be transported by these transporters, as for example shown for proton pump inhibitors [27].

From this brief description it is evident why these transporters are called poly-specific. The translational relevance of studies on OCs with laboratory animals should be cautiously inferred, since rodent and human transporter orthologs can differ in substrate specificity, tissue expression [28] and also regulation (see Chap. 5 by E. Schlatter of this book), even though the global substrate preference of the SLC22 family seems to be conserved over a long evolutionary time [7].

Integration of OC Transport

Since many transporters for OCs are expressed in liver and kidney, they play a pivotal role in drug and xenobiotic absorption and excretion [29]. In these organs, SLC22A and SLC47 transporters are expressed in hepatocytes and renal proximal tubules cells, which are highly polarized cells, and mediate the coordinated movement of OCs across the cell by a concerted activity, mainly resulting in excretion of OCs into bile or urine. The first step for hepatic and renal OC secretion is their absorption from the basolateral side into the cells. While in human kidney this process is mainly mediated by OCT2 (Fig. 1.1), in rodent kidney it is supported by Oct1 and Oct2. OCT3 shows only a tiny expression in the basolateral membrane of proximal tubule cells, and for this reason is probably less important than OCT2 under normal conditions. OCs are secreted in a second step from the tubular cell into the tubular lumen. In the kidney this process is mediated by different transporters: the Na⁺-carnitine cotransporter OCTN2, and *P*-glycoprotein (also named MDR1), an ATP-dependent transporter that probably mediates the efflux of bulky

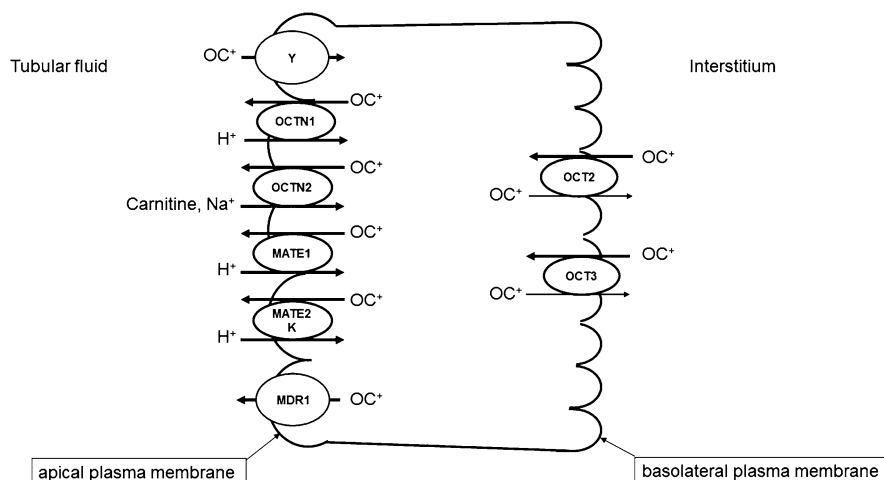


Fig. 1.1 Transport systems for organic cations in human renal proximal tubules. The basolateral uptake of organic cations (OCs) from interstitium is mainly mediated by hOCT2, where there is also a much lower expression of hOCT3. Secretion of OCs into the tubular fluid is mediated by MATE1, MATE2K, and OCTN1 in exchange with H⁺. The necessary H⁺ gradient is substrained by the activity of NH₃, an apically expressed Na⁺/H⁺ exchanger (not shown). Bulky OCs are secreted into the urine under energy consumption by the Multidrug Resistance protein 1 (MDR1). OCs can be also reabsorbed from the tubular fluid by an not yet identified transport system (Y), and then transported into the interstitium by OCT. Modified from Koepsell et al. [30] and Ciarimboli and Schlatter [31]

hydrophobic OCs, and other H⁺/organic cation antiporters (OCTN1, MATE1, and MATE2K in Fig. 1.1). According to their electrochemical gradient, in the kidney OCs can be also reabsorbed from the lumen into the interstitium. For this process, a polyspecific cation transport system mediating their uptake across the luminal membrane of proximal tubular cells has been proposed, but not yet molecularly identified (system Y in Fig. 1.1). The efflux across the basolateral membrane into the interstitium may be mediated by OCTs. The hepatic transport pathways of OCs in humans are illustrated in Fig. 1.2. The uptake of OCs into human hepatocytes is mediated by OCT1 present on the sinusoidal membrane. The extrusion of OCs in the canalicular space is mediated by *P*-glycoprotein (MDR1 in Fig. 1.2) and MATE1.

Genetic Organisation of Transporters for Organic Cations

Some of the *SLC22A* genes (e.g. the genes for OCT1 and 2, OCTN1 and 2, and also OAT1 and 3) are organized in the mouse and in humans as tightly linked pairs [32]. The gene coding for OCT3 is also in close proximity of the *SLC22A1-2* pair, and also *SLC47A1* and *SLC47A2* are adjacent. The gene pairing probably originates

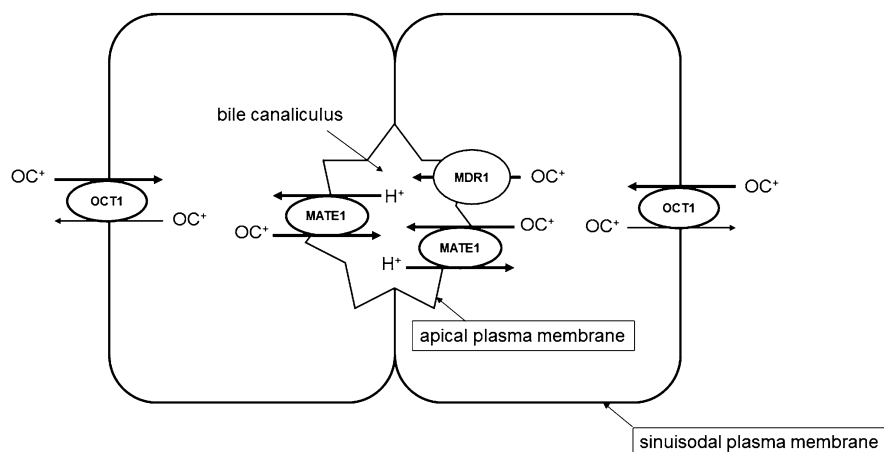


Fig. 1.2 Transport systems for organic cations in human hepatocytes. OCs are transported through the sinusoidal membrane (corresponding to the basolateral side) of hepatocytes by hOCT1. Secretion of OCs into the bile canaliculus is mediated by MATE1 expressed in the apical membrane in exchange with H⁺. Bulky OCs are secreted into the bile under energy consumption by MDR1. Modified from Koepsell et al. [30] and Ciarimboli and Schlatter [31]

from an evolutionary duplication event, aimed at conferring the advantages of redundancy or broader substrate specificity [33].

The genes encoding for Oct1-3 are clustered within a 300-kb genomic region between the insulin-like growth factor receptor 2 (*Igf2r*) and the *Plg* (plasminogen) genes on mouse chromosome 17 and on rat chromosome 1. Also in humans, the genes encoding for OCT1-3 are clustered in a region between the *IGF2R* and the *APO(a)-like* genes on chromosome 6 [34].

Interestingly, expression of *Slc22a2* and *Slc22a3* in mouse placenta is predominantly maternally imprinted, at least till embryonic day 15.5 for *Slc22a3* [35]. Imprinting is an epigenetic modification, which leads to preferential expression of a determined parental allele in somatic cells of the progeny. After evolutionary divergence, imprinting of only 29 transcripts has been conserved in mice and humans [36]. Imprinted genes often have key roles in embryonic development, but also in postnatal functions including energy homeostasis and behaviour [37]. In humans, imprinting of the *SLC22A2* and *SLC22A3* genes in the placenta is not a general phenomenon, but is present only in few subjects with a temporal expression pattern resembling that of the murine genes [38].

Topology of Transporters for Organic Cations

The transporters of the SLC22 family have a similar predicted membrane topology consisting of 12 alpha-helical transmembrane domains (TMDs), a large glycosylated extracellular loop between the first and the second TMD, and a large intracellular

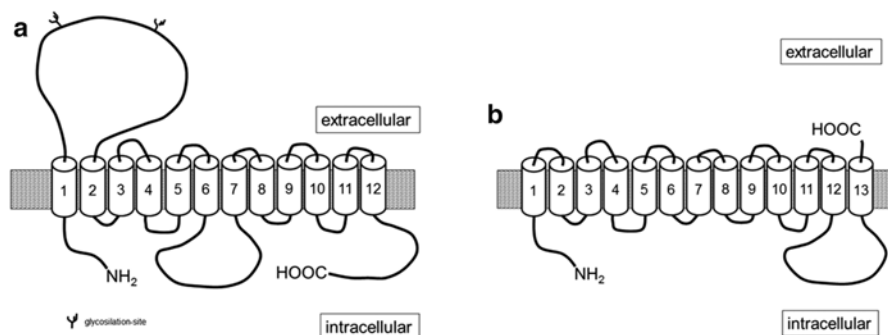


Fig. 1.3 Panel (a) shows the proposed secondary structure of OCTs and OCTNs. These transporters have 12 TMD, a big extracellular and a big intracellular loop with type and subtype specific glycosylation and phosphorylation sites, respectively. Amino- and carboxy-termini are intracellular. Panel (b) shows the proposed secondary structure of MATEs. These transporters have 13 TMD, an intracellular and an extracellular terminus. Modified from Ciarimboli and Schlatter [31]

loop between TMDs 6 and 7 with consensus sequences for phosphorylation (Fig. 1.3a). Both the amino- and carboxy-termini are intracellularly localized.

The topology of SLC47 transporters seems to be somewhat different, as these transporters possess 13 TMDs, an intracellular amino- and an extracellular carboxy-terminus, no glycosylation sites and few intracellularly located putative phosphorylation sites (Fig. 1.3b) [39, 40]. However, there are data demonstrating that the functional core of MATE1 consists of 12 TMDs [41].

In the following the basic information on OCT, OCTN, and MATE present in the literature will be summarized, focussing on human and rodent transporters, which will be separately described, because of the known differences between species.

Organic Cation Transporters (OCTs)

Transport of organic cations by the three OCT subtypes (OCT1, OCT2, and OCT3) is electrogenic, Na⁺- and H⁺-independent and bidirectional [29]. The driving force is supplied exclusively by the electrochemical gradient of the substrate. The first member of the SLC22 transporter family was isolated and identified by expression cloning from rat kidney and was named rat organic cation transporter 1 (rOct1) [42]. In this initial study, it was shown that rOct1 has functional characteristics resembling those of the organic cation transport processes previously described in the basolateral membrane of renal proximal tubule cells and of hepatocytes. Mammalian orthologs of *OCT1* have been cloned also from human [43, 44], rabbit [45], and mouse [46].

Mouse Organic Cation Transporter 1 (mOct1)

The gene *Slc22a1* encodes for a 556 amino acids (aa) protein, which is mainly expressed in the liver and the kidneys [46, 47]. Upstream sequences for *mOct1* contain putative binding motifs for hepatocyte (HNF5 and H-APF-1), and mammary (WAP and MGF) specific expression, and potential binding sites for metallothioneine-regulated gene expression (MBF-1, GR-MT-IIA, and AP-2) [48]. *Slc22a1* transcripts have been shown to turn up in the mouse kidney at midgestation, at the time when the proximal tubules begin to differentiate, and to increase gradually in the course of nephron maturation. *Slc22a1* transcripts are also transiently expressed in other tissues than the kidneys such as the ascending aorta and the atrium [49]. In the liver, ontogenic expression data showed that *Oct1-3* approach adult expression levels at an age of about 3 weeks [50]. The highest hepatic Oct1 mRNA labelling intensity was detected in the hepatocytes which are localized in the proximity of the vena centralis, while in the kidney Oct1 mRNA appeared to be unevenly distributed throughout the renal cortex but not in glomeruli [51]. The mOct1 has been found to be higher expressed than mOct2 and mOct3 in the S1, S2, and S3 segments of the proximal tubules (relative mRNA expression of Oct1/Oct2/Oct3: 1/0.3/0.01) [52]. Expression and function of mOct1 has been detected also in other organs: in the luminal blood-retina barrier [53] Oct1 and Oct2 have been found to be expressed in an age-dependent manner (with decreased expression in aged mice [54]) in endothelial cells of mouse brain microvessels (BMVs). Elevated Oct1 mRNA levels were measured in mammary glands of lactating mice, suggesting that this transporter may be involved in the transfer of drugs into milk [55].

Generally, when expressed in polarized cells, such as hepatocytes and proximal tubule cells, mOct1 localizes to the basolateral plasma membrane [56]. However, in enterocytes this transporter has been shown to be expressed on the apical plasma membrane [57].

The transport mediated by mOct1 has been demonstrated to be pH- and Na⁺-independent and potential dependent [58]. In mice, Oct1 and Oct2 have been identified also in the respiratory epithelium, where they seem to be involved in the acetylcholine (ACh) release [59]. Interestingly, Oct1 and Oct3 have been also found to be expressed in mouse urothelium, where they may mediate ACh secretion [60]. Transport studies showed that the mOct1 mediates the uptake of choline with a K_m of 42 μ M [61] and the low-affinity transport of serotonin [51]. Moreover, mOct1 accepts also exogenous OCs such as [¹⁴C]-TEA⁺ and MPP⁺ as substrates (K_m =38 μ M [47] and 10 μ M [62], respectively).

To better understand the physiological role of Oct1, Oct1 knockout (*Slc22a1*^{-/-}) mice were generated [63]. These mice were viable, healthy, and fertile and did not appear to have obvious phenotypic abnormalities; they only showed a decreased hepatic accumulation and intestinal excretion of exogenously administered TEA⁺ [63]. Further studies with *Slc22a1*^{-/-} mice showed that Oct1 is important for the hepatic and intestinal uptake of metformin, a hypoglycemic agent used for the oral treatment of type 2 diabetes mellitus, whereas its renal distribution and excretion are

mainly governed by other transport mechanisms [64, 65]. Since a high-fat diet for 19 weeks up-regulated the Oct1 expression in the mouse liver and this increased transporter expression was associated with a higher hepatic metformin uptake, it has been suggested that obesity might have an effect on the absorption or distribution pharmacokinetics of metformin through an up-regulation of hepatic Oct1 expression [66]. Also mice fed with a lithogenic diet, which increases the biliary cholesterol and phospholipid secretion and the probability of gallstone formation, showed an up-regulation of hepatic Oct1 mRNA levels during lithogenic bile formation, probably resulting in an increased uptake of choline, necessary to sustain phospholipid synthesis under conditions of biliary phospholipid hypersecretion [67]. The hepatic expression of mOct1 is transcriptionally increased by peroxisome proliferator agonist receptor (PPAR)- α and - γ agonists, which are commonly used agents able to regulate several hepatocellular transport functions [68]. Oct1 is not expressed in the mouse brain [51].

Rat Organic Cation Transporter 1 (rOct1)

The rOct1 is a 556 aa membrane protein [42] mapped to chromosome 1q11-12 [69], which has been identified by Northern blot analysis in rat kidney, small intestine, colon and liver [42]. In the kidney, rOct1 protein expression has been localized at the basolateral membrane of S1 and S2 segments of proximal tubule cells [70, 71]. rOct1 mRNA was detected primarily in the superficial and juxtamedullary proximal convoluted tubules [72]. Renal rOct1 mRNA levels are gradually up-regulated from day 0 through day 45 [73]. In the liver, rOct1 has been localized in the sinusoidal membranes of hepatocytes. Translational regulation is suggested since even though the mRNA of rOct1 is distributed throughout the liver lobules, the rOct1 protein is expressed only in hepatocytes surrounding the central veins [74]. rOct1 has been also identified in the airway epithelia in the luminal membrane of ciliated epithelial cells [75].

The transport mediated by rOct1 has been characterized as electrogenic, Na⁺- and pH-independent and bidirectional [76]. rOct1 can translocate organic cations like TEA⁺ (K_m = 38 μ M) [77] and choline [78], catecholamines and other biogenic amines [79], nucleosides like 2'-deoxytubercidin [80], while cations like tetrapentylammonium (TPA⁺) and cyanine 863 are nontransported inhibitors of the transporter [78, 81]. Superfusion of rOct1-expressing *Xenopus* oocytes with dopamine, serotonin, noradrenaline, histamine and acetylcholine induced saturable inwardly directed currents with K_m values ranging from 20 to 100 μ M [82]. Transport of dopamine was also demonstrated by uptake measurements in oocytes and in renal mammalian cells (human embryonic kidney cells, HEK293 cells) transfected with rOct1. The high uptake rates measured in rOct1-expressing oocytes and in transfected HEK293 cells suggested that rOct1 is a high capacity transporter, which in vivo mediates the first step in the excretion of monoamine neurotransmitters [82].

The cysteins of the large extracellular loop of rOct1 have been suggested to be involved in the formation of oligomers, which influence the insertion of the transporter in the plasma membrane, but not its affinity for the substrates [83].

An alternatively spliced variant of rOct1 (rOct1A), which lacks the first two TMDs has been cloned from rat kidney. This variant is also present in the intestine and liver [84] and encodes a 430 aa protein that is 92 % identical to rOct1. rOct1A exhibited similar functional characteristics to those of rOct1, implying that the first two transmembrane domains and the three potential glycosylation sites normally present in this protein domain are not essential for transport function, even though other characteristics such as synthesis, targeting, and sorting of the transporter have been supposed to be different between the two isoforms [84].

Rabbit Organic Cation Transporter 1 (rbOct1)

A cDNA encoding a 554 aa protein highly homologous to other mammalian OCTs has been isolated from rabbit kidney (rbOct1) [45]. rbOct1 mediated a [³H]MPP⁺ transport, which was saturable, sensitive to membrane potential, and inhibited by various OCs. rbOct1 mRNA transcripts were found to be expressed in the kidney, liver, and intestine.

Human Organic Cation Transporter 1 (hOCT1)

The *hOCT1* encodes a 553 aa protein with 80 % identity to rOct1. Northern blot analysis showed that *hOCT1* is mainly transcribed in the liver [43], differently to what was observed for *rOct1*, that is mainly transcribed in kidney, liver and small intestine [42]. *hOCT1* has been localized on chromosome 6q26 [85].

Functionally, hOCT1 together with hOCT3 have been also identified in the placenta [86], where they possibly mediate the release of non-neuronal ACh.

The presence of hOCT1 has been also demonstrated in bronchial tissue by immunofluorescence, where a luminal and also an intracellular staining of ciliated epithelial cells was observed [75].

hOCT1 expressed in oocytes of *Xenopus laevis* could translocate ACh in either direction. An almost complete inhibition of hOCT1-mediated TEA⁺ uptake was produced by the inhalational glucocorticoids beclomethasone and budesonide, suggesting that hOCT1 mediates a budesonide-inhibitable luminal ACh release in the respiratory epithelium [75].

Since hOCT1 is expressed in both subcutaneous and visceral adipose tissue, and its expression is significantly increased in obese subjects [87], it has been speculated that this is the reason for the increased metformin action in obese subjects [87].

The binding site of hOCT1 has been demonstrated to interact preferentially with elongated cationic molecules, which are able to interact with a “supraplanar

stabilizing structure” within a hydrophobic pocket of the transporter [88]. A balance between hydrophobic and hydrophilic properties was supposed to be necessary for binding and subsequent translocation by hOCT1 [89]. A comparative analysis of substrates of hOCT1 and hOCT2 showed that molecular volume was inversely correlated to transport by hOCT1, whereas H-bonding parameters like polar surface area dominated for hOCT2 mediated transport [90].

When expressed in polarized cells, hOCT1 was generally localized on the basolateral plasma membrane, except in airways and in the kidneys, where it seemed to be expressed on the apical cell membrane [75, 91]. These findings imply that the information for the insertion of the transporter in the suitable plasma membrane domain is not directly contained in the transporter aa sequence.

Both hOCT1 and mOct1 have been proposed to be high-capacity thiamine transporters, and for this reason, they are probably associated with its hepatic dietary uptake [92]. hOCT1 accepts TEA⁺ as a substrate: other OCs, including clonidine, quinine, quinidine and verapamil, but also the neutral compounds corticosterone and midazolam significantly inhibited [¹⁴C]-TEA⁺ uptake by hOCT1 [93]. The K_i values of several compounds for interaction with hOCT1 are different from the K_i values determined in previous studies for rOct1 and hOCT2 [93]. hOCT1 has been shown to mediate a specific and membrane potential sensitive uptake of MPP⁺, which showed remarkable differences in the interaction with some organic cations when compared to rOct1 [42, 43].

In the human glioma cell line SK-MG-1, four *hOCT1* isoforms were identified [94]. Two of these isoforms were also found in human liver cDNA. Several reports are dedicated to the study of drug interactions and how transporter mutations influence hOCT-drug interactions. These topics will be examined in the Chap. 4 by M. Tzvetkov, N. Dalila and F. Faltraco of this book. Here, it should be underlined that many drugs can interact with hOCT without being substrate of the transporters, as for example demonstrated for proton pump inhibitors, which potently inhibit hOCT but are not translocated into the cell [27]. Both the interaction as substrate and as inhibitor are of great pharmacological importance, in the first case determining drug tissue distribution and excretion and in the second case causing important drug–drug interaction effects.

The expression of hOCT1 in the sinusoidal membrane of hepatocytes is linked to drug hepatotoxicity, as shown for example for the quaternary alkaloid nitidine chloride [95] and the pyrrolizidine alkaloid retrorsine [96].

Mouse Organic Cation Transporter 2 (mOct2)

The mOct2 exhibits about 93 and 84 % amino acid identity with rat Oct2 and hOCT2, respectively, and only about 70 % amino acid identity with the mouse and rat Oct1 [48].

The promoter region of *mOct2* contains a TATA and a CCAAT box, and several binding sites for transcription factors (three E-box motifs, putative responsive

elements for interleukin 6 (IL-6), and tumour necrosis factor (TNF), and a putative histone H1 specific element HI-S) [48]. mOct2 is mainly expressed in the kidney, in the brain, ureter and in the developing embryo. In the central nervous system, mOct2 is expressed in the limbic system, where it plays a role in the control of the concentration of noradrenalin (NA) and serotonin (5-HT) [97]. For this reason, mOct2 may be implicated in anxiety and depression-related mouse behaviors. Indeed, genetic deletion of *mOct2* in mice produced a significant reduction in brain concentrations of NA and 5-HT [97]. Expression of mOct2 has been demonstrated in the cochlea in hair cells of the Corti organ and also in cells of the stria vascularis, where it is involved in the development of acute ototoxicity induced by cisplatin [98]. In the peripheral nervous system, mOct2 is expressed in dorsal root ganglia, where it plays a prominent role for the development of oxaliplatin-induced peripheral neurotoxicity [99]. Together with mOct1, mOct2 is expressed in respiratory epithelium, where they mediate ACh release [59]. mOct2 is highly expressed in alveolar cells, where it seems to catalyze the transport of choline [100]. Interestingly, since adenoviruses increased both choline release from biomembranes and steady-state mOct2 mRNA and protein expression, it has been speculated that adenoviruses might transcriptionally activate the *mOct2* promoter or directly interact with regulatory domains in the primary structure of the transporter [100].

The transport properties of mOct2 are in general similar to those of mOct1, even though several organic cations had weaker inhibitory effects on MPP⁺ uptake by mOct2 than by mOct1 [62].

mOct2 mediates the renal secretion of creatinine [101] and is involved in the renal uptake, secretion and toxicity of metals and metal containing compound such as Cd²⁺ [102] and cisplatin [98].

Rat Organic Cation Transporter 2 (rOct2)

rOct2 encodes a 593 aa protein with a calculated molecular mass of 66 kDa [103]. The rat *Oct2* cDNA (2205 bp) shows 67 % identity with the rat *Oct1*. Renal expression of rOct2 is evident in the basolateral cell membrane of S2 and S3 segments of the proximal tubule [71, 104, 105]. rOct2 mRNA has been found to be expressed in rat choroid plexus [106] and, together with rOct1 and rOct3, it was also identified in the luminal membrane of ciliated epithelial cells of respiratory tract, where, together with rOct1, it serves as an ACh transporter [75]. OCT-type transporters are probably responsible for non-neuronal ACh release [86]. A recent work demonstrates that rOct2 is expressed in cholinergic neurons, anterior horn motoneurons of the spinal cord, and in the neuromuscular junctions and plays a role in ACh recycling in pre-synaptic terminals, possibly acting as a low-affinity and high-capacity choline transporter at presynaptic terminals in cholinergic neurons [107].

rOct2 has been demonstrated to function also as a histamine transporter and thus to participate in histamine metabolism [108]. rOct2 transports dopamine, NA, adrenaline, 5-HT [104] and choline [106]. The IC₅₀ values of many inhibitors were

similar for both rOct1 and rOct2, whereas those of others (mepiperphenidol, *O*-methylisoprenaline, and corticosterone) were significantly different [81]. The substrate binding site of rOct2 is like a pocket containing overlapping binding domains for ligands, which may undergo separate structural changes, and which can change its affinity depending on membrane potential [109]. Also rOct2 has been demonstrated to mediate the cellular uptake and hence the nephrotoxicity of cisplatin [110].

Rabbit Organic Cation Transporter 2 (rbOct2)

The rabbit ortholog of OCT2 (rbOct2) cloned from rabbit kidney is 71 % identical to its paralog rbOct1 [111]. The structure of rbOct2 postulated by computer analysis revealed 2 large hydrophilic loops and 12 transmembrane-spanning α -helices similar to what was described for rbOct1 and all other members of the OCT family [112]. Moreover, five potential N-linked glycosylation sites and three potential PKC phosphorylation sites in the two large hydrophilic loops were also identified [111]. Asparagines 71, 96, and 112 in rbOct2 are glycosylated to target the transporter to the cell membrane [113]. Even though the functional characteristics of rbOct1 and rbOct2 are similar, they interact with different potency with specific substrates, such as cimetidine and 2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl trimethylammonium (NBD-TMA), rbOct2 showing a greater apparent affinity than rbOct1 [111]. The Glu447 residue in rbOct2 exerted a marked influence on substrate selectivity, being probably located in a putative docking region within a hydrophilic cleft of the protein [111]. In the rabbit kidneys, rbOct1 was found to be expressed mainly in the S1 segment of proximal tubules, while rbOct2 is the main Oct in S2- and S3-segments [114, 115]. Both rbOct1 and rbOct2 have been demonstrated to be involved in the development of Cd²⁺-induced nephrotoxicity [116].

Human Organic Cation Transporter 2 (hOCT2)

The *hOCT2* encodes a 555 aa protein with 81 % identity to rOct2 and 68 % identity to rOct1. *hOCT2* could be detected in kidneys, spleen, placenta, small intestine and brain [43], and human bronchi, mostly in the apical membrane of ciliated cells, where, together with *hOCT1* mediates cellular release of ACh [75]. In the kidneys, *hOCT2* is expressed in the basolateral membrane of proximal tubule cells [117]. A splice variant of *hOCT2*, *hOCT2-A*, mainly expressed in the kidneys and weakly in brain, liver, colon, skeletal muscle, bone marrow, spinal cord, testis, and placenta, has been also identified. *hOCT2-A* codes for a 483-amino acid protein, which is predicted to have nine transmembrane domains and, when expressed in HEK293 cells, was able to interact with organic cations, even though with different substrate affinities compared with *hOCT2* [118].

The *hOCT2* promoter region contains a combination of TATA box and Inr element, probably important as transcription start point and many other possible regulatory sites for specific factors, such as E boxes, GATA, IK2, and HFH-8, a member of the forkhead/winged helix class of regulators involved in specific tissue expression of proteins [119].

hOCT2 is a low affinity transporter of the monoamine neurotransmitters dopamine, NA, 5-HT, and histamine [120]. Since *hOCT2* has a broad expression in the human brain, it might co-operate with high affinity uptake mechanisms [120] to control the local concentration and action of aminergic neurotransmitters. Cerebral *hOCT2* and also *hOCT3* have been proposed as a complementary target of antidepressant action [15, 121, 122]. *hOCT2* together with *hMATE1* transports asymmetric dimethylarginine (ADMA) and also L-arginine (*hOCT2* has a much lower affinity for L-arginine than for ADMA, see Table 1.3) and are consequently involved in the renal elimination of these substances [143]. ADMA is an endogenous substance, which interferes with the synthesis of nitric oxide from L-arginine and with endothelium-relaxation and for this reason is a cardiovascular risk factor [143]. In an attempt to identify endogenous substrates of *hOCT2*, metabolites differentially present in urine samples collected from individuals carriers of wild type and mutated *SLC22A2* (*SLC22A2* wild type, *808GT* heterozygous, and *808TT* homozygous) were analyzed by gas chromatography–mass spectrometry. Tryptophan was the most significantly decreased compound associated with the *808GT* and *808TT* variants, compared with the levels in the individuals bearing the wild type *SLC22A2* [147].

Interestingly, *hOCT2* has been demonstrated to transport metals and metal compounds such as Cd^{2+} [8], cisplatin [9] and oxaliplatin [99]. These substances are well-known to be nephrotoxic and their *hOCT2*-mediated uptake seems to be critical for their toxic effects.

The binding of the substrates to *hOCT2* was demonstrated to be dependent on their ionization degree [151] and also on their hydrophobicity, molecular size, shape, and flexibility, as determined using quantitative structure activity relationship (QSAR) computational models [192]. The hydrophilic cleft, where the transporter interacts with its substrates, is supposed to be formed by several transmembrane helices, which contain cysteines at aa position 437, 451, 470, and 474, with cysteine 474 exposed to the aqueous milieu of the cleft [193] and being part of an interaction domain [194]. As already demonstrated for *rOct1* [195], also the binding region of *hOCT2* is characterized by multiple, possibly overlapping interaction sites [196]. The molecular basis of polyspecificity will be discussed in the Chap. 2 by T. Keller and H. Koepsell of this book. The six cysteines of the *hOCT2* extracellular loop have been demonstrated to be important for transporter oligomerization and its correct trafficking to the plasma membrane [197]. *hOCT2* is not only able to interact with itself to form oligomers [197], but it has also other proteinic interaction partners such as the lysosomal-associated protein transmembrane 4 α (LAPTM4A), which regulates the function of *hOCT2* by influencing its trafficking to/from the cell membrane and processing it via the intracellular sorting machinery [198].

Table 1.3 Summary of proteins size (in amino acids=aa), tissue distribution, and of the known endogenous and exogenous substrates with K_m , K_i , and IC_{50} values, when available from the literature, of human cloned OCTs, OCTNs, and MATEs

Transporter	Protein size (aa)	Tissue distribution	Endogenous substrates	Exogenous substrates
hOCT1 (SLC22A1)	553 [43]	Liver [43, 44], jejunum [123] (lateral membrane of enterocytes), luminal membrane of bronchial tissue epithelial cells [75], adipose tissue [87], peripheral blood mononuclear cells and CD4 ⁺ T-cells [124]	Acetylcholine (IC_{50} for MPP ⁺ 580 μ M) [75]	Acyclovir (K_m 151 μ M) [14]; amiloride [90]; agmatine (K_m 18.7 mM) [125]; ASP ⁺ [23]; amisulpride (K_m 31.3 μ M) [126]; ethidium bromide (K_m 0.8 μ M) [20]; DAPI (K_m 8.9 μ M) [127]; fenoterol [90]; furamide (K_m 6.1 μ M) [128]; ganciclovir (K_m 516 μ M) [14]; glycopyrrolate [90]; HPP ⁺ (K_m 0.99 μ M) [129]; imatinib [130, 131]; ipratropium [90]; irinotecan (IC_{50} for MPP ⁺ uptake 1.7 μ M) [132]; lamivudine (K_m 1.25 mM) [133]; metformin (IC_{50} for cimetidine uptake 2010 μ M) [134]; monocrotaline (K_m 25 μ M) [135]; morphine (K_m 3.4 μ M) [136]; MPP ⁺ (K_m 14.6 μ M) [44]; nitidine [95]; oxybutynin (K_m 8 μ M) [137]; oxyphenonium [90]; pacitaxel (IC_{50} for MPP ⁺ uptake 50 μ M) [132]; pentamidine (K_m 36.4 μ M) [128]; phenformin (IC_{50} for cimetidine uptake 10 μ M) [134]; pHTCU compounds [90]; procaterol [90]; quercetin [138]; ranitidine (K_m 70 μ M) [139]; retrorsine (IC_{50} for MPP ⁺ uptake 2.3 μ M [96]); RHPP ⁺ (K_m 5.2 μ M) [129]; rilpivirine (IC_{50} for TEA ⁺ uptake 28.5 μ M) [140]; rhodamine 123 (K_m 0.54 μ M) [24]; spermidine ($K_m \approx 1$ mM) [16]; sulpride (K_m 260 μ M) [126]; sumatriptan [90]; TEA ⁺ (K_m 229 μ M) [93]; thiamine (K_m 780 μ M) [92]; tiotropium [90]; trospium (K_m 17 μ M) [137]; xamoterol [90]; YM155 (K_m 22 μ M) [141]; zebularine (efflux transporter) [142]
hOCT2 (SLC22A2)	555 [43]	Kidney [43] (basolateral membrane of proximal tubules cells) [117]; small intestine [43, 120]; brain [43, 120]; luminal membrane of bronchial tissue epithelial cells [75]	Acetylcholine (K_m 150 μ M) [75]; ADMA (K_m 967 μ M) [143]; creatinine (K_m 4 mM) [101, 144]; cyclo(his-pro) (K_m 74 μ M) [145]; dopamine (K_m 0.4 mM) [120]; histamine (K_m 1.3 mM) [120]; L-arginine ($K_m > 10,000$ μ M) [143]; N-methylnicotinamide (K_m 318 μ M) [146]; norepinephrine (K_m 1.9 mM) [120]; salsolinol (K_m 130 μ M) [145]; serotonin (K_m 80 μ M) [120]; tryptophan [147]	Agmatine (K_m 1.4 [148] and 1.8 mM [125]); albuterol [90]; amantadine (K_m 27 μ M) [120]; amiloride (K_m 95 μ M) [22]; aminoguanidine (K_m 4.1 mM) [149]; amisulpride (K_m 168 μ M) [126]; ASP ⁺ (K_m 24 μ M) [22]; atenolol (IC_{50} for ASP ⁺ uptake 93 μ M) [150]; cadmium (K_m 54 μ M) [8]; cimetidine (K_i for TEA ⁺ uptake 13 μ M) [151]; cisplatin (K_m 11 μ M) [9] and [99]; ethidium bromide (K_m 1.7 μ M) [20]; fenoterol [90]; glycopyrrolate [90]; guanfacine (K_m 96 μ M) [152]; HPP ⁺ (K_m 2.79 μ M) [129]; ifosfamide (IC_{50} for ASP ⁺ uptake 624 μ M) [153]; lamivudine (K_m 1.90 mM) [133]; memantine (K_m 34 μ M) [120]; metformin (IC_{50} for cimetidine uptake 1.7 mM) [134]; MPP ⁺ (K_m 19 μ M) [43]; nitidine [95]; ofloxacin (IC_{50} for ASP ⁺ uptake 686 μ M) [150]; oxalipatin (IC_{50} for cimetidine uptake 1 μ M) [99, 154]; oxyphenonium [90]; paraquat [19]; phenformin (IC_{50} for cimetidine uptake 65 μ M) [134]; pindolol (IC_{50} for ASP ⁺ uptake 145 μ M) [150]; pramipexole (K_m 15.4 μ M) [155]; putrescine (K_m 11.3 mM) [125]; rhodamine 123 (K_m 0.61 μ M) [24]; spermidine ($K_m \approx 1$ mM) [16]; sulpride (K_m 187 μ M) [126]; TEA ⁺ (K_m 76 μ M) [43]; thiamine (K_m 750 μ M) [92], and 60 μ M [156]; tiotropium [90]; trospium (K_m 8 μ M) [137]; YM155 (K_m 2.7 μ M) [141]; zebularine (efflux transporter) [142]

hOCT3 (SLC22A3)	556 [157]	Brain cortex [157]; placenta, aorta, prostate, salivary gland, adrenal gland, fetal lung and skeletal muscle [34]; heart and liver [157]; submandibular salivary gland [158]; Jejunum [123]; monocytes [124]	Histamine [159]; monoamine neurotransmitters [148, 157]; norepinephrine (K_m 510 μ M) [157]	Agmatine (K_m 2.14 mM) [148]; amisulpride (K_m 192 μ M) [126]; antiarrhythmic drugs [160]; ethidium bromide (K_m 2 μ M) [20]; HPP ⁺ (K_m 2.23 μ M) [129]; lamivudine (K_m 2.14 mM) [133]; metformin (K_m 2.5 mM) [161]; MPP ⁺ ([157] and [161] K_m 157 μ M); nitidine [95]; pramipexole (K_m 138 μ M) [155]; RHPP ⁺ (K_m 8.21 μ M) [129]; spermidine ($K_m \approx$ 1 mM) [16]
hOCTN1 (SLC22A4)	551 [162]	Kidney, bone marrow and trachea and fetal liver [162]; skeletal muscle and placenta [163]; heart [164]; ocular epithelium [165]; lactating mammary epithelial cells [166]; monocytes [124]; mitochondria [167]	Acetylcholine [168]; L-carnitine (K_m 1.3 mM) [167]	Amisulpride (K_m 180 μ M) [126]; ergothieneine (ingredient of human food, K_m 21 μ M) [169]; gabapentin [170]; imatinib (IC_{50} for inhibition of ASP ⁺ uptake 31 μ M) [21]; ipratropium (K_m 444 μ M) [171]; pyrrolamine, quinidine [11]; TEA ⁺ (K_m 436 μ M) [162]; verapamil [11]

(continued)

Table 1.3 (continued)

Transporter	Protein size (aa)	Tissue distribution	Endogenous substrates	Exogenous substrates
hOCTN2 (SLC22A5)	557 [172]	Placenta, kidney, liver and brain [172]; heart and skeletal muscles [12]; brain capillary endothelial cells [173]; ocular epithelium [165]; peripheral blood mononuclear cells and CD4 ⁺ T-cells [124]; bronchial epithelial cells [171]; nasal epithelium [174]; colon [175]; sperm [176]; lactating mammary epithelial cells [166]	Acetyl-L-carnitine (K_m 8.5 μ M), D-carnitine (K_m 10.9 μ M), and L-carnitine (K_m 4.34 μ M), [12]	Amisulpride (K_m 185 μ M) [126]; betaine [177]; butyryl-L-carnitine (K_m 0.4 μ M) [178]; cephaloridine [179]; imatinib [180]; ipratropium (K_m 53 μ M) [171]; mildronate (K_m 26 μ M) [181]; pyrilamine, quimidine [182] spirinolactone [183]; TEA ⁺ , verapamil, and valproate [182]
hOCT6 (hCT2) (SLC22A16)		Sperm [176]	Carnitine (K_m 26 μ M) [176]	

hMATE1 (SLC47A1)	570 [184]	Liver, kidney, skeletal muscles [184]	ADMA [143]; creatinine [13]; L-arginine [143]; N-methylnicotinamide (K _m 301 μM) [146]	Acyclovir (K _m 2640 μM) [13]; agmatine (K _m 240 μM) [125]; chloroquine (K _i for inhibition of metformin uptake 2.8 μM) [185]; cimetidine (K _m 8 [186] and 170 [13] μM); cisplatin [187]; DAPI (K _m 1.1 μM) [188]; estrone sulfate (K _m 470 μM) [13]; fexofenadine [189]; ganciclovir (K _m 5120 μM), and guanidine (K _m 2100 μM) [13]; imatinib (IC ₅₀ for inhibition of ASP ⁺ uptake 118 nM) [21]; MPP ⁺ (K _m 16 [184] and 100 [13] μM); metformin (K _m 780 μM); nitidine [95]; oxaliplatin [190]; paraquat [19]; procainamide (K _m 1230 μM); TEA ⁺ (K _m 366 μM) [186]; thiamine (K _m 3.5 μM) [13, 156]; topotecan (K _m 70 μM) [13]
hMATE2-K (SLC47A2)	566 [191]	Kidney [191]	Creatinine [13]; N-methylnicotinamide (K _m 422 μM) [146]	Acyclovir (K _m 4320 μM) [13]; cimetidine (K _m 370 [191], 120 [13] and 18 [186] μM); cisplatin, [187, 190]; DAPI (K _m 3.2 μM) [188]; estrone sulfate (K _m 850 μM) [13]; ganciclovir (K _m 4280 μM) [13]; guanidine (K _m 4200 μM) [13]; metformin (K _m 1980 [13] and 1050 [191] μM); MPP ⁺ (K _m 93.5 μM) [191]; oxaliplatin [187, 190]; procainamide (K _m 1580 [13] and 4100 [191] μM); TEA ⁺ (K _m 830 [191] and 375 [186] μM); thiamine (K _m 3.9 μM) [13, 156]; topotecan (K _m 60 μM) [13]

ADMA asymmetric dimethylarginine, ASP⁺ 4,4-(dimethyl-aminostyryl)methylpyridinium, DAPI 4',6-diamidino-2-phenylindol, HPP⁺ 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxybutyl]pyridinium, MPP⁺ 1-methyl-4-phenylpyridinium, *phTCU* phenylthiophenecarboxamide ureas, *RHPP⁺* 4-(4-(chlorophenyl)-1-4-(fluorophenyl)-4-hydroxybutyl-pyridinium, TEA⁺ tetraethylammonium, YM155 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,3-d]imidazolium

Mouse Organic Cation Transporter 3 (mOct3)

The coding region of *Slc22a3* consists of 11 exons and 10 introns [199]. *Slc22a3* encodes a low-affinity transporter for the neurotransmitters 5-HT and histamine [15, 200]. Measuring mOct3 mRNA levels in different tissues, it was found that mOct3 was highly expressed in placenta, ovaries, and uterus [50], skeletal muscle and heart [201], and at low levels in most other tissues [50].

In the placenta, mOct3 expression was shown to depend on embryonic development, dramatically decreasing toward the end of gestation [34], analogously to what observed for the expression of the intracellular enzyme monoamine oxidase A (MAOA), which inactivates monoamines after transport into the cell [202]. In the placenta, mOct3 and MAOA were found to co-localize in the labyrinth layer, where trophoblast cells are in contact with both the maternal and the foetal circulation to allow an exchange of nutrients, gases and waste products [202]. mOct3 has been identified as a component of the uptake 2 system, a transport system responsible for clearing extracellular monoamines, which is active in tissues with high mOct3 expression such as skeletal muscle, heart, and uterus [201]. In an attempt to establish the importance of mOct3 as part of the uptake2 system, the effects of deletion of the *Slc22a3* gene in mice (*Slc22a3*^{-/-} mice) were evaluated. These mice were viable and fertile and showed no obvious physiological defect and no significant imbalance of NA and dopamine. However, uptake experiments with MPP⁺ revealed that *Slc22a3* is an essential component for uptake-2 function in the adult heart and placenta but not in other adult organs [201]. Further experiments with *Slc22a3*^{-/-} mice showed that these animals ingested an increased quantity of hypertonic saline under thirst and salt appetite conditions, and showed alterations of the neural response in the subfornical organ after Na⁺ deprivation [203]. In mouse brain, mOct3 was demonstrated to be expressed in dopaminergic neurons of the substantia nigra compacta, non-aminergic neurons of the ventral tegmental area, substantia nigra reticulata (SNr), locus coeruleus, hippocampus and cortex [204]. However, mOct3 expression was also occasionally detected in astrocytes in the SNr, hippocampus and several hypothalamic nuclei. *Slc22a3*^{-/-} mice showed a decreased intracellular content and increased turnover of aminergic transmitters in the brain, which resulted in subtle behavioral alterations, such as increased sensitivity to psychostimulants and increased levels of anxiety and stress [204]. In contrast with these findings, deletion of *mOct3* was found by another group to tendentially increase animal activity and diminish anxiety [205]. The reason for this discrepancy is not known.

Interestingly, it has been shown that mOct3 is able to transport 5-HT and that its expression is upregulated in the brain of mice with constitutively reduced 5-HT transporter [206]. Moreover, in these mice the OCT blocker decynium-22 diminished 5-HT clearance, exerting antidepressant-like effects. In this way, mOct3 may be an important transporter mediating serotonergic signaling when the expression or function of 5-HT transporters is compromised [206]. mOct3 has been found to be expressed also in basophils, cells able to synthesize and secrete huge amounts of histamine. Here, mOct3 seems to be involved in the control of histamine secretion [200].

Rat Organic Cation Transporter 3 (rOct3)

rOct3 encodes a 551 aa protein with a predicted molecular mass of 61 kDa and 48 % identity with rOct1 and rOct2 [207]. rOct3 is expressed most abundantly in the placenta and moderately in the intestine, heart, and brain [207, 208]. Its expression is comparatively low in the kidney and lung and is undetectable in liver [207]. In the brain, rOct3 is expressed widely in different regions, especially in the hippocampus, cerebellum, and cerebral cortex [208], in osmolarity-sensitive regions, and in relay regions. Discrete expression of rOct3 was observed in circumventricular organs such as area postrema and subfornical organ, which are located at the blood–brain interface [209]. In these two structures, rOct3 was found in neurons. In addition, ependymal cells of the subcommissural organ and the pinealocytes of the pineal gland express rOct3. The transporter is also expressed in the choroid plexus and in ependymal cells of some areas lining the ventricles and in ependymal and glial-like cells of the dorsomedial hypothalamus (DMH) of male rats [210].

DMH accumulates histamine and this uptake could be reduced by corticosterone, serotonin, estradiol, and the OCT inhibitor decynium22, supporting the hypothesis that corticosterone-mediated inhibition of rOct3 is responsible for stress-induced accumulation of serotonin in the DMH [210]. Also the toxicity of MPP⁺, a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, in cerebellar granule neurons is probably mediated by rOct3 [211].

In the placenta, rOct3 is expressed on the basolateral, i.e., fetus facing side of the placenta, while the rMate1 is expressed in the labyrinth area mainly on the apical (maternal) placenta region [212]. The OC metformin can be transported in the rat placenta in a fetal-to-maternal direction even against its concentration gradient [213] leading to the conclusion that rOct3 and rMate1 represent an “efficient trans-placental excretory pathway” responsible for protection and detoxication of the fetus [213].

It has been observed that at low concentration inhibitors and substrates stimulated rOct3-mediated MPP⁺ and noradrenaline, but not cimetidine transport. Basing on these observation, a kinetic model with two binding sites for substrate or inhibitor per transporter unit was proposed, where activation may serve to keep the transporter working for specific substrates in the face of inhibitors [214].

rOct3 has been identified in the luminal membrane of ciliated epithelial cells of the lung but it did not seem to be involved in the transport of ACh [75].

Human Organic Cation Transporter 3 (hOCT3)

The hOCT3 (also known as extraneuronal monoamine transporter, EMT) has been first cloned from Caki-1 cells, a human kidney carcinoma cell line [157]. The *SLC22A3* has been mapped to 6q27, the end of the long arm of chromosome 6 [157] and codes for a 556 aa long protein. Like in mouse *Slc22a3*, *SLC22A3* coding

regions consist of 11 exons and 10 introns [199]. The *SLC22A3* promoter is located within a CpG island and lacks a consensus TATA box, but contains a prototypical initiator element and a number of potential binding sites for the ubiquitous transcription factors Sp1 and NF-1 [119]. mRNA for hOCT3 was detected in liver, heart and brain cortex. HEK293 cells transfected with hOCT3 showed uptake of known substrates of the extraneuronal catecholamine transporter, such as tyramine, adrenaline, NA, 5-HT and the neurotoxin MPP⁺. Adrenaline was taken up three times more efficiently than NA, while TEA⁺, a prototypical substrate for the OCT proteins, was not accepted as a substrate by hOCT3 [15, 157]. The transport of the neurotransmitters dopamine, NA, and 5-HT by hOCT3 was determined to be of high-capacity and low-affinity [215]. Functionally, the hOCT3 has been proposed to be involved in the inactivation of released monoamine transmitters that escapes neuronal reuptake and thus in the prevention of uncontrolled signal spreading [157] and to be a new molecular target for the development of drugs that aim at an elevation of free monoamine transmitters [157, 216]. Loss of hOCT3 function may cause chronically elevation of sympathetic tone, which may induce vasoconstriction and vascular hypertrophy, leading to progressive increases in peripheral resistance and hypertension [157]. A genome-wide haplotype association study identified *SLC22A3-apolipoprotein(a)L2-apolipoprotein(a)* gene cluster as a strong susceptibility locus for coronary artery disease [217].

A recent study examined the relationship between catecholamine gene expression in peripheral blood and tic severity in Tourette syndrome (TS), a heritable disorder characterized by tics and by dysregulation of neurotransmitters (such as dopamine, histamine, 5-HT, and NA) [218]. *SLC22A3* was highly associated with TS severity, underlining the potential importance of hOCT3 in regulating neurotransmitter balance [218].

hOCT3 has been localized in the basolateral membrane of acinar and ductal cells of human submandibular salivary gland, where it is supposed to play a role in the secretion of histamine by such non-professional histamine-producing cells. Interestingly, hOCT3 expression in these cells is strongly reduced in Sjögren's syndrome patients, suggesting that impaired histamine transport may contribute to glandular pathology in these patients [158].

Also at the example of hOCT3 it has been shown that sensitivity of tumor cells to chemotherapeutic treatment with e.g. irinotecan, vincristine, melphalan [219], and oxaliplatin [220] depends on the expression of transporter proteins mediating specific drug accumulation into target cells [219].

hOCT3 is more widely expressed than its mouse ortholog [34].

A functional discrimination of the three hOCTs has been demonstrated to be attained using the following substances: hOCT1 is selectively inhibited by prazosin, reversibly inhibited by phenoxybenzamine (PbA) and it is not sensitive to inhibition by (9-fluorenyl)-*N*-methyl-beta-chloroethylamine (SKF550) and *O*-methyl-isoprenaline (OMI); hOCT2 is reversibly inhibited by SKF550, irreversibly by PbA and not by prazosin, β -estradiol and OMI, whereas hOCT3 is selectively inhibited by corticosterone, OMI and decynium22 [221].

Organic Cation Transporter Novel 1

Mouse Octn1

Mouse Octn1 (mOctn1) has a low, Na⁺-dependent, carnitine transport activity [222]. mOctn1 is expressed in kidney, smooth muscle, and hematopoietic tissues, such as spleen and bone marrow. Here, its expression was shown to be associated with myeloid cells of the erythroid-lineage at the differentiation stage from immature erythroid cells to peripheral mature erythrocytes [223]. In mouse kidney, mOctn1 is expressed predominantly in the luminal membrane of cortical proximal tubule cells [163]. Moreover, mOctn1 is expressed in inflammatory joints of mice with collagen-induced arthritis, a model of human arthritis, but not in the joints of normal mice [224]. Together with mOctn2 and -3, mOctn1 is widespread expressed across the mouse central nervous system with a distribution pattern indicating a role in modulating cerebral bioenergetics and in ACh production for neurotransmission in olfactory, satiety, limbic, memory, motor and sensory functions [225]. Indeed, mOctn1 has been demonstrated to play a role in neuronal differentiation and proliferation, which are required for brain development [226].

The mOctn transporters are also expressed in epithelial ducts, specialized myo-epithelial cells and fatty stroma of mammary glands. In pregnant and lactating mice the expression of these transporters has been shown to be up-regulated compared with virginal females, and to be down-regulated 15 days after cessation of lactation, probably to provide the suckling infant with adequate carnitine [227]. Mice with genetic deletion of *mOctn1* (*Slc22a4*^{-/-}) developed normally and did not display any gross phenotypic abnormalities [228]. However, they developed an ergothioneine deficiency (for ergothioneine function see the paragraph human OCTN1 below) and were more prone to intestinal inflammation in the ischemia and reperfusion model. Moreover, using *Slc22a4*^{-/-} mice it has been demonstrated that mOctn1 transports the antidiabetic drug metformin [229], being involved in its oral absorption in small intestine, and phenformin, influencing its accumulation in mitochondria [230].

mOctn1-mediated uptake of ergothioneine in neural progenitor cells was shown to inhibit cell proliferation while promoting cellular differentiation by regulating the expression of basic helix-loop-helix transcription factors through a still unknown process, which is different from antioxidant action [231].

Rat Octn1

Rat *Octn1* gene codes for a protein of 553 aa with a high homology to human OCTN1 (85 % identity) [232]. rOctn1 has been demonstrated to be a pH-dependent polyspecific transporter for organic cations, which is expressed in a wide variety of tissues in the rat, principally in the liver, intestine, kidney, brain, heart, placenta [232], choroid plexus [233], and testis Sertoli cells [234]. Renal rOctn1 mRNA

levels were shown to increase gradually from postnatal day 0 through day 45 in both genders [73]. Also rOctn1 was shown to be involved in the Na⁺-dependent transport of ergothioneine [235]. rOctn1 and also rOctn2 accept oxaliplatin as a substrate and are functionally expressed in dorsal root ganglia neurons. rOctn1-mediated transport of oxaliplatin was suggested to contribute to its neuronal accumulation and treatment-limiting neurotoxicity [236].

Human OCTN1

Human *OCTN1* gene encodes a 551 aa protein with 11 transmembrane domains and one nucleotide binding site motif [162]. hOCTN1 works as a polyspecific, bidirectional proton antiporter [11], transporting several cationic compounds, including ACh, TEA⁺, pyrilamine, quinidine, verapamil, donepezil, betonicine, ergothioneine and stachydrine [11, 168, 169]. Human OCTN1 is strongly expressed in kidney, trachea, bone marrow and fetal liver and in several human cancer cell lines, but not in adult liver [162]. A discrete expression of hOCTN1 has been also detected in cardiomyocytes [164]. In the intestine, hOCTN1 is expressed at the same level in all gut sections [175]. hOCTN1 is highly expressed in ocular tissues, especially in the iris-ciliary-body [237] and in the apical membrane of human corneal and conjunctival epithelial cells [165]. hOCTN1 together with hOCTN2 is also expressed in human airway epithelia, with a predominant localization to the apical portion of epithelial cells [238]. Lactating mammary epithelial cells (MEC) express more than fourfold higher RNA levels of hOCTN1 relative to nonlactating MEC [166]. hOCTN1, together with hOCTN2 and hOCTN3 is expressed in sperm, where they possibly mediate carnitine uptake [176]. hOCTN1 is expressed in immunological and hematological organs and tissues, and expression of its mRNA is induced by proinflammatory stimuli [224], suggesting that SLC22A4 functions as a transporter in lymphoid organs or inflammatory milieu. Interestingly, polymorphisms of *hOCTN1* in a gene region, where the transcription factor RUNX1 binds, and polymorphisms of RUNX1 itself were suggested to be associated with susceptibility to rheumatoid arthritis [224]. However, these findings are strongly debated (see for example [239]). A localization of hOCTN1 in mitochondria has been also demonstrated [167]. Here, the transporter should mediate the uptake of L-carnitine [167]. Ergothioneine (ET), a substance that is biosynthesized exclusively by fungi and mycobacteria and is captured by plants through their roots, has been identified as key substrate of hOCTN1 ($K_m=21\text{ }\mu\text{M}$) [169]. In humans, ET is of dietary origin (high levels of ET are present in mushrooms) and accumulates in erythrocytes, bone marrow, and seminal fluid [169]. ET is considered to be an intracellular antioxidant [169]. Indeed, epidermal keratinocytes express hOCTN1, which enables them to internalize and accumulate L-ergothioneine conferring to the cells a resistance to oxidative damage [240]. In blood cells, hOCTN1 is involved in the transport of physiological compounds that are important for cell proliferation and erythroid differentiation [241]. hOCTN1 mediates the cellular extrusion of ACh [168].

Mouse Octn2

Because of the function of hOCTN2 as a carnitine transporter (see below), expression of *Octn2* has been investigated in wild-type (WT)- and in the juvenile visceral steatosis (JVS)-mice, so called because of accumulation of fat in viscera due to defects in fatty acid oxidation caused by carnitine deficiency (for this reason this is an animal model of human systemic carnitine deficiency) [242]. In JVS mice, a mis-sense mutation in *mOctn2* gene from CTG (Leu) to CGG (Arg) at codon 352 located within the sixth transmembrane transporter domain was identified, suggesting that *mOctn2* is a candidate gene responsible for the JVS and that JVS mice can be considered to be *mOctn2*^{-/-} mice [242]. The JVS mice spontaneously develop intestinal villous atrophy, breakdown and inflammation with intense lymphocytic and macrophage infiltration, leading to ulcer formation and gut perforation [243]. Using this animal model, it has been demonstrated that the Na⁺-dependent carnitine transporter *Octn2* is responsible for carnitine transfer from the mother to the fetus and to supply the placenta with carnitine for its own metabolic needs [244] and that carnitine is necessary to maintain normal intestinal and colonic structure and morphology [243]. *mOctn2* was found to be also expressed in pancreatic A-cells [245]. In mice, PPAR α transcriptionally up-regulates *mOctn2* and enzymes involved in hepatic carnitine biosynthesis [246]. Also fasting and caloric restriction were demonstrated to activate PPAR α leading to an upregulation of *mOctn2* in several tissues [247]. The pharmacological importance of *mOctn2* has been suggested in studies with mice, demonstrating that the uptake of the organic cation ipratropium bromide, an anti-cholinergic drug used to treat chronic obstructive pulmonary disease, is mediated by *mOctn2* expressed at the apical portions of ciliated epithelial cells of trachea [248].

Rat Octn2

Rat *Octn2* encodes a 557 amino acid protein with 12 putative membrane-spanning domains, which mediates the high-affinity, Na⁺-dependent transport of L-carnitine ($K_m = 25 \mu\text{M}$) [249]. rOctn2 is expressed in the testis, colon, kidney (in the proximal and distal tubules and in the glomeruli), heart (in myocardium, valves, and arterioles) and liver and also in the skeletal muscle (where it is involved in the import of carnitine for fatty acid oxidation, especially in highly oxidative muscles [250]), in the labyrinthine layer of the placenta, small intestine, and brain (in the cortex, hippocampus, and cerebellum) [249, 251]. Rat *Octn2* is also expressed in the basolateral membrane of epithelial cells in the distal caput, corpus, and proximal cauda epididymides, where it is likely to be responsible for the transport of L-carnitine into the cells of the epididymal epithelium [252] and also in the Sertoli cells, which are part of the blood-testis barrier [253]. Expression of rOctn2 has been also detected in astrocytes [254] and in brain capillary endothelial cells at the basolateral membrane and in the cytoplasmic region [255], suggesting an important role of rOctn2 in

removal of carnitine esters from the brain. Renal mRNA expression of rOcn2 increased by a factor of 1.7 in carnitine deficient rats, whereas rOcn2 mRNA expression remained unchanged in gut, liver or skeletal muscle [256]. Muscle contraction facilitated carnitine uptake in skeletal muscles, possibly via the contraction-induced translocation of rOcn2 to the plasma membrane [257]. Two splicing variants of rOcn2 have been identified in rats [258].

Human OCTN2

Human *OCTN2* encodes for a polyspecific, pH-dependent transporter for OCs of 557 aa with twelve putative transmembrane domains and with 75.8 % similarity to OCTN1 [12, 172]. The physiological importance of OCTN2 derives from its function as a high affinity Na⁺-carnitine cotransporter [12]. Indeed, mutations, which produced a missfunctioning hOCTN2, have been associated with primary systemic carnitine deficiency, an autosomal recessive disease, whose manifestations are progressive cardiomyopathy, skeletal myopathy, hypoglycaemia and hyperammonaemia [259–261]. Importantly, Na⁺ does not change the affinity of hOCTN2 for OCs, but it strongly increases the hOCTN2 affinity for carnitine [251, 262]. hOCTN2 showed a stereospecific transport activity: D-carnitine was transported with slightly lower affinity ($K_m = 10.9 \mu\text{M}$) than the L-isomer ($K_m = 4.3 \mu\text{M}$) [182]. The fluxes of L-carnitine and Na⁺ have been demonstrated to be coupled with 1:1 stoichiometry and to be electrogenic processes [263]. The transmembrane domains 1–7 of hOCTN2 were shown to be responsible for organic cation transport and for Na⁺ dependence in carnitine transport, with glutamines 180 and 207 as critical amino acids for the Na⁺ dependence [264]. The hOCTN2 is physiologically glycosylated but glycosylation does not affect maturation of hOCTN2 to the plasma membrane. The three asparagines that have been demonstrated to be normally glycosylated are located in a region important for substrate recognition and turnover rate [265]. The C terminus of hOCTN2 has been shown to interact directly with PSD-95-Dlg-ZO-1 domain-containing protein K1 and 2 (PDZK1 and 2), which resulted to be functional regulators of hOCTN2 [266, 267].

hOCTN2 is strongly expressed in kidneys on the apical membrane of renal tubular epithelial cells, skeletal muscle, heart, and placenta in adult humans [12, 263]. Expression of hOCTN2 has been also detected in primary cultured brain capillary endothelial cells [173], on the apical side of nasal epithelium [174], in the brush-border membrane of intestinal Caco-2 cells [268] and in the colon [175]. In the intestine, hOCTN2 was shown to transport the “competence and sporulation factor” from *Bacillus subtilis* into intestinal epithelial cells, protecting these cells from oxidative stress [269]. The production of such a factor by *Bacillus subtilis* probably provides the host with the ability to respond or adapt to changes in the microbiome in order to maintain intestinal homeostasis [269].

Because of its expression in brain capillary endothelial cells, hOCTN2 is suggested to be involved in transport of L-carnitine and acetyl-L-carnitine from the circulating blood to the brain across the blood brain barrier [173].

hOCTN2 seems also to be expressed in sperm, where carnitine is important for spermatozoan maturation, motility, and fertility [176]. hOCTN2 is also present in brush border membranes from human term placentas, where it may mediate most maternofetal carnitine transport [270].

Insulin can acutely increase muscle total carnitine content in humans during hypercarnitinemia, which is associated with an increase in *hOCTN2* transcription [271].

hOCTN2 is expressed in the endothelial cells of human heart and its expression can be modulated by drug administration. Moreover, hOCTN2 can contribute to the cardiac uptake of cardiovascular drugs [183]. hOCTN2 is also expressed in cultured human limbal corneal and conjunctival epithelial cells, where it mediates carnitine uptake [272].

Therapeutic use of cephaloridine, a beta-lactam antibiotic, in humans is associated with carnitine deficiency. This fact has been explained by inhibition of hOCTN2-mediated carnitine transport by beta-lactam antibiotics such as cephaloridine, cefoselis, cefepime, and cefuprenam. These antibiotics possess a quaternary nitrogen as carnitine does. Several other beta-lactam antibiotics that do not possess this structural feature did not interact with hOCTN2 [179]. Even though hOCTN2 is a transporter for organic cations, it is highly specific for carnitine and closely related molecules, such as the cardioprotective agent mildronate [181].

The high carbohydrate consumption observed in vegetarians was associated with a significant stimulation of hOCTN2 expression in oral mucosa, probably to compensate lower carnitine levels in the alimentation [273].

Lactating mammary epithelial cells (MEC) express more than fourfold lower RNA levels of hOCTN2 relative to nonlactating MEC [166].

Mouse and rat Octn3, Human OCT6

An *Octn3* clustered on mouse chromosome 11 with *mOctn1* and *mOctn2* has been identified in the mouse brain (mainly in the grey matter, specifically in anterior horn cell bodies) [225], kidney and testis, where carnitine is required to maintain sperm cell motility [222]. The mouse *Octn3* mediates a specific, Na^+ independent uptake of carnitine with a K_m of 3 μM [222] in peroxisomes [274], suggesting a role of carnitine in peroxisomal lipid metabolism. PPAR α mediates transcriptional upregulation of *mOctn3* [246]. In the rat, *Octn3* protein is mainly expressed in the basolateral membrane of enterocytes [275], in the apical membrane of the kidney epithelia [276], and in astrocytes [277]. In humans, *Octn3* has not been found. However, another high-affinity carnitine transporter called hCT2 or hOCT6 has been identified in sperm, where it mediates the uptake of carnitine with a K_m of 26 μM [176].

Multidrug and Toxin Extrusion Transporters (MATEs)

Multidrug and toxic compound extrusion (MATE) proteins are widely distributed in all kingdoms of living organisms. Plant MATE-type transporters are involved in the detoxification of secondary metabolites, including alkaloids [278]. Human and mouse MATE1 have been identified as the transporters involved in the H⁺-coupled electroneutral exchange of endogenous and exogenous OCs as their final excretion step in the luminal membranes of the renal tubules and bile canaliculi [184].

MATE1 (SLC47A1)

Mouse Mate1

In addition to the localization in kidneys and liver, mMate1 is also expressed in other cells, including brain glia-like cells and capillaries, pancreatic duct cells, urinary bladder epithelium, adrenal gland cortex, α cells of the islets of Langerhans, Leydig cells, and vitamin A-storing Ito cells [279]. The expression of mMate1 mRNA in the kidneys of both male and female mice has been shown to increase steadily from prenatal day -2 to 45 days of age. Pregnancy significantly reduced mMate1 renal expression by 20–40 %. At day 30 appeared a gender difference, with higher expression in kidneys from male than female animals [280]. Pyrimethamine has revealed as a potent and specific inhibitor of mMate1 (K_i = 145 nM) and can be useful to discriminate transport of OCs by this transporter [281]. mMate1 was suggested to play an important role for cisplatin nephrotoxicity, since its genetic deletion (*Slc47a1*^{-/-}) or inhibition by pyrimethamine in mice increases renal toxicity of cisplatin, probably because of decreased cisplatin efflux from the tubular cells into the urine [282]. Since the treatment with the antidiabetic drug metformin can cause the fatal adverse effect lactic acidosis, and since metformin is an organic cation, which is substrate for mOct1 and also mOct2, it has been investigated whether mMate1 is linked to lactic acidosis. Indeed, treatment of *Slc47a1*^{-/-} mice with metformin resulted in a high hepatic metformin concentration and in lactic acidosis, suggesting that the homozygous *mMate1* variant could be one of the risk factors for metformin-induced lactic acidosis [283]. [¹¹C]metformin has been synthesized as a positron emission tomography (PET) probe and used to study the role of mMate1 for its hepatobiliary transport in mice treated or not with pyrimethamine. Indeed, an increased concentration of [¹¹C]metformin was observed in the livers of mice pretreated with pyrimethamine, confirming the importance of mMate1 for hepatic secretion of metformin [284].

A variant of mMate1, mMate1b, with a long carboxyl terminal hydrophobic tail, but with similar transport characteristics, organ and cell distribution has been identified [285]. The carboxyl terminal hydrophobic tail seemed not to determine transport properties of mMate1b.

Rat Mate1

The rat Mate1 has been demonstrated to be abundantly expressed in the renal proximal convoluted and straight tubules, and in the placenta and to a lower level also in the spleen, while it is not expressed in the liver [286]. Also rMate1 works as a pH-dependent transporter and accepts organic cations such as cimetidine ($K_m=3\text{ }\mu\text{M}$) [287] and metformin but also the zwitterionic compound cephalixin as substrates [286]. The Cys-62 and Cys-126 residues of rMate1, which are located in the first and the third transmembrane domain, respectively, were shown to play an important role as substrate-interaction sites, while the His-385 residue in the fifth extracellular loop functions as an H^+ -binding site [288].

Transcriptional regulation of the human and rat *MATE1* gene is mediated by binding of Sp1 to the gene regions spanning $-65/-25$ and $-146/-38$ [289]. rMate1 is also expressed in the rat placenta in the labyrinth area predominantly on the apical, i.e., maternal side of the placenta, where it can mediate the efflux of OCs from placenta to the maternal circulation [212].

Human MATE1

In human kidney, hMATE1 is expressed at the brush-border membranes of proximal tubular epithelial cells and in human liver on the apical membrane of hepatocytes [184, 290]. As already proposed for rOct1 [195], also the hMATE1 seems to interact with its ligands at multiple sites within a larger binding surface [291]. It has been suggested that the kinetics of interaction between inward-facing hMATE1 and intracellular H^+ are not significantly different from the kinetics of the interaction between outward-facing hMATE1 and extracellular H^+ [292]. In hMATE1, the conserved Glu273, Glu278, Glu300, and Glu389 of transmembrane regions seem to be involved with different individual roles in binding and/or transport of TEA^+ and cimetidine [293]. hMATE1 and also hMATE2 (K_m 3.5 and 3.9 μM , respectively) are responsible for the renal efflux of thiamine and perhaps carnitine as well as drugs into the urine [156]. Immunofluorescence analysis suggested that hMATE1 is primarily expressed in the plasma, endoplasmic reticulum, and peroxisomal membranes in cultured hepatocytes, hMATE1 cells, and both mouse and human liver tissues [294].

MATE2 (SLC47A2)

Mouse Mate2

mMate2 mediates a pH-dependent transport with substrate specificity similar to, but distinct from that of mMate1 [295]. In male mice, mMate2 is specifically expressed in testicular Leydig [295] and Sertoli [280] cells. In female mice, mMate2 mRNA levels are expressed most highly in the colon [280].

Human MATE2

hMATE2 is a kidney-specific isoform of *hMATE1* [184], which has a splicing variant, *hMATE2K*, where an exon of *hMATE2* is deleted [191]. Both *hMATE2* and *hMATE2K* are expressed in the kidneys, with *hMATE2K* having the higher expression level [296]. *hMATE2K* cDNA encodes a 566 aa protein, which shows 94, 52, and 52 % identity with the *hMATE2*, *hMATE1*, and rat *MATE1*, respectively [191]. The affinities of several OCs for *hMATE1*, *hMATE2*, and *hMATE2K* are similar [13, 296]; however, the zwitterionic cephalixin and cephradine are specific substrates of *hMATE1*, but not of *hMATE2K* [13]. Pyrimethamine is a potent competitive inhibitor also of the uptake by *hMATE2K* with an inhibition constant (K_i) of 56 nM [146].

In conclusion, OCTs play an important physiological role for neurotransmitter balance in the body. Moreover, they are also important uptake routes for intracellular drug delivery and, considering their high expression in excretory organs, together with MATEs are responsible for drug excretion. For this reason, OCTs and MATEs can be important determinants of drug efficacies and also toxicities. OCTNs are transporters involved in the cellular uptake of substances, which are important in cell metabolism and in signal transmission, such as the antioxidant ergothionein and the neurotransmitter ACh (substrates of OCNT1) and the quaternary ammonium compound L-carnitine (substrate of OCNT2), which is of pivotal importance for cell energy production. Even though the expression and function of orthologs of transporters for OCs is generally similar, still there are important differences (e.g. hOCT1 is not expressed in the basolateral membrane of renal proximal tubules, in contrast with what observed for mOct1 and rOct1) that have to be considered for a proper interpretation of translational studies. Paralogs of transporters for organic cations often display similar characteristics, however they show also important differences e.g. with regard to interaction with substrates and to regulation. Other important functional aspects of transporters for organic cations, such as the molecular correlates of polyspecificity, regulation, interaction with drugs, genetic variations, role in the central nervous system, and distribution in the plants are discussed in the other sections of this book.

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Chapter 2

Functional Properties of Organic Cation Transporter OCT1, Binding of Substrates and Inhibitors, and Presumed Transport Mechanism

Hermann Koepsell and Thorsten Keller

Abstract Organic cation transporters (OCTs) of the SLC22 family mediate absorption, distribution and excretion of cationic drugs. The OCTs belong to the major facilitator superfamily (MFS) containing transporters with 12 pseudosymmetrically arranged transmembrane α -helices. Whereas most transporters of the MFS are substrate selective and secondary active, most transporters of the SLC22 family are polyspecific facilitative diffusion systems. Recently resolved crystal structures of MFS transporters indicate translocation via alternating access surpassing a state with substrate occlusion. After cloning of the rat transporters rOCT1 and rOCT2, the functional properties of these transporters have been investigated employing tracer uptake measurements, electrical measurements, voltage clamp fluorometry, and substrate binding measurements. Extensive mutagenesis studies in rOCT1 were interpreted in frame of tertiary structures that were modeled according to lactose permease which belongs to the MFS. Considering rOCT1 and rOCT2 as OCT prototypes, and assuming that all transporters of the MFS undergo similar interhelical movements during transport, a model for the translocation mechanism of OCTs is proposed. The model suggests that two small organic cations bind to the innermost cleft of the outward-facing conformation of OCTs and that translocation can be performed when either one or two cations are loaded per transporter monomer. With this model recent experimental results concerning interaction of ligands at OCTs can be explained that have high biomedical impact for in vitro testing.

Keywords Organic cation transporters • OCT1 • SLC22 • MFS • Transport mechanism • Polyspecificity • Mutagenesis • Tertiary structure • Modeling

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Introduction

The organic cation transporters OCT1, OCT2 and OCT3 of the *SLC22* transporter family are polyspecific facilitative diffusion systems with overlapping substrate specificities [1–3]. The *SLC22* family belongs to the major facilitator superfamily (MFS) which represents the second largest superfamily of transporters following the superfamily of ABC transporters [4]. The MFS contains secondary active cotransporters and antiporters and facilitative diffusion systems including the OCTs. The driving force for transport by OCTs is provided by the concentration difference of the respective cationic substrate across the plasma membrane and the electrical membrane potential. Because OCTs can mediate uniport of organic cations across the plasma membrane, their translocation of cations can be analysed by electrical measurements.

The purpose of the present article is to discuss the current knowledge concerning substrate recognition and transport mechanism of OCTs and to provide a comprehensible hypothesis for substrate recognition and transport mechanism of OCTs that includes very recent findings. In a first part we summarize the characterization of transport by rat organic cation transporters rOCT1 and rOCT2 and present experimental evidence which indicates that translocation by OCTs occurs by an alternating access transport mechanism. Because the current molecular understanding of functions of OCTs is mainly based on extensive mutagenesis of rOCT1 and on crystal structures of transporters of the MFS in different conformations, we included a second part in which we discuss the interpretation of mutagenesis experiments in general and the benefits and limitations of structural models of OCTs that are performed on the basis of crystal structures of MFS transporters. In this part we also present the current concept of alternating access transport mechanism by transporters of the MFS. In a third part of the review, current knowledge about structure-function relationship of rOCT1 is presented which is derived from functional characterizations of rOCT1 mutants in combination with models of tertiary structures in the outward-facing and inward-facing conformation. In this part we describe mapping of cation binding sites, elucidation of quaternary structure and transporting unit, measurements of transport-related structural changes, identification of a structural key element for translocation, and identification of high affinity binding sites. We also mention some of our very recent data concerning the identification of MPP binding sites in the innermost part of the outward-facing binding cleft that have not been published so far. In the fourth part of the review we provide a model for substrate binding and translocation that is derived from the described functional data. In this part we point out how recent experiments indicating that inhibitor affinities are dependent on substrate concentration and substrate structure, can be explained within the new model.

Functional Properties of rOCT1 and rOCT2

Substrate and Inhibitor Selectivity

OCT1, OCT2 and OCT3 have been cloned from different species including human and have been characterized functionally [1–3]. Different selectivities of substrates and inhibitors were observed between OCT1, OCT2 and/or OCT3 within individual species and for the individual OCT subtypes between different species. The functional characterizations are consistent with the view that independent of species the three OCT subtypes have the same basic properties. This includes the independence from sodium gradient, transport in both directions, and the possibility to function as electrogenic uniporter or electroneutral exchanger. This may be an over-simplified view because an in depth characterization of transporter properties has only been performed for rOCT1 and rOCT2, and data were obtained suggesting that the stoichiometry between translocation of charges and organic cations is different between rOCT1 and rOCT2 [5]. Similar to all OCTs characterized so far, rOCT1 and rOCT2 translocate organic cations with widely differing molecular structures and are inhibited by a large number of compounds that are not transported [6]. Tetraethylammonium (TEA), 1-methyl-4-phenyl-pyridinium (MPP), ¹N-methylnicotinamide, histamine, choline and *d*-tubercidine are transported by rOCT1 and rOCT2 with similar apparent K_m values [7, 8]. At variance guanidine and dopamine are common substrates of rOCT1 and rOCT2, however, 10-fold and 40-fold lower apparent K_m values have been determined for rOCT1 versus rOCT2, respectively [7, 9]. Quinine and serotonin are transported by rOCT1 but not by rOCT2 [6]. Whereas tetrapropylammonium (TPPrA), tetrapentylammonium (TPeA) and desipramine are common inhibitors of rOCT1 and rOCT2 with similar IC_{50} values, corticosterone inhibits rOCT2 with a 38-fold lower IC_{50} value compared to rOCT1, and procainamide inhibits rOCT2 with a 68-fold higher IC_{50} [7]. Evidence has been presented that both rOCT1 and rOCT2 translocate organic cations in an electrogenic manner when they operate under *trans*-zero conditions as uniporters, whereas cation transport is electroneutral when they operate in the cation exchange mode [7, 10–13]. It has been shown that both rOCT1 and rOCT2 operate independently from sodium and chloride, and data were obtained suggesting that they function independently of proton gradients [10, 12–14].

Stoichiometry Between Translocation of Charge and Cations

The stoichiometry of rOCT1 between translocated cations and charges appears to be different compared to rOCT2. For tracer uptake of TEA, choline and MPP by rOCT1 and rOCT2 similar V_{max} values were obtained, however, the currents induced

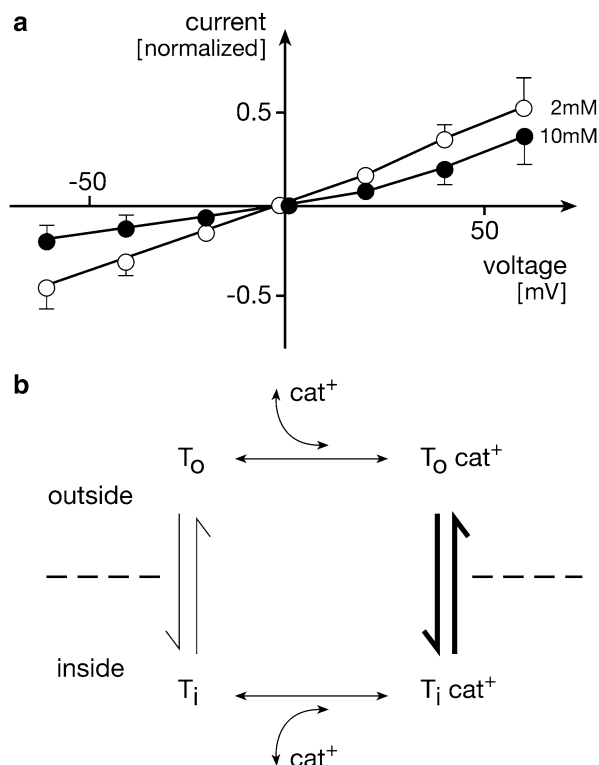
by saturating concentrations of these compounds were up to ten times smaller in oocytes expressing rOCT1 versus rOCT2. This difference is supposed to be due to a translocation of small cations together with organic cation substrates by rOCT2. Measuring rOCT2 mediated translocation of organic cations in parallel with translocation of positive charge, translocation of a surplus of positive charge was observed at low membrane potential [5]. This surplus of charge translocation could not be explained by leakage for small ions during the transport cycle. It was dependent on the presence of a negatively charged amino acid within the innermost part of the modeled outward-open binding pocket of rOCT2 which is not conserved in rOCT1 [5].

Demonstration of Transporter Function

The findings that one subtype of the SLC5 family, SLC5A4 also called SGLT3, is a Na^+ -D-glucose cotransporter in pig and a glucose-ligated ion channel in human [15], and that protein EcClC from *Escherichia coli* which has a channel like structure containing a transmembrane path and a selectivity filter functions as Cl^-/H^+ antiporter [16–18], indicate that the structural differences between channels and transporters may be small. However, the functional distinction between channel and transport activity is useful and of theoretical and practical relevance. During transport substrate translocation is stoichiometrically linked to structural changes of the protein which are typically relatively large. Transport always includes binding and dissociation of the substrate which allows a subtle differentiation of substrate selectivity between transporters. In contrast channel activity comprises opening of transmembrane pathways that are controlled by gating mechanisms which include conformational changes in the protein that may be small. The selectivity of channels is determined by selectivity filters. Because open channel pathways may allow passage of a restricted number of ions and may contain binding sites for the translocated ions, channel activity may show saturation similar to transporters. Since rOCT1 and rOCT2 facilitate diffusion of structurally different compounds across the membrane it was important to clarify whether they function as transporters as generally assumed, or as poorly selective channels with short opening times which would explain the relatively low rates of cation translocation.

For rOCT2 mediated TEA^+ induced inward currents in oocytes measured under *trans-zero* condition at -50 mV, a high activation energy of 39 kJ/mol was determined indicating that transporter typical large conformational changes are associated with TEA^+ translocation [14]. Electrical measurements performed with inside-out oriented giant patches from oocytes in which rOCT2 was expressed, strongly support transport function [12]. When 2 mM choline or 10 mM choline were present on both sides of the giant patches and different membrane potentials were applied, symmetrical and potential dependent inward or outward currents were measured (Fig. 2.1a). Importantly, the currents observed with 2 mM choline were higher compared to the currents observed with 10 mM choline. This observation

Fig. 2.1 Higher voltage induced currents mediated by rOCT2 with 2 mM choline versus 10 mM choline on both membrane sides indicate electrogenic transport. **(a)** Current–voltage relationship in giant patches from *Xenopus laevis* oocytes in which rOCT2 was expressed and 2 or 10 mM choline were present at both sides of the plasma membrane. **(b)** Simple transporter model allowing electrogenic cation uniport and more rapid electroneutral cation exchange



contradicts channel-like activity because in a channel a higher concentration of choline would lead to higher voltage dependent currents. In contrast the data are consistent with the alternating access transporter model shown in Fig. 2.1b. The model implies two possible transport modes, electrogenic uniport and electroneutral cation exchange. In both modes a cation binds to the outside-facing substrate binding site, the bound cation is exposed to the intracellular side and released. Finally the empty inward-facing substrate binding site is transposed to the extracellular side and a second uniport cycle can be started. In the electroneutral cation exchange mode an intracellular cation binds to the inward-facing substrate binding site and the cation-loaded substrate binding site is exposed to the extracellular site where the counter-transported cation is released. Because the electrogenic transport mode is preferred at low substrate concentration, the higher currents observed with 2 mM choline compared to 10 mM choline are consistent with transport function. Experimental evidence for transport has been also provided by demonstrating *trans-stimulation* of rOCT1 mediated uptake under voltage-clamp condition [13]. These experiments were performed with proteoliposomes containing purified rOCT1 in which the membrane potential was clamped to zero by valinomycin in the presence of equal concentrations of potassium on both sides of the membrane. Under these conditions uptake of radioactive labeled MPP was stimulated when the proteoliposomes were

loaded with choline. This observation is not consistent with channel activity. However, it can be explained by the transport model assuming that the conformational changes of the transporters leading to the in–out orientation of the choline-loaded substrate binding site occurs more rapidly than the structural changes leading to in–out orientation of the empty transporter (Fig. 2.1b).

Experimental support that rOCT2 is a transporter that operates according to the alternating access model [19, 20] was provided by experiments in which rOCT2 mediated transport was inhibited by corticosterone or TBUA that were applied either from the extracellular side of the plasma membrane or from the intracellular side [21]. Competition experiments indicated that both compounds interact within the substrate binding pocket. We characterized the interaction of corticosterone and TBUA with the outward-facing substrate binding pocket of rOCT2 by measuring the short-term inhibition of TEA induced inward-currents in intact oocytes expressing rOCT2, and the interaction with the inward-facing substrate binding pocket by measuring short-term inhibition of TEA induced outward-currents in giant patches obtained from rOCT2 expressing oocytes [21]. Corticosterone had a higher affinity when applied from extracellular compared to intracellular whereas TBUA had a lower affinity from extracellular compared to intracellular. The data indicate that the substrate binding region of rOCT2 can be exposed to both sides of the plasma membrane and exhibits different affinities for substrates and inhibitors in both conformations as postulated for the alternating access transport mode.

Considerations about Interpretation of Functional Effects of Mutations

To unravel functional mechanisms of individual transporters, crystal structures of different functional states and functional characterization of point mutations in critical positions are required. In addition the quaternary structure of the transporter must be known. If the transporter forms dimers or oligomers, it must be known whether the monomers function independently or not. The interpretation of functional effects of point mutations may be straight forward if crystal structures of the same transporter have been solved, whereas the interpretation must remain speculative if no crystal structure is available. In this situation modeling of tertiary structures according to known structures from transporters of the same family or superfamily are helpful for interpretation.

Changes of $V_{\max}$ values measured after overexpression of mutants in cells are difficult to interpret if the exchanged amino acid is not located in a functionally relevant domain of the crystallized or modeled transporter. Reasons are that the exchange of amino acids within parts of transporters that are not directly involved in substrate recognition and/or transport but are important for the formation of the correct tertiary structure, may result in ineffective targeting to the plasma membrane and/or to an inactive or partially active misfolded transporter within the membrane. An additional difficulty is that the available methods to determine the amount of

transporter incorporated into the plasma membrane do not allow the identification of small differences. Measurements of $V_{\max}$ values after reconstitution of transporters in proteoliposomes circumvent the problem of the potential effect of mutations on targeting.

Similar to $V_{\max}$, changes of apparent K_m values observed after point mutations are difficult to interpret when the crystal structure has not been solved. One reason is that the apparent K_m is a bulk constant that may be influenced by rate constants of different steps during translocation including substrate binding at the extracellular side and substrate release at the intracellular side. In case of a cotransporter in which the binding of the cosubstrate increases the affinity for substrate binding, effects of mutations on the binding of the cosubstrate or on the allosteric effect of cosubstrate binding may change the apparent K_m . In addition to these obstacles for correct interpretation it must be kept in mind that point mutations in peripheral parts of a transporter may induce structural changes that lead to alterations of the apparent K_m . In case of polyspecific transporters effects of point mutations on K_m values of different substrates can be determined in order to evaluate effects on selectivity, however, also in this case a solid interpretation is only possible in combination with a crystal structure and a modeled structure is required to raise educated hypotheses.

If point mutations alter IC_{50} values determined for non-transported competitive inhibitors measured in cells, it can be concluded that the structure of the substrate binding site has been altered directly or indirectly. In case of polyspecific transporters differential effects of point mutations on IC_{50} values for different substrates are of specific interest. Such type of investigations may provide indirect support for the interaction of substrates and inhibitors with different binding sites in a binding region that may be overlapping (see below). In such a scenario it has to be kept in mind that allosteric interactions between binding of substrates and inhibitors may occur. Again a modeled tertiary structure of the binding cleft may help to raise hypotheses.

The analysis of partial reactions of the transport cycles after point mutations has high impact for the elucidation of transport mechanisms. These studies include direct measurements of binding of substrates, cosubstrates and inhibitors, determinations of stoichiometries between substrate and cosubstrate or translocation of charged substrate and charge, effects of substrate induced conformational changes etc. In combination with crystal structures these types of studies are required to unravel transport mechanism. In combination with modeled structures such types of studies are helpful for generation of hypotheses.

Considerations about Modeling of OCT Structures According to Crystal Structures of MFS Transporters

So far 16 crystal structures of transporters from MFS have been reported. Although the different transporters have less than 20 % amino acid identity between each other, they all exhibit the same pseudosymmetric structure consisting of an

N-terminal and a C-terminal part, each comprising six TMHs. Five different structural states of MFS transporters have been crystallized; an open outward-facing state (fucose transporter FucP from *E. coli* [22]), a ligand-bound, outward-facing, partially occluded state (xylose transporter XylE from *E. coli* [23]), an occluded state (oxalate transporter OxlT from *Oxalobacter formigenes* [24], multidrug transporter EmrD from *E. coli* [25], nitrate/nitrite transporter NarU from *E. coli* [26]), an inward-facing occluded state (phosphate transporter PiPT from *Piriformospora indica* [27], peptide transporter PepT_{so} from *Shevanella oneidensis* [28]), and an open inward-facing state (the lactose permease from LacY *E. coli* [29–32], glycerol-3-phosphate transporter GlpT from *E. coli* [33], peptide transporter PepT_{st} from *Streptococcus thermophilus* [34], peptide transporter PepT_{so2} from *S. oneidensis* [35], peptide transporter GkPOT from *Geobacillus kaustophilus* [36], nitrate/nitrite transporter NarK from *E. coli* [37], and nitrate transporter NRT1.1 from *Arabidopsis* [38]). Whereas the open outward-facing and the open inward-facing state are mainly formed by en-block movements of the pseudosymmetric transporter halves without significant bending of individual TMHs, the formation of the occluded states required bending and movements of individual helices in addition to en bloc movements of transporter halves. The large overall similarities between the crystal structures of different transporters including proton-cotransporters, a sodium-cotransporter and polyspecific facilitative diffusion systems suggest that the MFS transporters have a common basic structure that allows the alternating access related conformational transitions that have been described as rocker-switch movement of the two transporter halves [39]. The functional difference between the transporters are supposed to be due to transporter specific structural differences in the binding sites, in regions that are responsible for energy coupling and in regions that form the translocation pathways. In conclusion a modeling of the tertiary structure of OCTs on the basis of MFS transporters is expected to provide the structural backbone of OCTs allowing alternating access but cannot provide details explaining polyspecific translocation of cations by facilitative diffusion.

Theoretical Considerations About Polyspecificity of OCTs

Accepting that OCTs are transporters that operate in the alternating access mode, polyspecificity must be explained by polyspecific cation binding that induces transporter related conformational changes rather than by poorly discriminative selectivity filters. Although allosteric changes within the substrate binding regions of polyspecific transporters are expected after binding of a specific substrate, it can be excluded that the potential of rOCT1 and rOCT2 to bind structurally different cations like TEA and MPP is mainly due to an induced fit mechanism. In the absence of pre-existing binding sites for TEA and MPP, the interaction energy between the cations and the binding region is supposed to be too low to induce a structural change within the binding pocket that could form a selective binding site exhibiting tight interaction with the respective cation. In addition the tertiary structure of the

transporters is supposed to restrict the degrees of freedom allowing substrate induced structural changes that would be required to induce polyspecificity. In conclusion, polyspecific binding to OCTs is supposed to require cation binding to several binding sites that interact with different ligand structures. These binding sites may be located within a binding region as has been demonstrated for p-glycoprotein [40, 41]. An intriguing question is how binding at different sites can induce the conformational changes that mediate cation translocation or in other words how ligand protein interaction at different sites can provide the activation energy to overcome the activation barrier for translocation [42, 43].

Initial Mutagenesis Experiments in rOCT1

We started our mutagenesis analysis of rOCT1 by exchanging amino acids of rOCT1 which are conserved in OCTs but not found in the organic anion transporters (OATs) of the *SLC22* family [44]. In another study we replaced all amino acids of the fourth TMH that contains many amino acids that are conserved in the OCTs [45]. For functional characterization we expressed the rOCT1 mutants in oocytes of *Xenopus laevis* and compared K_m and V_{max} values for different substrates as well as IC_{50} values for inhibition of TEA and/or MPP uptake by transported as well as non-transported inhibitors. To identify effects of mutations on transporter targeting to the plasma membrane we performed Western blots of isolated plasma membranes. The experiments revealed that the replacement of aspartate 475 in the 11th TMH of rOCT1 by glutamate reduced the amount of transporter in the plasma membrane by 90 % and decreased the apparent K_m value for TEA eightfold whereas the apparent K_m value for MPP was not changed. Similar to the K_m for TEA the IC_{50} values for the competitive non transported inhibitors TBuA, tetrapropylammonium (TPrA) and tetrapentylammonium (TPeA) were decreased. We interpreted that aspartate 475 is probably directly involved in binding of TEA, TBuA, TPrA and TPeA. Because rOCT1 mediated transport of TEA is competitively inhibited by MPP and vice versa, we interpreted that rOCT1 probably contains a substrate binding region with overlapping binding domains for TEA and MPP. Conservative replacements of 18 amino acids in the fourth TMH revealed that after replacement of Trp218 by tyrosine (W218Y) and Tyr222 by leucine (Y222L) the K_m values of both TEA and MPP were decreased whereas after replacement of Tyr222 by phenylalanine (Y222F) only the K_m value for TEA and after replacement of Thr226 by alanine (T226A) only the K_m value for MPP was decreased. Presuming that the K_m values were reflecting the affinity of substrate binding to the outward-facing conformation of rOCT1, these data supported the existence of an outward-facing binding region with overlapping binding domains for TEA and MPP. The data did not allow to differentiate whether Trp218, Tyr222 or Thr226 are directly involved in binding of TEA and/or MPP or whether the mutations in these positions altered the structure of the substrate binding domains.

Employing the nearly 40-fold higher affinity of corticosterone for inhibition of cation uptake by rOCT2 versus rOCT1, we identified the amino acids which are responsible for this difference. When amino acids Ala443, Leu447 and Gln448 of rOCT1 were replaced by the respective amino acids of rOCT2 (Ile443, Tyr447, Glu448), corticosterone inhibited cation uptake of the rOCT1 mutant with the same affinity as rOCT2 wildtype [46]. The measurements were performed by expressing the transporters in *X. oocytes* and measuring the effects of corticosterone on the uptake of 10 μM TEA or 0.1 μM MPP using an incubation time of 30 min. Although corticosterone is not transported by rOCT1 or rOCT2 we could not differentiate whether the observed inhibition occurred from extracellular or intracellular, because corticosterone passively permeates the plasma membrane. Considering also that the amino acid exchanges may exhibit indirect effects on the extracellular and/or intracellular corticosterone binding site(s), the data did not provide unequivocal information about the binding site(s) for corticosterone. Because the triple rOCT1 mutant (A443I,L447Y,Q448E) showed decreased apparent K_m values for TEA and MPP, and the IC_{50} values for corticosterone inhibition of TEA uptake versus MPP uptake were different, the data suggested complex interactions between substrate and corticosterone binding sites.

Modeling and Mapping of the Open, Inward-Facing Binding Cleft of rOCT1

2003 the first two high-resolution crystal structures of transporters of the MFS family were published; the glycerol-3-phosphate transporter GlpT from *E. coli* [33] and lactose permease LacY from *E. coli* [29]. Both transporters were crystallized in the open, inward-facing conformation. Using the crystal structure of LacY as template, we modeled rOCT1 in this conformation. The modeled open, inward-facing cleft was formed by TMHs 1, 2, 4 and 5 of the left transporter half and TMHs 7, 8, 10 and 11 of the pseudosymmetric right transporter half (Fig. 2.2c). TMHs 1, 4, 7 and 8 contained distinct bends whereas the other cleft forming TMHs were straight or showed minor bending. In the model Asp475 in TMH 11, Phe160 in TMH 2, Trp218 in TMH 4, and Arg440 in TMH 10 are localized relatively close together in the innermost part of the inward-facing cleft whereas Tyr222 in TMH 4 and Gln448 in TMH 10 are located more peripheral close to the cytosol (Fig. 2.2c). According to the modeled cleft all identified amino acids with the exception of Gln448 have contact with the aqueous phase. Viewing from intracellular into the inward-facing cleft suggests different compartments within the cleft (Fig. 2.2d).

For characterization of the inward-facing cleft by mutagenesis, we measured whether the inhibition of TEA induced inward-currents by intracellular corticosterone was changed after replacement of Leu447 by tyrosine or of Gln448 by glutamate which had been shown to influence the affinity of corticosterone [46, 47]. The mutations in both positions decreased the IC_{50} values for corticosterone suggesting

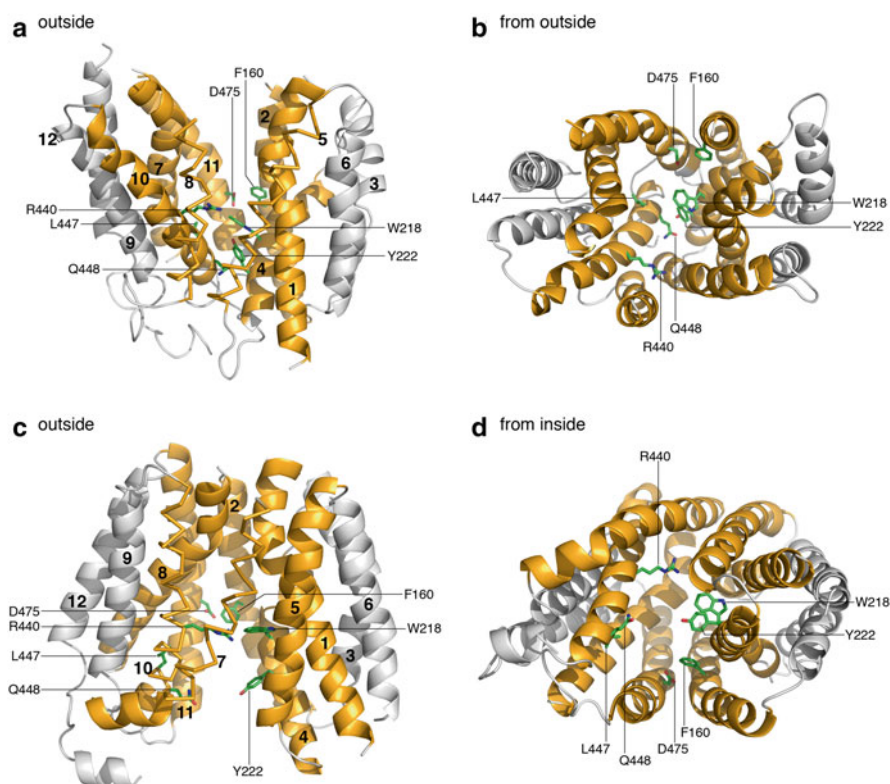


Fig. 2.2 Modeled rOCT1 conformations with outward-facing or inward-facing substrate binding clefts. (a) Outward-facing conformation, side view, (b) Outward-facing conformation, view from extracellular, (c) Inward-facing conformation (side view), (d) Inward-facing conformation, view from intracellular. The TMHs forming the cleft are indicated in orange. Amino acids that have been identified to be critical substrate affinity, affinity of inhibitors and/or transport are indicated. The TMHs are numbered

that corticosterone interacts with the substrate binding domain. These mutations were performed in mutant rOCT1(C451M) which showed higher cation induced currents compared to rOCT1 wildtype [48]. To measure inhibition of TEA induced inward currents by intracellular corticosterone, oocytes were clamped to -50 mV, equilibrated for 10 min with corticosterone, washed for 1 min, and TEA-induced inward currents were measured by superfusion for 45 s with TEA. Trying to further define the interaction site for corticosterone we performed docking experiments of corticosterone with the modeled inward-facing cleft [47]. The obtained data suggest interaction of corticosterone with Phe160 (TMH 2), Trp218 (TMH 4), Arg440 (TMH 10) Leu447 (TMH 10), and Asp475 (TMH 11). Mutagenesis of Phe160, Trp218, Arg440 or Asp475 revealed that the affinity for inhibition of TEA uptake by intracellular corticosterone was increased after replacement of Phe160 by alanine and after replacement of Asp475 by glutamate, whereas maximal inhibition of

TEA uptake by intracellular corticosterone was decreased after replacement of Trp218 by phenylalanine and Arg440 by lysine. Taking together the data indicate that the mutations of Phe160, Trp218, Arg440, Leu447 and Asp475 change the interaction of corticosterone from intracellular either directly or by short-distance allosteric effects. They support the modeled inward-facing cleft. Relevance of Phe160, Trp218, Arg440 and Leu447 for cation translocation was shown by demonstrating that the affinity of MPP to inhibit TEA uptake was changed by mutations in these positions. Noteworthy Phe160 and Arg440 are located within the inner most part of the cleft in about the same plane as Trp218 and Asp475 (Fig. 2.2c).

Modeling and Mapping of the Open, Outward-Facing Binding Cleft of rOCT1

The open, outward-facing cleft of rOCT1 was modeled by applying the en-bloc rearrangement mechanism of the two pseudosymmetric halves of the modeled inward-facing conformation of rOCT1 as has been proposed for conformational change of LacY during transport [49, 50] (Fig. 2.2a, b). In the outward-facing cleft Phe160, Arg440 and Asp475 are located within the inner third of the outward-facing cleft about in the center of the presumed plasma membrane. Noteworthy, Gln448 and Tyr222 are located at the bottom of the cleft about 20 Å closer to the cytosolic surface of the plasma membrane (Fig. 2.2a) suggesting that cation occlusion may occur in the space between these two different groups of amino acids. Trp218 is located within this space. The top view from extracellular into the cleft (Fig. 2.2b) suggests a division into three compartments lined by Asp475, Trp218 and Phe160, by Asp475, Trp218 and Leu447, or by Tyr222, Glu448 and Arg440.

For characterization of the outward-facing cleft we measured the effects of mutations of Phe160, Trp218, Arg440, Leu447 or Asp475 on the inhibition of TEA or MPP uptake by extracellular corticosterone [47]. The K_i values for inhibition were changed after replacement of Phe160 by alanine, Leu447 by tyrosine and Asp475 by glutamate, whereas the maximal inhibition was reduced after replacement of Trp218 by phenylalanine and Arg440 by lysine. The data indicate that the mutations alter the interaction of corticosterone within the outward-facing binding cleft. The data support the modeled outward-facing cleft.

Elucidation of Quaternary Structure and Transporting Unit of rOCT1

To interpret the effects of the point mutations in rOCT1 on affinities and maximal inhibition of cation uptake correctly, the quaternary structure of rOCT1 and the functional unit for cation transport must be known. MFS transporters may exist as

monomers in the plasma membrane or may form dimers or oligomers which may function independently or in concert. LacY and the sugar/phosphate antiporter UhpT from *E. coli* are present as monomers [51, 52] whereas the reduced folate carrier RFC from human is present as homooligomer in which the monomers function independently [53]. Lactose transport protein LacS from *Streptococcus thermophilus* forms dimers which are required for proton driven lactose uptake [54]. Homo-oligomerization was demonstrated for human organic anion transporter hOAT1 [55] for rat OAT1, rOCT1, rOCT2 [56, 57] and human OCT2 (hOCT2) [58]. It has been also reported that surface expression of hOAT1 and hOCT2 was reduced when oligomerization was disturbed [58, 59]. In addition it was shown that the tertiary structure of the large extracellular loop between TMH 1 and 2 of rOCT1 or hOCT2, which is stabilized by disulfide bridges, is essential for oligomerization [57, 58]. We provided evidence that both monomers of rOCT1 may transport independently under *trans*-zero conditions by showing that the apparent K_m values for TEA and MPP uptake remained unchanged when oligomerization was prevented, and that a tandem protein containing two rOCT1 monomers showed about 50 % transport activity with unchanged K_m values when one monomer was blocked [57]. Cooperativity of monomers concerning transport of other substrates, cation exchange or inhibition of transport has not been excluded.

Analysis of Transport-Related Structural Changes of rOCT1

Employing capacitance measurements with oocytes expressing rOCT2 or an rOCT1 variant with increased electrical activity, substrate and inhibitor dependent movement of charges within the proteins were detected [14, 50]. Since the capacitance changes were also observed with the uncharged inhibitor corticosterone, they are due to charge movements of charged amino acids of the transporter proteins indicating structural changes. Similar capacitance changes were observed with the non-transported inhibitor TBA and transported cations. This suggests that the observed structural changes in response to transported cations are induced by the initial cation binding step during transport.

To determine whether individual TMHs of rOCT1 move in response to changes of the membrane potential, during cation binding and/or cation translocation we performed voltage-clamp fluorometry with transporter variants that were fluorescence-labeled at amino acids of individual TMHs that are located at the boundary between plasma membrane and extracellular aqueous phase [50, 60]. The experiments were performed with a rOCT1 variant in which all cysteine residues with exception of those forming the disulfide bridges in the large extracellular loop had been removed [48]. In this variant individual amino acids were replaced by cysteine and the introduced cysteine residue was labeled with the covalently binding fluorescent dye tetramethylrhodamine-6-maleimide. We observed membrane potential-dependent movements of TMHs 5, 8 and 11 which were partially or totally reversed by addition of transported cations or of the non-transported cationic inhibitor

TBuA. Potential-dependent movements of TMH 11 were strongly effected by choline, MPP and only slightly influenced by TBuA. At variance choline, MPP and TBuA induced differential effects on potential-dependent movements of TMH 8. The data indicate potential-dependent structural changes of rOCT1 that involve TMHs 5, 8 and 11. The open, outward-facing conformation of rOCT1 is supposed to be the predominant conformation at the physiological membrane potential around -50 mV because it allowed rapid and complete inhibition of rOCT1 by extracellular TBuA. This conformation—or equilibrium of conformations—is changed after binding of TBuA and probably also after binding of choline and MPP. The similar movements of TMH 11 observed with choline, MPP and TBuA suggest that TMH 11 is involved in transport related conformational changes independently of the structure of the interacting cation. The cation specific movements of TMH 5 and 8 indicate cation specific structural movements.

Identification of a Substrate Binding Hinge Domain in OCTs

Our data suggested an important role of TMH 11 during cation translocation. First, movements of TMH 11 were induced by the three tested compounds choline, MPP and TBuA [60]. Second, exchange of Asp475 in the middle of TMH 11 by glutamate decreased $V_{\max}$ for TEA and MPP and changed substrate selectivity [44]. Third, also the exchange of Phe483 at the transition of TMH 11 to the extracellular space changed cation selectivity [60]. Fourth, exchange of Cys474 in hOCT2 by alanine increased the affinity for TEA [61], and fifth, the Gly447–Gly448 motif in TMH11 suggests mobility for bending of the α -helix that may be required for the transition between open and closed states of the transporter [60].

When the freedom for bending of TMH 11 was reduced by exchange of Gly448 with cysteine, uptake of MPP was decreased whereas the K_m for MPP was not changed [60]. In addition, the voltage dependent movement of TMH was blocked [60]. The data suggest that a decrease of flexibility in the middle of TMH 11 impairs movement of TMH 11 and transport activity of structurally different cations. The open outward-facing model of rOCT1 suggests that Asp475 and Phe160 form part of the entrance to the innermost cavity of the outward-facing cleft (Fig. 2.3a, b). Trying to verify this aspect of the structural model, we investigated whether access of cations can be blocked by covalent labeling of a cysteine residue that was introduced in position 448, with the covalent binding substrate analog tetramethylrhodamine-6-maleimide. The location of Gly448 within or close to a transport-relevant cation binding center was indicated by the observation that the covalent labeling of rOCT1(G478C) mutant was partially blocked by transported cations. After covalent labeling of Cys478 with tetramethylrhodamine-6-maleimide cation transport was largely reduced. This can be explained by blockage of cation access to the innermost cavity of the outward-facing cleft or by blockage of transporter related conformational changes. Taken together the data indicate that the motif CDXGGI that is conserved in OCT1-3 but not in OCTNs or OATs represents an OCT specific substrate binding hinge domain.

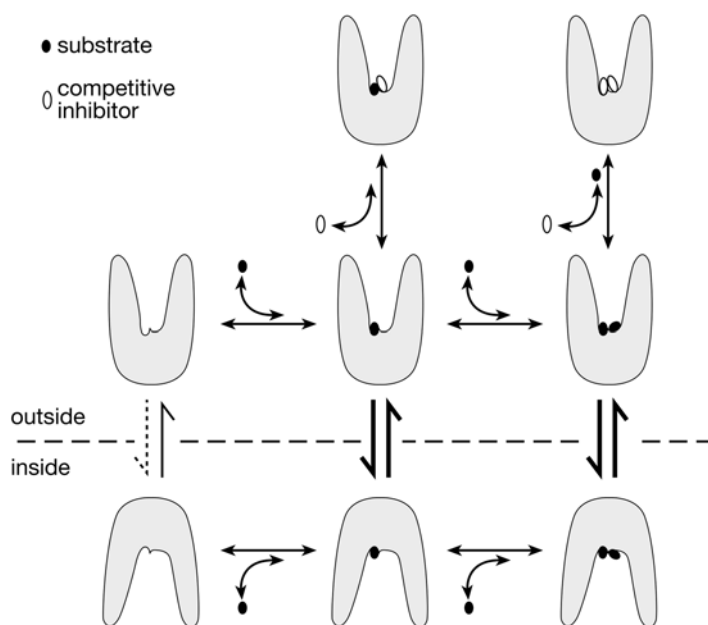


Fig. 2.3 Proposed mechanism for translocation of small organic cations by rOCT1 in the one cation/monomer and two cations/monomer modes, and for inhibition of transport by a competitive inhibitor. The two low-affinity transport-relevant cation binding sites are indicated whereas the high-affinity binding site is not shown. Both low-affinity sites are closely associated. After loading of the outward-facing cleft with one or two cationic substrates the equilibrium of rOCT1 conformations may change in favour of an inward open conformation. In the inward open conformation one or both cationic substrates may be released. The inward open transporter conformation may change to the outward open state in loaded forms or in the unloaded state. A competitive inhibitor applied from extracellular may inhibit cation uptake with different affinities if the transporter operates in one cation/monomer mode or two cations/monomer mode. The affinity for inhibition of the one cation/monomer mode may be also dependent on the structure of the employed substrate

Effect of Mutations on the Binding of MPP to rOCT1 Reconstituted into Nanodiscs

Recently we succeeded to measure binding of radioactively labeled MPP to purified rOCT1 which was reconstituted into nanodiscs consisting of a lipid bilayer that is framed by the amphiphilic membrane scaffold protein MSP1 (T. Keller, F. Bernhard, V. Doetsch, V. Gorboulev, H. Koepsell, unpublished data) [62]. Cell-free expressed rOCT1 was co-translationally incorporated into the nanodiscs [56, 62, 63], and binding of different concentrations of MPP was measured using a filter assay. The lipid bilayer consisted of di-myristoyl phosphatidylcholine (DMPG) or palmitoyl-oleyl phosphatidylcholine (POPC). The amount of rOCT1 protein in the nanodiscs was measured by quantification of Western blots, and dissociation constants (K_D) and maximal binding (B_{max}) were determined. In parallel, cell-free expressed rOCT1

was reconstituted into proteoliposomes formed from phosphatidylcholine, phosphatidylserine and cholesterol [56], and the apparent K_m and V_{max} values were calculated.

After reconstitution of rOCT1 wildtype into nanodiscs formed from DMPG or after reconstitution of a rOCT1 variant in which oligomerization was prevented by replacement of the cysteine residues in the large extracellular loop (rOCT1-6 Δ C) [57], binding of two MPP molecules per rOCT1 monomer and one K_D of about 30 μ M was determined (T. Keller, F. Bernhard, V. Doetsch, V. Gorboulev, H. Koepsell, unpublished data). However, when rOCT1 or rOCT1-6 Δ C was reconstituted in nanodiscs that were formed with POPC, binding of three molecules MPP per rOCT1 monomer was observed. For binding of two MPP molecules a common low affinity K_D of 36 μ M was obtained whereas for binding of the third MPP molecule a high affinity K_D of 0.24 μ M were determined. For MPP transport into proteoliposomes reconstituted with rOCT1 wildtype only one K_m of 19 μ M was resolved. The data suggest that the interaction of MPP with two low affinity MPP binding sites per rOCT1 monomer is relevant for transport.

We also measured MPP binding to rOCT1 mutants that were reconstituted into nanodiscs formed with POPC as well as MPP uptake by rOCT1 mutants that were reconstituted into proteoliposomes. First we exchanged Asp475 or Trp218 which are located in the same region of the modeled outward-facing cleft of rOCT1 (Fig. 2.2a, b). We observed that only two MPP molecules bound per OCT1 monomer when either Trp218 was replaced by tyrosine (rOCT1-W218Y) or when Asp475 was replaced by glutamate (rOCT1-D475E). Whereas in both mutants the K_D value of the high affinity site remained unchanged, the K_D value of the low affinity site was decreased by 50 % in rOCT1-W218Y and by 25 % in rOCT1-D475E. The K_m values determined for mutants rOCT1-W218F and rOCT1-D475E were similar to rOCT1 wildtype, however, the turnover numbers were decreased by 50 % in rOCT1-W218F and by 80 % in rOCT1-D475E.

We also investigated the effects of replacements of Leu447 by phenylalanine or tyrosine (T. Keller, F. Bernhard, V. Doetsch, V. Gorboulev, H. Koepsell, unpublished data). Leu447 is located in a different region of the outward-facing binding cleft of rOCT1 than Asp475 and Trp218 (Fig. 2.2a, b). The mutations of Leu447 did not change total binding of three MPP molecules per transporter monomer observed in rOCT1 wildtype. Also the K_D value for high affinity MPP binding was not significantly different compared to rOCT1 wildtype. At variance, the K_D of the low affinity site was 2.5-fold decreased in rOCT1-L447F and 4.6-fold decreased in rOCT1-L477Y. For MPP transport by rOCT1-L447F and rOCT1-L447Y twofold lower K_m values compared to rOCT1 wildtype were observed whereas turnover numbers were similar.

The data indicate that the structure of the substrate binding region of rOCT1 is dependent on the lipid environment of rOCT1. Because the K_m value for rOCT1 mediated MPP transport measured in proteoliposomes is about fourfold higher compared to the K_m value of rOCT1 mediated MPP transport measured in cells, and the K_D value for low-affinity MPP binding to rOCT1 determined in nanodiscs is 40-fold higher compared to high-affinity MPP binding to a cysteine-less rOCT1

determined by voltage-clamp fluorometry (see below), the structure of the cation binding region may be modulated by various factors in cells that have not been mimicked after reconstitution in nanodiscs and/or proteoliposomes. In addition to a different lipid microenvironment in cellular membranes the structure of the binding region may be influenced by phosphorylation of rOCT1 in cells [64] and by interaction with cellular proteins [65]. Also the replacements of endogenous cysteine residues in rOCT1, the exchange of Phe483 by cysteine, and fluorescence labeling of cysteine in position 483 performed in the rOCT1 variant used for voltage-clamp fluorometry (see below), may have had an effect on the binding regions. However, in spite of the differences in absolute binding affinities observed with the different methods, the cell-free expressed transporter reconstituted in lipid bilayers of appropriate phospholipids provides an excellent model to study structure-function relationship of binding and transport.

After reconstitution into nanodiscs rOCT1 can bind MPP in the outward-facing and inward-facing conformation. After binding to the outward-facing conformation or after binding to the inward conformation MPP may be transported to the other side of the nanodiscs, however, since MPP in the aqueous phase is removed during the filter binding-assay, only MPP molecules are detected that are bound to the transporter. Because the outward- and inward-facing conformations are mutually exclusive, the determined stoichiometry indicates that three MPP molecules bind per binding cleft of rOCT1 monomer. We interpret that the high-affinity and low-affinity binding sites for MPP characterized in nanodiscs are located in the outward-facing binding cleft. One reason is that rOCT1 is mainly involved in cellular cation uptake, and that the cation binding sites for MPP in cellular uptake systems are supposed to have a higher affinity in the outward-facing compared to the inward-facing conformation. Other reasons are that we detected high-affinity and low-affinity cation binding sites in rOCT1 after application of the non-transported cationic inhibitor TBuA from extracellular [50] and that we observed high-affinity and low-affinity binding sites for inhibition of cation uptake by human OCTs after application of transported cationic drugs from extracellular [66].

From the observed effects of mutations of amino acids located in the innermost part of the outward-facing binding cleft important conclusions can be drawn. The data indicate that Tryp218 and Asp475 are directly or indirectly involved in MPP binding to one transport relevant low-affinity MPP binding site. After mutations in both positions the number of MPP molecules bound per monomer was decreased from three to two, and the K_D for low-affinity MPP binding was changed. In addition both mutants showed a decreased turnover number for MPP transport. Leu447 appears to be located close to the second transport relevant low-affinity MPP binding site. Mutations in this position decreased the affinity of low-affinity MPP binding and the K_m of MPP transport. Because mutations in this position did not reduce the number of MPP molecules bound per rOCT1 monomer Leu447 is probably not directly involved in MPP binding to the second low-affinity MPP binding site. The observation that the K_D of high-affinity MPP binding was not altered after the performed mutation suggests that the high-affinity site is not located within the innermost part of the outward-facing cleft. Our data do not allow to distinguish whether the high-affinity site is regulatory or directly involved in transport.

Identification of High-Affinity Cation Binding Sites of rOCT1 by Voltage-Clamp Fluorometry

We employed cation induced voltage-dependent fluorescence changes of a fluorescent labeled rOCT1 variant to determine affinities for the binding of the substrates choline, TEA, MPP, and the non-transported inhibitor TBuA [50]. In the rOCT1 variant all cysteine residues with exception of the six cysteine residues in the large extracellular loop were removed and one reactive cysteine residue was introduced in position 483 that was covalently labeled with the fluorescent sulfhydryl reagent tetramethylrhodamine-6-maleimide. For choline, TEA and MPP two individual binding sites with low and high affinities were titrated [50]. The apparent K_D values of the low affinity sites (choline 0.35 mM, TEA 57 μ M, MPP 0.87 μ M) were in the range of the apparent K_m values determined for transport of the respective cations or the K_i values determined for inhibition of transport measured in cells in which rOCT1 was overexpressed. For the high affinity sites, K_D values of 12 nM (choline), 57 nM (TEA) and 41 pM (MPP) were determined by voltage-clamp fluorometry. Notably the high affinity K_D value for MPP is 6000-fold lower compared to the high affinity K_D value determined for rOCT1 wildtype in nanodiscs. For the non-transported inhibitor TBuA three different binding sites were distinguished. One apparent K_D value (0.3 μ M) is ten times lower than the apparent K_i value for inhibition of TEA uptake measured in oocytes [44] and three times lower than the apparent K_i value for inhibition of MPP uptake measured in HEK293 cells (H. Koepsell and V. Gorboulev, unpublished data). The other apparent K_D values determined by voltage-clamp fluorometry ($\sim$ 0.4 nM and $\sim$ 2 pM) indicate binding sites with very high affinities.

Hypothesis on Mechanism of Substrate Binding and Translocation by rOCT1

The above described data indicate that each rOCT1 monomer is capable to bind three molecules of MPP. The changes in binding and transport observed in rOCT1 mutants suggest that two low-affinity MPP binding sites located in close proximity within the innermost part of the outward-facing cleft are involved in transport. This interpretation is based on the following observations. First, mutations of Trp218 and Asp475 that are located in one region within the innermost part of the modeled outward-facing cleft (Fig. 2.2a, b), destroy one low affinity MPP binding site, change the K_D for MPP binding to the remaining low-affinity site, and halve the turnover for MPP uptake. Second, mutations of Leu447 which is located in a different niche of the inner part of the modeled outward-facing cleft (Fig. 2.2a, b), do not destroy a low-affinity binding site but also change the K_D for low-affinity binding which is supposed to represent a lumped constant for binding to both low-affinity MPP binding sites. Our data suggest that rOCT1 mediated translocation of MPP

may occur when one or both low-affinity MPP binding sites of the monomer are loaded i.e. in a one cation/monomer transport mode and in the two cation/monomer transport mode. This hypothesis is depicted in Fig. 2.3. The capability of rOCT1 to operate in a one cation/monomer mode is supported by the observation that transport was observed when one low-affinity MPP binding site was blocked after mutation of D475 or W218. The capability of rOCT1 to operate in a two cation/monomer transport mode is suggested by the observation that the optimal turnover of rOCT1 was only observed when both low-affinity binding sites were intact. Operation in the one cation/monomer mode at substrate concentrations far below the lumped K_D value of both low-affinity binding sites and operation in the two cation/monomer mode at saturating substrate concentration is suggested by the observation that the substrate dependence of MPP uptake by rOCT1 wildtype and by the L447 mutants was hyperbolic rather than exponential as would be expected if the transporter would only function in the two cation/monomer mode.

Considering the complex structure of the transport relevant cation binding region one can imagine that also two different organic cations may be transported together. It appears to be also possible that larger substrates are only transported in the one/monomer transport mode. The proposed one/monomer and two/monomer transport mode for small cations increases the versatile capability of rOCT1 to mediate uptake at largely differing concentrations of extracellular substrates.

Potential Mechanisms for Inhibition of rOCT1 Mediated Transport

Based on the complex mechanism of organic cation transport by OCTs involving one cation/monomer and two cation/monomer transport modes and the existence of high affinity cation binding sites that may be regulatory, various ways can be imagined how transport can be slowed down or blocked by transported or nontransported inhibitors. Because all transported cations have to bind at transport relevant binding sites within the innermost part of the outward-facing binding cleft, which represent low-affinity binding sites in case of MPP and rOCT1, mutual competition at these sites should be an important mechanism for inhibition. At substrate concentrations above K_m , when small substrates may be translocated predominantly in the two cation/monomer mode and the high-affinity substrate binding site(s) should be loaded, the K_i values of transported inhibitors and competitive nontransported inhibitors should be relatively independent from the substrate employed for uptake measurements because inhibition is supposed to be mainly due to substrate replacement at the transport sites (Fig. 2.3). At variance, when very small substrate concentrations are used for inhibition studies i.e. when mainly inhibition of the one cation/monomer transport mode is measured, inhibitors may not only compete with the substrate loaded low-affinity binding site but may also interact with the second unoccupied low-affinity binding site and inhibit transport without replacement of

the substrate (Fig. 2.3). If the substrate bound to one low-affinity site contains hydrophobic aromatic rings such as MPP, the interaction of hydrophobic competitive inhibitors may include interaction with the bound substrate (Fig. 2.3). Due to the proximity of both cation binding sites allowing short range effects of substrate binding to one low-affinity site on the structure of the second low-affinity site and/or interaction of the inhibitor with the bound hydrophobic cation, the affinity of inhibitor binding to the transporter monomer containing one substrate may be higher compared to the unloaded monomer. At very low substrate concentrations transport inhibition via interaction of inhibitors with high affinity cation binding sites may be possible via allosteric effects on transport.

In the frame of the proposed model for transport in two modes recent experimental data of biomedical impact that appeared to be incomprehensible on the first view can be explained. Measuring inhibition of MPP uptake mediated by human OCT1, OCT2 and OCT3 with the antiviral drug lamuvidine, monophasic inhibition curves were obtained when MPP concentrations near the K_m of MPP were used for uptake measurements, whereas biphasic inhibition curves indicating high-affinity and low-affinity inhibitor binding sites were resolved when the MPP concentration employed for the uptake measurements was far below the K_m of MPP [66]. Lamuvidine which is transported by human OCT1-3 with apparent K_m values between 1.3 and 2.1 mM inhibited the uptake of 1.3 nM MPP by human OCT1-3 about 50 % with a half maximal effective concentrations (EC_{50}) values between 8 and 20 pM and inhibited the remaining 50 % with EC_{50} values between 1.9 and 3.5 mM [66]. The transport model depicted in Fig. 2.3 also provides an intuitive plausible explanation for the recently reported observation that largely different affinities of OCT inhibitors were determined when very low concentrations of different substrates were used for the uptake measurements [67–69]. This property of OCTs is of high biomedical importance for the prediction of clinical drug–drug interactions. In the one cation/monomer transport mode prevailing at very low substrate concentration, the inhibitors may interact with a transport relevant low-affinity binding site that has been differentially modified due to binding of different substrates to the second transport relevant binding site, and/or the affinity may be increased by hydrophobic interaction with bound substrate.

Concluding Remarks

The outlined concept concerning polyspecific substrate binding to OCTs, translocation of substrates and inhibition of transport has been derived from recent reported crystal structures of transporters of the MFS, detailed functional characterization of rOCT1 and rOCT2, extensive mutagenesis, and recent MPP binding measurements that allowed the identification of three MPP binding sites per rOCT1 monomer. This transport model provides intuitively convincing explanations for the observations that largely different inhibitor sensitivities were determined for transport at very low substrate concentrations compared to transport at substrate concentrations around

the K_m values and that inhibitor sensitivities measured at very low substrate concentrations were largely different when different substrates were used for the uptake measurements. Providing a comprehensible model for these observations will help pharmacists to accept these new alarming properties that have to be considered for preclinical in vitro testing of drug–drug interactions. The molecular understanding how the polyspecific organic cation transporters of the SLC22 family operate in general and which specific features can be attributed to the three human OCTs is of high theoretical interest and has high biomedical impact. The presented model provides a frame for future mutagenesis experiments. The observed functional effects of the mutations will help to develop a more defined and less hypothetical model when crystal structures of different functional states of transporters of the SLC22 family, the SLC22 subfamily of OCTs and of individual OCT subtypes will be available. Considering the fact that so far no crystal structure of a mammalian transporter of the MFS has been reported it will be still a long way to go. However, this is the only possibility to develop a solid basis for in silico drug design and to establish more predictive high throughput procedures for drug testing.

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Chapter 3

Pharmacological and Toxicological Significance of the Organic Cation Transporters OCT and MATE: Drug Disposition, Interaction and Toxicity

Hideyuki Motohashi and Ken-ichi Inui

Abstract Organic cation transporter OCTs and multidrug and toxin extrusion (MATE) are involved in pharmacokinetics of various drugs. In the renal proximal epithelia, OCT2 mediates uptake of the drugs such as metformin or cimetidine at basolateral membrane, and MATE1 and MATE2-K mediated the secretion of cationic and zwitterionic drugs at brush-border membrane. In the liver, OCT1 are expressed on the sinusoidal membrane and MATE1 are expressed on the canalicular membrane. These transporters mediate the biliary excretion of drugs. The change of these transporter activities, caused by genetic alteration or drug–drug interaction, affected the pharmacokinetics of substrates. Inhibitors of the transporters reduce the biliary or urinary secretion of substrate drugs. In addition, OCTs and MATEs are involved in adverse drug reactions. For example, it was considered that renal toxicities of platinum agents cisplatin or oxaliplatin were affected by substrate specificities of renal OCT2 and MATEs. OCTs and MATEs play important roles for drug efficacies and toxicities especially in the liver and the kidney.

Keywords Drug–drug interaction • Kidney • Liver • Renal secretion • Renal toxicity • Platinum agents

Introduction

Membrane transporters play pharmacokinetic important roles in various tissues, such as the brain, intestine, liver and kidney. The liver and kidney are the main organs responsible for the excretion of drugs and their metabolites. Renal excretion consists of glomerular filtration, tubular secretion and re-absorption. Organic cation transporters are involved in tubular secretion [1, 2]. The tubular secretion of drugs

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is mediated by a two-step membrane transport process [3] involving the basolateral membrane and the brush-border membrane. Organic cation transporters (OCTs) are expressed on the basolateral membranes of proximal tubular cells, and multidrug and toxin extrusion (MATE) proteins are expressed on the brush-border membrane. On the other hand, in the liver, OCTs are expressed on the sinusoidal membrane and MATEs are expressed on the canalicular membrane. These transporters are involved in the biliary excretion of cationic compounds.

General information about OCTs and MATEs was presented in the introduction Chap. 1 by G. Ciarimboli. This chapter highlights the pharmacological and toxicological significance of the organic cation transporters SLC22 (OCTs) and SLC47 (MATEs), specifically in the kidney and the liver. In addition, the membrane transport of victim drugs (substrate) by OCTs and MATEs can be inhibited by perpetrators (inhibitor), which is the one of the main mechanisms of drug–drug interaction in the kidney and the liver.

Pharmacokinetic Significance of Organic Cation Transporter SLC22 (OCTs) [4–14]

The transport characteristics of OCTs are similar in various species. OCT1, 2 and 3 mediated the facilitated transport of a broad range of structurally diverse organic cations and are inhibited by many additional compounds, which are not transported [5, 15]. The molecular size of the typical OCT substrate is under 500, and the OCTs mediate the bidirectional transport of small hydrophilic compounds. OCTs transport organic cations and weak bases, which are positively charged at physiological pH, in an electrogenic manner. In addition to these cations, zwitterionic compounds may also be the substrates transported by OCTs. The transported substrates and non-transported inhibitors of individual OCT transporters overlap broadly (Table 3.1).

OCT1 were originally isolated from rat kidney [50] and using knockout mice [51] it was demonstrated that OCT1 is an influential transporter in the renal secretion of organic cations in rodents. In contrast to rodents, OCT1 expression levels are quite low in the human kidney [52]. There are also species difference in the tissue distribution and expression level of OCT1. One of main organs, in which OCT1 mediates the membrane transports of drugs in humans, is the liver [53]. Here, OCT1 is expressed on the sinusoidal membranes of hepatocytes around the central veins. Therefore, considering its hepatic localization, OCT1 plays pivotal roles in the uptake of drugs by hepatocytes. The substrate specificities of OCTs, including OCT1, are reviewed extensively elsewhere [5]. For several drugs, which inhibit OCTs but are not transported, a highly inhibitory potency for OCT1 was observed compared with hOCT2 or hOCT3. These drugs include the glutamate receptor antagonist phencyclidine, the histamine receptors antagonists diphenylhydramine and ranitidine, the muscarinic acetylcholine receptor antagonist atropine, and the antidepressant desipramine. Some cations that are transported by OCT2 and OCT3 (e.g., epinephrine, norepinephrine, and histamine) are not transported by hOCT1.

Table 3.1 Drugs interact with OCT1, OCT2, OCT3, MATE1 and MATE2-K

Class	Compound	Km or (Ki or IC50)				MATE1	MATE2-K	References
		OCT1	OCT2	OCT3				
Receptor antagonists								
Acetylcholine receptor (muscarinic)	Atropine	(1.2)	(29)	(466)				[16]
Acetylcholine receptor (muscarinic)	Butylscopolamine	(16)	(764)					
Acetylcholine receptor (muscarinic)	Mepiperphenidol		(4.8)					[17]
α-Adrenoceptor	Phenoxybenzamine	(2.7)	(4.9)	(6.1)				
α-Adrenoceptor	Prazosin	(1.8)	(>100)	(13)				[18]
β-Adrenoceptor	Acebutolol	(96)						[19]
Histamine H ₁ receptor	Cetirizine				(371)	(817)		[20]
Histamine H ₁ receptor	Chlorpheniramine				(87.6)	(191)		[20]
Histamine H ₁ receptor	Diphenylhydramine	(3.4)	(15)	(695)	(87.0)	(266)		[16, 20]
Histamine H ₂ receptor	Cimetidine	(101–223)	8.6, 73 (95–146)	(17)	170 (1.1–3.8)	120, 370 (2.1–7.3)		[5, 19–25]
Histamine H ₂ receptor	Famotidine		56.1, 204 (111)		(0.6)	(9.7)		[5, 16, 20, 24, 25]
Histamine H ₂ receptor	Ranitidine	(28)	65.2, 265 (40, 1617)	(372)	(25.4)	(25.0)		[5, 16, 20, 24, 25]
NMDA receptor	Amantadine	(236)	20, 27 (18, 28)	(>1000)	(112)	(1167)		[20, 24, 26, 27]
NMDA receptor	Ketamine	(115)	(23)	(226)				[27]
NMDA receptor	Memantine	(3.7)	34 (7.3)	(236)				[26, 27]
(continued)								

(continued)

Table 3.1 (continued)

Class	Compound	Km or (Ki or IC50)					References
		OCT1	OCT2	OCT3	MATE1	MATE2-K	
NMDA receptor	Phencyclidine	(4.4)	(25)	(333)			[27]
<i>Receptor agonists</i>							
Acetylcholine receptor (nicotinic)	Nicotine	(186)	(22, 42)	(101)		(>500)	[5, 24, 28, 29]
α-Adrenoceptor	Clonidine	(0.6–6.5)	(23)	(110, 373)			[16, 19, 30]
α-Adrenoceptor	Etilefrine	(447)	(4009)	2800 (4450)			[16]
β-Adrenoceptor	O-Methylnisoprenaline	(>100)	(570)	(4.4)			[17, 18]
NMDA receptor	Dizocilpine	(81)	(22)	(224)			[27]
Dopamine receptor	Pramipexole		15.4	138	(141)	(24.1)	[20, 31]
Dopamine receptor	Talipexole				(66)	(120)	[20]
<i>Ion channel and transporter blocker</i>							
Ca ²⁺ channel	Verapamil	(2.9)	(206)	(24)	(27.5)	(32.1)	[5, 19–21, 28]
Ca ²⁺ channel	Diltiazem				(12.5)	(117)	[20]
Na ⁺ channel	Disopyramide	(15, 30)			(83.8)	(292)	[5, 19, 20]
Na ⁺ channel	Procainamide	(74)	(50, 58)	(738)	1230 (217)	1580, 4100 (178)	[5, 17, 19–22, 28, 30]
Na ⁺ channel	Quinidine	(18)			(29.2)	(23.1)	[19–21, 28]
Noradrenaline transporter	Cocaine	(85)	(113, 277)	(>1000)			[5, 27]
Serotonin transporter	Citalopram	(2.8)	(21)	(158)			[5]

<i>Drugs and Xenobiotics</i>									
Anesthetic	Midazolam	(3.7)							[19]
Anesthsthetic	Beclometasone		(4.4)						[29]
Antiasthmatic	Budesonide		(7.3)						[29]
Antibiotic	Cephalexin					5900 (6500)		(>10,000)	[22, 32]
Antibiotic	Cephadrine					(4040)		(10,400)	[22]
Antibiotic	Cefazolin					(>10,000)		(>10,000)	[22]
Antibiotic	Ciprofloxacin	(>1000)	(>1000)	(>1000)		(231)		(98.7)	[22, 33]
Antibiotic	Levofloxacin	(>1000)	(>1000)	(>1000)		(38.2)		(81.7)	[22, 33]
Antibiotic	Pentamidine	(0.4)	(3.8)						[34]
Antibiotic	Trimethoprim	(20, 36.7)	(51, 27.2)			(6.3)		(28.9)	[34, 35]
Antidepressant	Desipramine	(5.4)	(16)	(14)		(55.7)		(283)	[17, 19, 20, 30, 36, 37]
Antidepressant	Imipramine			(42)		(42.0)		(182)	[20, 30, 38]
Antidiabetic	Metformin	1470 (2010)	990, 1066 (339, 1700)			253–780 (667)		362–1980 (6515)	[20–22, 24, 39–41]
Antidiabetic	Phenformin	(10, 15)	(65)						[24, 40]
Antiemetic	Granisetron	(<100)	(<100)	(<100)					[5]
Antiemetic	Ondansetron		(3.85)			(0.035)		(0.015)	[42]
Antiemetic	Tropisetron	(<100)	(<100)	(<100)					[5]
Antihypertensive	Debrisoquine		(7.3)						[5]
Antimalarial	Quinine	(13, 23)	(3.4, 23, 34)	(37)					[16, 17, 19, 21, 43, 44]

(continued)

Table 3.1 (continued)

Class	Compound	Km or (Ki or IC50)				MATE1	MATE2-K	References
		OCT1	OCT2	OCT3				
Antimalarial	Pyrimethamine		(10)			(0.093)	(0.059)	[39]
Antiviral	Aciclovir	151				2640	4320	[22, 37]
Antiviral	Ganciclovir	516				5120	4280	[22, 37]
Antiviral	Indinavir	(37, 62)	(275)					[34, 45]
Antiviral	Saquinavir	(8.3, 37)	(205)					[34, 45]
Antiviral	Zalcitabine	242 (24)	232 (131)					[34]
Antiviral	Lamivudine	249 (17)	248 (33)					[34]
Antiviral	Ritonavir	(5.2, 14)	(25)					[34, 45]
Antiviral	Nelfinavir	(7, 22)	(13)					[34, 45]
Cytostatic	Cisplatin	(>100)	(1.5)					[46]
Cytostatic	Mitoxantrone	(16)	(800)	(440)				[5]
Cytostatic	Topotecan					70	60	[22]
Muscle relaxant	Vecuronium	(127, 232)						[19, 43]
Psychostimulant	3,4Methylenedioxymetamphetamine	(24)	(1.6)	(74)				[27]
Psychostimulant	D-Amphetamine	(202)	(11)	(460)				[27]
Serine protease inhibitor	Nafamostat mesilate		(20)					[47]
Tyrosine kinase inhibitor	Dasatinib	(1.07)	(2.11)	(4.50)		(0.843)	(0.844)	[48]
Tyrosine kinase inhibitor	Erlotinib	(0.356)	(5.24)	(4.21)		(7.93)	(3.45)	[48]
Tyrosine kinase inhibitor	Gefitinib	(1.07)	(24.4)	(5.47)		(1.82)	(0.194)	[48]

Tyrosine kinase inhibitor	Imatinib	(1.47)	(5.81, 6.7)	(4.36)	(0.0479, 1.0)	(0.478, 4.3)	[48, 49]
Tyrosine kinase inhibitor	Lapatinib	(>30)	(>30)	(>30)	(>30)	(>30)	[48]
Tyrosine kinase inhibitor	Nilotinib	(2.92)	(>30)	(0.345)	(3.38)	(1.76)	[48]
Tyrosine kinase inhibitor	Sorafenib	(>30)	(>30)	(20.1)	(>30)	(>30)	[48]
Tyrosine kinase inhibitor	Sunitinib	(0.330)	(1.73)	(5.22)	(0.275)	(0.864)	[48]

OCT2 was cloned after OCT1 from the rat kidney and was later also cloned in human [17, 43, 54]. OCT2 is highly expressed in the rodent and human kidney. In the human kidney in particular, OCT2 is the most highly expressed among cation transporters, and OCT2 protein is expressed in all segments of proximal tubules [52, 55]. Hence, OCT2 is thought to uptake organic cations at the basolateral membrane and play pivotal roles in the tubular secretion of organic cations. OCT2 and OCT1 shares various substrate cations [5]. For example, OCT2 transports MPP, TEA, quinine, and metformin with similar K_m values as OCT1 and transports acetylcholine with an approximately fourfold lower K_m value compared with OCT1 [5, 15]. Uptake by OCT2 has also been demonstrated for choline; the neurotransmitters dopamine, norepinephrine, epinephrine, serotonin, histamine, agmatine; the glutamate receptor antagonists amantadine and memantine; the histamine H_2 receptor antagonists cimetidine, famotidine and ranitidine; the cytostatic cisplatin; and the antihypertensive drug debrisoquine.

In contrast to OCT1 and OCT2, OCT3 is expressed ubiquitously [30, 56]. OCT3 mRNA was detected at high levels in the aorta, skeletal muscle, prostate, adrenal gland, salivary gland, liver, placenta, and fetal lung. OCT3 also transports substrates, such as MPP, with similar K_m values as OCT1 and OCT2, whereas a much higher K_m value was measured for the translocation of TEA by OCT3 compared with OCT1 and OCT2 [5]. Amantadine, memantine, phenylcyclidine, clonidine, diphenylhydramine, atropine, procainamide and cocaine were observed to inhibit OCT3 with much lower affinity compared to OCT1 and OCT2. High-affinity inhibitors of hOCT3 include disprocynium, decynium, and corticosterone.

Multidrug and Toxin Extrusion (MATE) Proteins [4–6, 57–60]

MATE1 was isolated as a mammalian orthologue of the bacterial multidrug and toxin extrusion family, which confers the multidrug resistance [28]. Human MATE1 and MATE2 were identified in 2005, followed by the isolation of the kidney-specific multidrug and toxin extrusion isoform MATE2-K [21]. In humans, the MATE1 is strongly expressed in liver, kidney and skeletal muscle and was also detected in the heart. [21, 28] MATE1 localizes to the brush-border membrane of renal proximal tubules in the kidney [21, 55] and to the luminal membranes of bile canalicular epithelial cells in the liver. TEA uptake by MATE1 was independent of the membrane potential and the extracellular sodium concentration. Since MATE1-mediated uptake of TEA was stimulated by opposite-directed proton gradients, MATE1 is supposed to be a H^+ /cation antiporter as suggested in the TEA uptake by renal brush-border membrane vesicles. TEA uptake by MATE1 is inhibited by a large variety of organic cations such as MPP, serotonin, cimetidine, quinidine, and verapamil, suggesting that, much like OCTs, substrate recognition by MATE1 is poly-specific [22].

MATE2-K is highly expressed in the human kidney and is localized to the brush-border membrane in proximal tubules [21, 55]. When expressed in HEK293 cells,

MATE2-K behaves as a H^+ gradient-dependent TEA antiporter. MATE2-K is considered to be the active MATE2 variant in the human kidney. MATE2-K is a poly-specific H^+ /cation antiporter that transports TEA, cimetidine, MPP, procainamide, metformin and NMN, creatinine, guanidine, quinidine, thiamine, and verapamil.

MATE1 and MATE2-K transport typical organic cations such as TEA, cimetidine, MPP, and the antiarrhythmic drug procainamide. All of these compounds were demonstrated to be substrates for H^+ /organic cation antiporters characterized in brush-border membrane vesicles. Zwitterionic β -lactam antibiotics such as cephalixin and cephradine are effectively transported by MATE1 [32], and the results were also consistent with the transport experiments performed using renal brush-border membrane vesicles. A platinum anticancer agent, oxaliplatin, was a better substrate for MATE2-K rather than for MATE1. The pharmacokinetic and toxicokinetic significance of oxaliplatin transport is discussed below in the section “Renal Toxicity of Platinum Agents”. With a few exceptions, the substrate specificities of MATE1 and MATE2-K are generally similar.

Pharmacokinetic Roles of Organic Cation Transporters in the Liver [5, 53, 61–63]

Many endogenous or exogenous organic compounds, which are positively charged at physiological pH, are handled in the liver. Many of these compounds are highly hydrophilic, and therefore cannot passively diffuse across the plasma membrane. Hence, it has been considered that the membrane transport system is thought to be necessary for the translocation of these compounds. Based on their structural characteristics, organic cations are classified into two categories. Type I organic cations are small and highly hydrophilic, usually below 500 Da. Several quaternary ammonium compounds, such as TEA and MPP^+ , are considered as typical type I cations. Type II organic cations are bulky and less hydrophilic, and are often polyvalent compounds. D-tubocurarine and quinine are the typical type II cations.

OCT1, as well as other OCT isoforms, translocates organic cations in an electrogenic manner that is independent of Na^+ or H^+ gradients. OCT1 is localized to the sinusoidal membrane of bile canalicular epithelial cells, and mediates the uptake of endogenous and xenobiotic cations into hepatocytes, which is the first step in the excretion or detoxification of many drugs. Importantly, OCT1 transports metformin, a biguanide antidiabetic agent that is widely used to treat type 2 diabetes. Metformin is a hydrophilic organic cation and OCT1 is responsible for the uptake of metformin into the hepatocytes. The main pharmacological activity of metformin is to decrease gluconeogenesis in the liver, which reduces blood glucose levels. Therefore, the transport activity of OCT1 in the sinusoidal membrane is important for the clinical efficacy of metformin. Another example of drug transported by OCT1 is lamivudine, a cytidine analog whose active metabolites prevent hepatitis B virus replication in the liver. Lamivudine is efficiently taken into hepatocytes by OCT1.

O-Desmethyltramadol is an opioid analgesic and the main active metabolite of tramadol, which is demethylated by CYP2D6 in the liver. *O*-Desmethyltramadol, but not tramadol, is transported by OCT1. Volunteers with loss-of-function OCT1 polymorphisms were reported to have significantly higher plasma concentrations of *O*-desmethyltramadol and significantly prolonged miosis, a surrogate marker of opioidergic effects. Interindividual differences observed in the OCT1 expression and/or activity levels affect pharmacokinetics of *O*-desmethyltramadol and thus the efficacy of tramadol treatment [62].

The serotonin receptor antagonists, ondansetron and tropisetron, are used to treat nausea and vomiting caused by chemotherapy. These compounds are taken up into hepatocytes by OCT1 and are subsequently metabolized and inactivated by CYP enzymes, mainly CYP2D6. This pathway is a major detoxification route for ondansetron and tropisetron. OCT1 plays a crucial role in the first-pass effect through the liver and hence in the bioavailability of other cationic drugs such as amantadine, levodopa and pramipexole; cimetidine, ciprofloxacin and other fluoroquinolones, furamidine and pentamidine; lamotrigine, sulpiride, and zalcitabine. Most type II organic cations, which are typically hydrophobic, bulky, and polyvalent, such as atropine, decynium-22, prazosin, quinine, and D-tubocurarine, are inhibitors but not substrate of OCT1. Some exceptions include the clinically used type II organic cations quinidine, pancuronium, and rocuronium, which are all transported by OCT1.

MATE1 mediates the excretion of organic cations across the biliary membrane into the bile. MATE1 deficiency in the biliary membrane will result in increased intracellular concentrations of cationic drugs that are substrates of OCT1. This may lead to the increased hepatotoxicity of drugs. Hume et al. developed a new positron emission tomography probe, ¹¹C-labelled metformin, to study hepatobiliary transport mediated by MATE1 [63]. This probe may be useful for non-invasively studies aimed at evaluating the in vivo function of hepatobiliary transport and drug–drug interactions, mediated by MATE1 in clinical investigations. Toyama et al. [64] reported that MATE1 dysfunction caused a marked elevation in the metformin concentration in the liver and led to lactic acidosis, suggesting that this homozygous MATE1 variant could be one of the risk factors for metformin-induced lactic acidosis.

Pharmacokinetic Roles of Organic Cation Transporters in the Kidney [1, 4, 57–59]

The kidney is one of the main organs responsible for the excretion of drugs and xenobiotics. Renal excretion is one of the determinants for the pharmacokinetics of cationic drugs and consists of three steps; glomerular filtration, tubular secretion and re-absorption. The secretion of xenobiotics is an important physiological function of the renal proximal tubules. Drugs are actively secreted via two distinct systems at the brush-border and basolateral membranes of tubular epithelial cells. Transport studies using isolated membrane vesicles and cultured renal epithelial cells characterized two distinct classes of organic cation transporters. Electrogenic

transporter is facilitated by an internal negative membrane potential at the basolateral membranes and electroneutral transporter is driven by the transmembrane H^+ gradient (H^+ /organic cation antiporter) at the brush-border membranes, which is dominated by a H^+ /organic cation antiport process. A prototype substrate, TEA, has been used for the functional characterization of these organic cation transport systems in the kidney. There are two SLC protein families involved in cationic drug secretion, including OCT2 at the basolateral membrane and MATEs at the brush-border membrane. OCT2 and MATEs can transport a variety of structurally unrelated organic cations.

The substrate specificity, membrane localization and driving forces indicated that OCT2 and MATE1 or MATE2-K coordinate the tubular secretion of cationic drugs from blood to urine. Double-transfected Madin-Darby canine kidney cells have been used as an *in vitro* model for the vectorial transport of cationic drugs across human epithelial cells [65]. In these cells, OCTs are expressed on the basolateral membrane and MATEs are on the apical membrane. Indeed, TEA was transported unidirectionally from the basolateral to apical side of the membrane in these double transfectants. In addition to TEA, the clear directional transport of procainamide and quinidine was also shown in the double transfectants. Procainamide and quinidine are actively secreted via the renal tubules into the urine suggesting that these drugs are substrates of membrane transporters. However, due to the lipophilicity of these compounds, it was difficult to detect the uptake of procainamide and quinidine by OCT- or MATE-expressing cells. Therefore, the double-transfected cells overcome the technical limitations of prior uptake experiments. These cells are convenient for *in vitro* examination to clarify the renal tubular secretion of cationic drugs in humans. Furthermore, OCT2 and MATEs in the proximal tubule are the sites of clinically important drug–drug interactions. For example, therapeutic doses of cimetidine decrease the renal elimination of procainamide. Double-transfected cells are thus also useful for the examining of drug–drug interactions. Details of drug–drug interactions are described below in section “Drug–Drug Interactions of Cationic Compounds in the Kidney”.

OCT2 may be involved in the renal excretion of a variety of drugs, such as the neurotransmitters dopamine, epinephrine and serotonin; agonists and antagonists of various receptors; various blockers of ion channels and transporters; and various other drugs, including a variety of psychoactive compounds. For example, OCT2 is important for the renal excretion of metformin, a biguanide that is used in the treatment of type-II diabetes and in polycystic ovary syndrome. Metformin is almost entirely excreted into the urine without being metabolized, but a portion of metformin is also excreted in bile. Metformin is mainly eliminated by glomerular filtration and tubular secretion, and OCT2, MATE1 and MATE2-K are involved in the tubular secretion of metformin. Lactic acidosis is a fatal adverse effect of metformin and its occurrence cannot be predicted in patients presymptomatically. Decreased renal excretion of metformin may lead to increased plasma levels. Increased concentration of metformin in the liver may lead to the excessive inhibition of mitochondrial respiratory enzymes and may cause lactic acidosis. Reduced OCT2 and/or MATEs activity *in vivo*, for example, through inhibition by the concomitant drugs or through renal impairments, may increase the risk of lactic acidosis.

To clarify the pharmacokinetic role of MATE1 *in vivo*, Tsuda et al. carried out the targeted disruption of the murine *Mate1* gene [66]. After a single intravenous administration of metformin, the area under the blood concentration-time curve of metformin in *Mate1*-knockout mice showed a twofold increase. The urinary excretion of metformin after intravenous administration was also significantly decreased in *Mate1*-knockout mice. The report demonstrated an essential role for MATE1 in the systemic clearance of metformin. [66] Toyama et al. reported that significantly higher blood lactate levels and lower pH and HCO_3^- levels were observed in *Mate1*-knockout mice 7 days after metformin administration in the drinking water [64, 67]. In the knockout mice, dysfunctional *Mate1* caused a remarkable elevation in the concentration of metformin in the liver and led to lactic acidosis. These results suggested that homozygous but not heterozygous MATE1 variants are risk factors for metformin-induced lactic acidosis.

Choi et al. characterized genetic variants of MATE2-K and determined their association with the response to metformin. [68] Four nonsynonymous variants and four variants in the MATE2-K basal promoter region were identified from ethnically diverse populations. Two nonsynonymous variants, including c.485C>T (Pro162Leu) and c.1177G>A (Gly393Arg), were shown to be associated with significantly lower metformin uptake and a reduction in protein levels when expressed in HEK293 cells. MATE2-K basal promoter haplotypes containing the most common variant, g.-130G>A (>26 % allele frequency), were associated with significantly increase in promoter activity and reduced binding to the transcriptional repressor myeloid zinc finger 1. Diabetic patients who were homozygous for g.-130A had significantly poorer responses to metformin treatment than carriers of the reference allele when assessed for the relative change in glycated hemoglobin (HbA1c). Choi et al. suggested that MATE2-K plays a role in the antidiabetic response to metformin and that the next challenge in pharmacogenomic research is to improve the outcome for patients through this pathway.

Tzvetkov et al. examined the effects of genetic polymorphisms in OCT1, OCT2, OCT3, OCTN1, and MATE1 on the pharmacokinetics of metformin in healthy male Caucasians [69]. Low-activity genotypes of OCT1 were related to an increase in the renal clearance of metformin. It is possible that OCT1 dysfunction indirectly affects the renal clearance of organic cations in humans.

Drug–Drug Interactions of Cationic Compounds in the Kidney [23, 39, 65, 70]

Drug–drug interactions are among the serious disturbances that render pharmacotherapies unsuccessful. In renal excretion, cationic perpetrators can reduce the renal secretion of victim drugs, resulting in severe adverse reactions due to elevations in the plasma concentrations. OCTs and MATEs are independent sites for drug–drug interactions between cationic drugs (Fig. 3.1). Perpetrator drugs exhibit considerable differences in the inhibitory potency with respect to MATE and OCT2

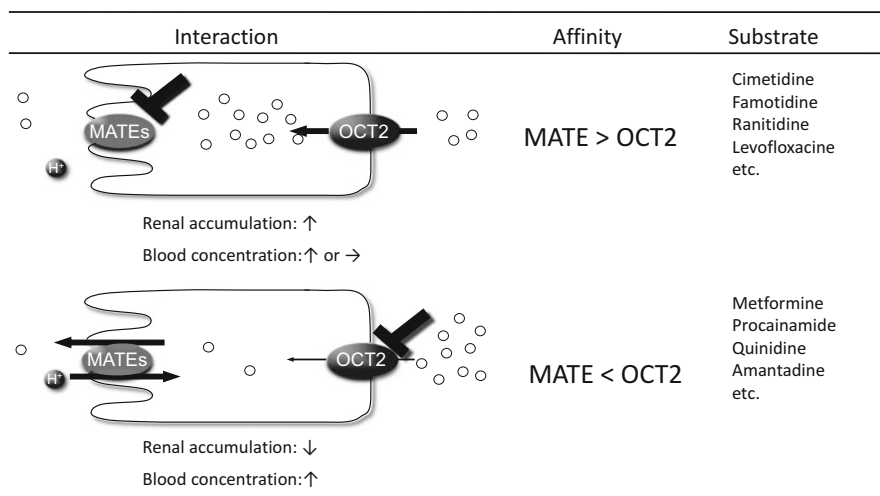


Fig. 3.1 Scheme of drug–drug interaction at renal proximal epithelia

(Table 3.1). For example, pyrimethamine markedly inhibits MATEs, whereas it negligibly inhibited OCT transport activity at its clinically relevant dose.

Cimetidine has been well known to reduce the renal excretion of other cationic drugs. For example, the excretion of procainamide was inhibited by concomitant cimetidine administration. The drug interactions between cimetidine and metformin were assessed using double-transfected kidney cells that stably expressed OCT2 and MATE1 as an *in vitro* model of proximal tubular epithelial cells [20]. The results from the investigation of the blood concentrations at clinical doses suggested that apical MATE1 is the site of drug interactions whereby cimetidine inhibits other cationic compounds in proximal tubular epithelial cells in clinical situations. Similar conclusions were reached by other groups. Recently, Ito et al. concluded that competitive OCT2 inhibition is unlikely to underlie the drug–drug interaction caused by cimetidine in the renal elimination of cationic drugs and that the competitive inhibition of MATEs by cimetidine is likely to be important *in vivo* at clinical doses [23, 70]. The conclusion based on the reason that cimetidine interacts with higher affinity with MATEs than with OCT1.

Kusuhara et al. conducted a microdose study of metformin to investigate the predictability of drug–drug interactions at the therapeutic doses [39]. Healthy subjects received microdoses and therapeutic doses of metformin, along with a potent and competitive MATE inhibitor, pyrimethamine, in a crossover fashion. Pyrimethamine significantly reduced the renal clearance of metformin and caused significant increases in the plasma concentrations of metformin. Pyrimethamine also increased the plasma concentration of creatinine, which is an endogenous substrate secreted by proximal tubules. They considered that the drug–drug interaction was attributed to the inhibition of MATE proteins by pyrimethamine. Pyrimethamine is considered to be a useful tool as an *in vivo* inhibitor of MATE proteins. Ito et al.

reported the usefulness of *N*-methylnicotinamide as an endogenous probe for evaluating drug–drug interactions involving MATE1 and MATE2-K. NMN is an endogenous substrate of MATE1 and MATE2-K, as well as OCT2. NMN uptake by human brush-border membrane vesicles with proton gradients was reported to be saturable and completely inhibited by low concentrations of pyrimethamine. NMN has been suggested for use as an internal probe to evaluate drug–drug interactions in renal tubular secretion.

Minematu and Giacomini reported interactions of tyrosine kinase inhibitors with OCTs and MATEs [48]. In their report, IC_{50} values were estimated for eight TKIs (imatinib, dasatinib, nilotinib, gefitinib, erlotinib, sunitinib, lapatinib, and sorafenib) on metformin transport by OCT1, OCT2, OCT3, MATE1 and MATE2-K. The estimated IC_{50} values were comparable to the maximum clinical concentrations of unbound TKIs in plasma. Imatinib, nilotinib, gefitinib, and erlotinib exerted selectively potent inhibitory effects on MATE1, OCT3, MATE2-K, and OCT1, respectively. Major metabolites of several TKIs showed IC_{50} values similar to those for unmodified TKIs. TKIs may therefore possibly affect the disposition, efficacy, and toxicity of drugs that are substrates of these transporters.

The International Transporter Consortium (ITC), which includes members from academia, industry, and the US Food and Drug Administration (FDA), was formed in 2007. The ITC aimed to determine which transporters are determinants of pharmacokinetics, to discuss method for characterizing drug-transporter interactions, and to propose preclinical and clinical studies of transporter-mediated drug interactions for drug development. In 2010, the ITC published a white paper and the white paper identified seven transporters involved in clinical drug–drug interactions. OCTs were included among these transporters of white paper. The white paper recommended that these transporters should be studied *in vitro* to determine the potential of clinical drug–drug interactions. The European Medicines Agency also included recommendations on transporters in its published drug interaction guidelines. Based on the second ITC transporter workshop in 2012, the ITC identified additional transporters of emerging importance in pharmacokinetics, drug interference with the transport of endogenous compounds, and drug–drug interactions in humans. MATEs were added because of their importance in the excretion of organic cations into bile and urine. A comparison of the concentration of an inhibitor and its inhibitory potency toward a transporter predicts the likelihood of clinical drug–drug interactions [71, 72].

Renal Toxicity of Platinum Agents [58, 73, 74]

Due to the vast renal blood flow and accumulation via uptake mechanisms, the kidney experiences far greater exposure to the xenobiotics than other organs. Therefore, the cytotoxic effects of these drugs easily damage the renal cells. Platinum-based drugs are anticancer agents and are used individually or in combination with other antitumor and/or radiation therapies for the many human malignancies.

Platinum-based chemotherapies have widely been used to treat solid tumors since the 1970s. However, renal impairment induced by cisplatin, the first of these compounds and typical platinum antitumor agent, is severe and limits cisplatin based chemotherapy.

Cisplatin, carboplatin, oxaliplatin, and nedaplatin are currently used to treat solid tumors. Of these drugs, only cisplatin induces nephrotoxicity with a higher accumulation in the kidney. Many groups have reported differential transport of platinum compounds by OCT2 [75]. OCT2 significantly increases the accumulation of oxaliplatin, ormaplatin, tetraplatin, transplatin and cisplatin but not carboplatin and nedaplatin [36]. Furthermore, OCT2-mediated oxaliplatin and cisplatin accumulation was time and concentration dependent, and OCT2 expression enhanced the sensitivity to oxaliplatin and cisplatin cytotoxicity. Many studies indicated that a kidney-specific OCT2 was the determining factor in cisplatin-induced nephrotoxicity, mediating the renal uptake of cisplatin. In contrast, the low-nephrotoxic platinum agents, carboplatin and nedaplatin, are not transported by OCT2. However, whereas oxaliplatin was revealed to be a good substrate for OCT2, it is not nephrotoxic. MATEs have been postulated to protect against oxaliplatin-induced nephrotoxicity by mediating the efflux of this agent from intracellular compartments. Marked transport of oxaliplatin by MATE2-K has been observed. MATE2-K may mediate the efflux of oxaliplatin from renal epithelial cells and protect these cells from platinum toxicity. These results clearly demonstrate the relationship between the renal pharmacokinetics and nephrotoxicity of platinum agents.

The nephrotoxicity of platinum agents is related to MATE and OCT (Fig. 3.2) [58]. Nakamura et al. investigated the role of MATE1 in the nephrotoxicity of cisplatin both in vivo and in vitro [74]. The intraperitoneal administration of cisplatin significantly reduced lifespan of MATE1-knockout mice compared with wild-type mice. The plasma concentration and renal accumulation of cisplatin were also

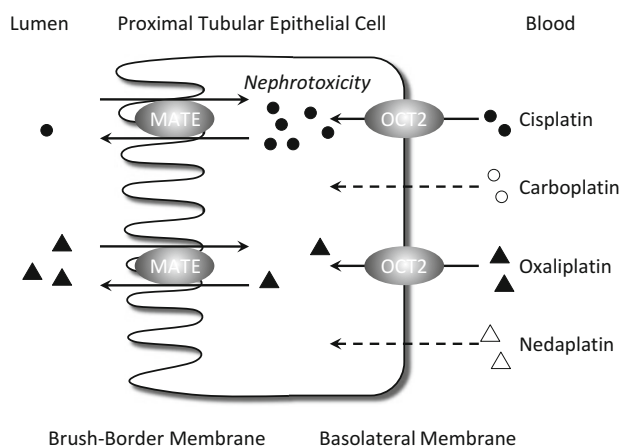


Fig. 3.2 Renal transport of platinum agents at renal proximal tubules

higher in the knockout mice. Furthermore, the combination of pyrimethamine with cisplatin elevated serum creatinine and BUN levels. MATE1 is thus thought to mediate the translocation of cisplatin and is involved in attenuating cisplatin-induced nephrotoxicity via disposition of cisplatin.

Li et al. also reported corresponding results showing that ondansetron enhanced cisplatin-induced nephrotoxicity by inhibiting MATEs as well as pyrimethamine [42]. In cells that stably express OCT2 and MATEs, ondansetron was shown to inhibit both OCT2 and MATEs. Ondansetron significantly increased the renal accumulation of cisplatin and induced more severe pathohistological damage. Increased serum levels of creatinine and BUN, as well as changes in two molecular biomarkers of kidney injury, were indicative of cisplatin-induced nephrotoxicity in ondansetron-treated mice. Therefore, the potent inhibition of MATEs likely enhances the nephrotoxicity associated with cisplatin treatment. The potential nephrotoxic effects of combining the chemotherapeutic cisplatin with MATE inhibitors such as the antiemetic 5-hydroxytryptamine-3 receptor antagonists, should be investigated in patients.

However, whether the efficacy and/or toxicity of platinum agents are influenced by OCT or MATE transport activity *in vivo* has remained controversial. OCT2 mRNA expression in clinical ovarian cancer specimens was low and was not correlated with the treatment outcomes of platinum-based regimens. OCT2 is a critical determinant in the uptake and cytotoxicity of various platinum compounds, particularly oxaliplatin *in vitro*. However, the effects of OCT2 expression on the results of chemotherapy should be carefully considered. Sprowl et al. reported the influence of the OCT2 inhibitor cimetidine on the antitumor efficacy and systemic clearance of cisplatin [76]. In their reports, cimetidine affected the uptake and cytotoxicity of cisplatin in cultured ovarian cancer cells with highly OCT2 expression. In contrast, the antitumor efficacy of cisplatin in mice bearing luciferase-tagged IGROV-1 xenografts was unaffected by cimetidine. Data obtained from 18 patients receiving cisplatin in a randomized crossover study with or without cimetidine revealed that cimetidine did not alter exposure to unbound cisplatin, a marker of antitumor efficacy. Iwata et al. reported the effects of genetic variants of OCT2 and MATE1 on cisplatin-induced adverse events in patients [77]. They concluded that the 808G>T SNP in OCT2 ameliorated CDDP-induced nephrotoxicity without altering disposition, whereas the rs2289669 G>A SNP in MATE1 had no effect on CDDP toxicity. These results support the future clinical exploration of OCT2 and MATEs inhibitors as specific modifiers of cisplatin-induced nephrotoxicity.

In addition to the kidney, Sprowl et al. reported that oxaliplatin-induced neurotoxicity is also dependent on OCT2 [78]. Peripheral neurotoxicity is one of dose-limiting factors in the clinical use of oxaliplatin. OCT2 was found to be expressed on dorsal root ganglia cells in the nervous system, where oxaliplatin is known to accumulate. The cellular uptake of oxaliplatin was stimulated by the overexpression of human OCT2, and DNA platination and oxaliplatin-induced cytotoxicity were increased. Furthermore, genetic or pharmacologic knockout of Oct2 protected mice from acute oxaliplatin-induced neurotoxicity. These findings provide a rationale for the development of targeted approaches to mitigate this debilitating toxicity.

Summary

Among organic cation transporters OCT2, MATE1 and MATE2-K play pivotal roles in the renal tubular handling of various drugs. On the other hand, OCT1 and MATE1 are among the determinants of biliary excretion as well as hepatic detoxification. These transporters are the sites of drug–drug interaction, and differences in substrate affinities between OCTs and MATEs lead to different outcomes for each DDI. In addition, drug-induced kidney impairments were affected by OCT2 and MATE1 or MATE2-K activity. Information on these transporters is critical for the developing effective and safe pharmacotherapies.

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Chapter 4

Genetic Variability in Organic Cation Transporters: Pathophysiological Manifestations and Consequences for Drug Pharmacokinetics and Efficacy

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Abstract Naturally occurring genetic variants may affect expression and function of organic cation transporters. This may result in inter-individual variations of plasma and organ concentrations of endogenous molecules and drugs. Hence, genetic variants in the organic cation transporters may confer susceptibility to diseases and may cause variations in drug pharmacokinetics, efficacy and toxicity.

This chapter gives a structured overview of the genetic variability in the human organic cation transporters and its consequences for disease susceptibility and therapeutic drug response. We present in a special depth the available information about genetically-determined loss of OCT1 activity and the consequences for the pharmacokinetics, efficacy and toxicity of drugs like metformin, morphine, tramadol and tropisetron. Another special focus is on polymorphisms in *OCTN1* and *OCTN2* as risk factors for Crohn's disease. The third focus is on the accumulating data about the effects of regulatory polymorphisms in the *MATE1* and *MATE2K* genes on metformin pharmacokinetics and efficacy. We also present meta-analyses of the currently available studies about the effects of the *OCT2* polymorphism Ala270Ser on metformin pharmacokinetics.

Keywords Organic cation transporter • Genetic polymorphisms • SNPs • Crohn's disease • Pharmacokinetics • Drug efficacy • Metformin • Morphine • Tramadol • Tropisetron • Cisplatin

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Introduction

Naturally occurring genetic variants can affect the expression and the function of organic cation transporters. This may cause inter-individual variations in the plasma and organ concentrations of endogenous molecules or drugs and may confer disease susceptibility or drug treatment failures. This chapter gives a structured overview of the genetic variability of the human organic cation transporters and summarizes the information about the relationship between genetic variations and disease susceptibility or therapeutic drug response. We collect and present the available data of in vitro models (typically HEK293 cells overexpressing the transporter variant, but also other cell lines and mRNA-injected *Xenopus laevis* oocytes) about functional assessment of the effects of genetic variants. Furthermore, we summarized the data from clinical studies in humans about associations between genetic variants in the organic cation transporters and disease susceptibility, pharmacokinetics, or drug efficacy.

OCT1

The High Genetic Variability in OCT1

Human organic cation transporter *OCT1* (alternative name *SLC22A1*) is localized at the long arm of chromosome 6 (6q26–6q27) together with two other organic cation transporters *OCT2* (*SLC22A2*) and *OCT3* (*SLC22A3*). *OCT1* is highly genetically polymorphic, showing particularly high genetic variability in its coding region. Analyses of 1511 individuals from the 1000 Genomes project [1] showed 51 non-synonymous and 31 synonymous polymorphisms in *OCT1*, which is by far the highest number of coding polymorphisms among the organic cation transporters (Table 4.1). The average heterozygosity of the non-synonymous polymorphisms and the ratio of average heterozygosity between non-synonymous to synonymous was by far the highest in *OCT1* compared to the other genes encoding for organic cation transporters (Table 4.1). This shows that not only the number, but also the frequency of the non-synonymous polymorphisms is the highest in *OCT1* among the organic cation transporter and indicates potential deviations from the neutral hypothesis.

Importantly, there are a high number of common amino acid polymorphisms that affect the function of *OCT1*. Systematic resequencing analyses, pioneered by the studies of Kerb et al. in 2002 and Shu et al. in 2003, identified 14 amino acid polymorphisms that lead to a partial or complete loss of *OCT1* activity [2–6]. Five of these functional polymorphisms are more frequent and are of special interest: a deletion of Met420 (rs202220802) and the amino acid substitutions Arg61Cys (rs12208357), Cys88Arg (rs55918055), Gly401Ser (rs34130495), and Gly465Arg (rs34130495).

Table 4.1 Comparative analyses of genetic variability in organic cation transporters

Gene	Number of polymorphisms		π ($\times 10^{-4}$)		$\frac{\pi_{\text{non-synonymous}}}{\pi_{\text{synonymous}}}$
	Non-synonymous	Synonymous	Non-synonymous	Synonymous	
OCT1/ SLC22A1	51	31	7.9	3.4	2.3
OCT2/ SLC22A2	23	16	1.2	3.4	0.4
OCT3/ SLC22A3	16	15	0.0	1.7	0.03
OCTN1/ SLC22A4	13	18	2.4	2.0	1.2
OCTN2/ SLC22A5	31	14	1.0	5.8	0.2
MATE1/ SLC47A1	22	13	0.4	0.7	0.6
MATE2/ SLC47A2	24	13	0.2	4.6	0.04

Shown is the variability in the coding regions of the transporters both in terms of number of non-monomorphic polymorphisms and average heterozygosity (π). Average heterozygosity accounts for both the number of polymorphisms and their frequency. The values are stratified to synonymous and non-synonymous polymorphisms

The analyses were based on 1000 Genomes data (GRCh37). The analyses include 1511 unrelated individuals from 15 populations worldwide. The average heterozygosity (π) presented is calculated per coding bp. of the corresponding gene

Met420 Deletion

A deletion of methionine at codon 420 (Met420del) is the most common functional variant in OCT1. In contrast to most of the other functional OCT1 variants that are population specific, Met420 deletion can be observed in different world regions (Table 4.2). Methionine₄₂₀ is located in the ninth transmembrane domain of OCT1 (Fig. 4.1a). Met420del does not change the membrane localization of the OCT1 protein [7] and the exact molecular mechanism for how Met420del affects OCT1 function is unknown.

The effects of Met420del on OCT1 function depend on the substrate (Table 4.3). Met420del does not affect the uptake of MPP⁺ (1-methyl-4-phenylpyridinium) and only marginally affects the uptake of ASP⁺ (4-(dimethylamino)styryl)-*N*-methylpyridinium) and debrisoquine [4, 7, 12]. In contrast, Met420del causes a more than 60 % reduction in the uptake of metformin and morphine [5, 8], a more than 80 % reduction in the uptake of tropisetron and a complete lack of *O*-desmethyltramadol uptake [9, 10]. Therefore, the *OCT1**2 allele (which carries Met420del alone, Fig. 4.1b) should be regarded as either active, with reduced activity, or completely inactive depending on the substrate analyzed. However, if the Met420 deletion is present in combination with Cys88Arg (*OCT1**6 allele) or

Table 4.2 Very important polymorphisms (VIPs)

Polymorphism name (alternative name)	rs Number	Minor allele frequency (%) ^a			Effects on transporter activity (in vitro data)	References	Associations with disease susceptibility, or effect on drugs pharmacokinetics and susceptibility (in vivo data)	References
		Caucasians	Africans	Asians				
<i>OCT1/SLC22A1</i>								
Arg61Cys (R61C)	rs12208357	6	0	0	Strong, substrate-unspecific reduction of OCT1 activity	[3–5, 7–10]	Associated with ^b • Changes in pharmacokinetics and effects of morphine and <i>O</i>-desmethyltramadol • Changes in the plasma concentration and efficacy of tropisetron and ondansetron	[4, 8, 10, 11] [9]
Cys88Arg (C88R)	rs55918055	0.30	0	0	Complete substrate-unspecific loss of OCT1 activity	[3, 8–10]	Associated with ^b • Changes in pharmacokinetics and effects of morphine and <i>O</i>-desmethyltramadol • Changes in the plasma concentration and efficacy of tropisetron and ondansetron	[8, 10, 11] [9]

Gly401Ser (G401S)	rs34130495	2	0	0	Strong, substrate- unspecific reduction of OCT1 activity	[3–5, 8–10, 12]	Associated with ^b	
							• Changes in pharmacokinetics and effects of morphine and <i>O</i> -desmethyltramadol	[4, 8, 10, 11]
							• Changes in the plasma concentration and efficacy of tropisetron and ondansetron	[9]
Met408Val	rs628031	41	27	26	Minor effects on OCT1 activity	[4, 13]	Associated with gastrointestinal side effects of metformin	[14]
Met420del (M420del)	rs202220802 (rs72552763)	18	4	1	Substrate-dependent decrease (with some substrates, up to a complete loss) of activity	[3–5, 7–10, 12, 15]	Associated with ^b	
							• Changes in pharmacokinetics and effects of morphine and <i>O</i> -desmethyltramadol	[4, 8, 10, 11]
							• Changes in the plasma concentration and efficacy of tropisetron and ondansetron	[9]
Exon 7–intron 7 border ^c	rs36056065	41	0.50	28	Potential effects on splicing	[16]	Associated with gastrointestinal side effects of metformin	[14]
Intron 7 polymorphism	rs662138	18	3	1	Not known		Associated with plasma concentrations of isobutyl- L-carnitine in a GWAS study	[17]

(continued)

Table 4.2 (continued)

Polymorphism name (alternative name)	rs Number	Minor allele frequency (%) ^a			Effects on transporter activity (in vitro data)	References	Associations with disease susceptibility, or effect on drugs pharmacokinetics and susceptibility (in vivo data)	References
		Caucasians	Africans	Asians				
Intron 8 polymorphism	rs622342	38	18	15	Not known		Associated with • Prescribed dose of levodopa and with mortality • Response to metformin in diabetes mellitus • Progression of primary biliary cirrhosis	[18–21]
Gly465Arg	rs34059508	1.50	0	0	Complete, substrate-unspecific loss of OCT1 activity	[3–5, 8–10, 12]	Associated with ^b • Changes in pharmacokinetics and effects of morphine and <i>O</i>-desmethyltramadol • Changes in the plasma concentration and efficacy of tropisetron and ondansetron	[4, 8, 10, 11] [9]
<i>OCT2/SLC22A2</i>								
Ala270Ser (c.88G>T)	rs316019	11	19	14	Substrate- and cell-model-dependent decrease in OCT2 activity	[22–26]	Associated with: - Changes in the renal clearance of creatinine and metformin. - Reduced amount elimination of tryptophan in urine	[26–28] [29]

Thr201Met (c.602C>T)	rs145450955	0	0	1	Limited decrease in OCT2 activity	[30]	Associated with renal secretory clearance of mefloquin.	[26]
Thr199Ile ^d (c.596C>T)		0	Not available	0.70	Limited decrease in OCT2 activity	[30]	Associated with renal secretory clearance of mefloquin.	[26]
OCT3/SLC22A3								
c.1233C>G (Ala411Ala)	rs2292334	35	10	46	Associated with reduced mRNA expression in the liver, assumed splice defect	[31, 32]		
Val432Phe	Not available	0	0	6.80	Strong substrate-dependent decrease in affinity	[33]		
Intron 5	rs9364554	28	2	33	Not known		T-allele was associated with 20 % higher risk for developing prostate cancer	[34]
	rs2048327	35	3	46	Not known		Haplotypes of OCT3-LPAL2-LPA region were associated with up to an 80 % increased risk of coronary disease	[35]
	rs7758229	32	5	25	Not known		Associated with about a 30 % increased risk of developing colorectal cancer in Asians	[36]

(continued)

Table 4.2 (continued)

Polymorphism name (alternative name)	rs Number	Minor allele frequency (%) ^a			Effects on transporter activity (in vitro data)	References	Associations with disease susceptibility, or effect on drugs pharmacokinetics and susceptibility (in vivo data)	References
		Caucasians	Africans	Asians				
<i>OCTN1/SLC22A4</i>								
Leu503Phe (c.1672C>T)	rs1050152	39	2	0	Decreased uptake of carnitine, but increased uptake of TEA ⁺	[37]	Associated with an increased risk of Crohn's disease	[37]
g. 6607C>T (intron 1)	rs3792876	9	4	30 ^d	Increased binding to the transcriptional factor RUNX1 leading to lower promoter activity	[38]	Associated with an increased risk of rheumatoid arthritis in Japanese individuals, but not in other populations	[38, 39]
<i>OCTN4/SLC22A5</i>								
-207G>C	rs2631367	44 ⁺	42	0	Destroys a HSE and reduced the expression of OCTN2	[37]	Associated with an increased risk of Crohn's disease	[37]
<i>MATE1/SLC47A1</i>								
-66T>C	rs2252281	41	21	41	Decreased promoter activity and reduced MATE1 expression in kidney	[40]	Associated with increased efficacy of metformin	[41]

G>A (intron 10)	rs2289669	42	2	49	Not known	Associated with improved efficacy of metformin (measured as a reduction in HgA1C levels). No changes in metformin pharmacokinetics were observed	[11, 42]
MATE2-K/SLC47A2							
-130G>A	rs12943590	27	18	45	Increased promoter activity	Associated with reduced response to metformin in patients	[43]
-396G>A	rs34834489	35	5	32	Increased promoter activity	Associated with reduced renal secretion of metformin ^e	[44]

Listed are polymorphisms in organic cation transporters that affect the transporter expression and function and/or that were associated with pathophysiological or pharmacological changes in humans

GWAS genome wide association study, TEA⁺ tetraethylammonium, HSE heat shock element

^aExcept when otherwise stated, the minor allele frequencies were obtained from the 1000 Genomes Project database

^bThe association was observed when the polymorphism was analyzed in combination with other functional OCT1 alleles. For details about the possible combinations see Fig. 4.1 and the text

^cThe minor allele frequencies were obtained from dbSNP database

^dThe minor allele frequencies were obtained from Kang et al. 2007 [30]

^eThe association was observed when the polymorphism was analyzed in combination with -130G>A polymorphism

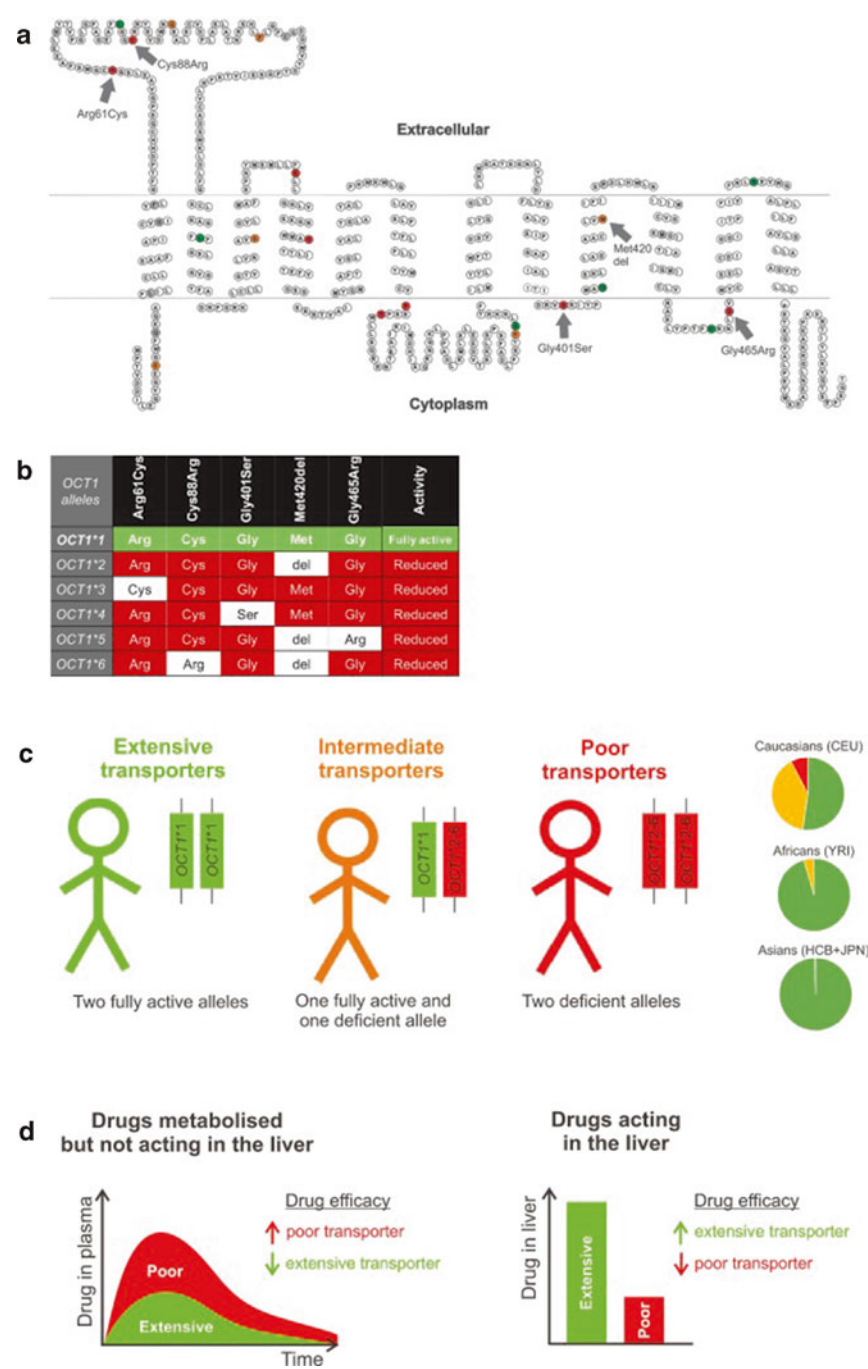


Fig. 4.1 Common amino acid substitutions leading to loss of OCT1 activity. (a) Polymorphic amino acids are shown on the secondary structure of the OCT1 protein. Amino acid substitutions with strong effects on OCT1 function are given in red, those with moderate effects in orange and those with no effects in green. Grey indicates amino acid substitutions for which function has not

Table 4.3 Substrate-specific effects of the Met420 deletion on OCT1 activity

Substance	Concentration used (μM)	Model system	Activity of the 420del allele (% of the WT)	References
MPP ⁺	1	<i>X. laevis</i> oocytes, HEK293 cells	100	[3, 4]
ASP ⁺	1	HEK293 cells	>87	[7, 12]
Debrisoquine	1	HEK293 cells	73.4	[12]
Metformin	250	HEK293 cells	37	[5, 7]
Morphine	1	HEK293 cells	15.6	[8]
Tropisetron	1	HEK293 cells	7.8	[9]
<i>O</i> -desmethyltramadol	1	HEK293 cells	0	[10]

Gly465Arg (*OCT1**5 allele), due to the effects of these two polymorphisms, the encoded OCT1 will be inactive regardless of the substrate used [3–5, 9, 10].

Another very intriguing observation made by Ahlin et al. was that although Met420del did not substantially change the uptake of some substrates, it did change the potential of most drugs to inhibit the uptake of these substrates [7]. ASP⁺ uptake was only non-significantly reduced by the Met420del variant. However, the deletion of Met420 increased the inhibition potency of some drugs by up to 13-fold. This study demonstrates that interactions between the genetic variants and drug–drug interactions that may be much more complex than currently expected. The observations made in this study may be relevant beyond the Met420del polymorphism. Other common coding *OCT1* variants that were not shown to affect OCT1 activity, like Leu160Phe, Pro341Leu and Arg488Met, may be also affected.

Arg61Cys

The amino acid substitution arginine to cysteine at codon 61 (Arg61Cys, R61C) leads to decrease in a OCT1-mediated uptake by more than 70 % for all substrates tested including MPP⁺, ASP⁺, metformin, morphine, tropisetron and *O*-desmethyltramadol (the active metabolite of tramadol) and debrisoquine [3–5, 7–10, 12]. The substitution is common in Caucasians, but not present in Asians and Africans (Table 4.2). Arg61Cys is not linked with the other functional OCT1 variants and alone constitutes the allele *OCT1**3, conferring substantially reduced or even a complete loss of OCT1 transporter activity (Fig. 4.1).

←
Fig. 4.1 (continued) yet been analyzed. The five common amino acid substitutions with strong effects on OCT1 are highlighted with *arrows*. **(b)** Allelic combinations of the five common functional amino acid substitutions in OCT1. The fully active allele *1 is shown in *green* and the deficient alleles in *red*. **(c)** Extensive, intermediate and poor OCT1 transporters and their frequency in Africans, Asians, and Caucasians. **(d)** Illustration of the opposite effects of loss of OCT1 activity between drugs metabolized (*left side*) and drugs acting in the liver (*right side*)

Arg61Cys is located in the large extracellular loop of OCT1. The Arg61Cys substitution substantially, but not completely, impairs the correct membrane localization of OCT1 [5, 7]. This may be explained by the generation of an additional cysteine residue that highly probably disturbs the formation of the correct disulfide bonds within the large extracellular loop of OCT1. Disulfide bonds in the large extracellular loop are essential for oligomerization and correct membrane localization of OCT1 [45].

Cys88Arg

Among the functional *OCT1* variants, Cys88Arg is the rarest one. Its frequency is below 1 % in Caucasians and it is not observed in other populations. Cys88Arg is observed only in combination with the Met420 deletion. The combination is designated allele *OCT1**6 and encodes a completely inactive protein [3, 8–10].

Cys88Arg is located in the large extracellular loop. Cys88Arg completely impairs the correct localization of OCT1 in the cytoplasmic membrane [46]. This is in line with the involvement of Cys88 in formation of disulfide bonds in the large extracellular loop [45].

Gly401Ser

The amino acid substitution glycine to serine at codon 401 leads to a strong, substrate-independent loss of OCT1 activity [3–5, 8–10, 12]. The reduction in OCT1 activity caused by Gly401Ser is more than 80 % with all the substrates tested. Gly401Ser is the third most common functional variant in Caucasians, with a frequency of about 2 % (*OCT1**4 allele). The substitution is observed only in Caucasians.

Gly401 is localized in the intracellular loop between transmembrane domains 8 and 9. There is no information about the effects of Gly401Ser on the subcellular localization of OCT1 or on any other mechanism by which this variant may cause loss or strong reduction of OCT1 function.

A recent study reported a lack of functional effects of Gly401Ser in both the transport of the model OCT1 substrate TEA⁺ and the toxicity of sorafenib [46]. However, this result is a clear contradiction with all other published in vitro analyses [3–5, 8–10, 12] and requires validation.

Gly465Arg

The amino acid substitution glycine to arginine at codon 465 leads to a complete inactivation of OCT1 [4, 5, 8–10, 12]. The Gly465Arg polymorphism was observed only in Caucasians with a frequency between 1 and 5 %. Gly465Arg was observed only in combination with the Met420 deletion (*OCT1* allele *5, Fig. 4.1b).

Gly465Arg is located in the intracellular loop between transmembrane domains 10 and 11. The Gly465Arg substitution completely impairs the correct localization of OCT1 in the cytoplasmic membrane [4, 7]. The exact mechanism leading to this impairment remains unclear, but it was shown that the substitution of Gly₄₆₅ with alanine impairs neither OCT1 localization nor function [4].

***OCT1* Alleles with Decreased Function and Categorization as Poor, Intermediate and Extensive OCT1 Transporters**

Analyzing the combinations of the functional OCT1 polymorphisms that can appear on a single chromosome, so called alleles, is an essential step to understand the combined effects of these variants. Six alleles were reported (Fig. 4.1b): *OCT1* alleles *OCT1**1 to *OCT1**6 [5, 9, 10]. Based on in vitro assessments of metformin, morphine, *O*-desmethyiltramadol (the active metabolite of tramadol) and tropisetron uptake, the alleles *OCT1**2 to *6 were identified as completely inactive or with substantially reduced activity [5, 8–10].

Based on the number of fully active *OCT1* alleles, each individual (patient or healthy volunteer) can be categorized as extensive, intermediate and poor OCT1 transporter. Extensive transporters have two, intermediate transporters have one, and poor transporters lack fully active *OCT1* alleles (Fig. 4.1c). Using such categorization will increase the power of clinical studies and make it easier to understand the functional mechanisms underlying the observed associations.

This categorization extensive, intermediate and poor transporters is dependent on the OCT1 substrate. This is due to the substrate-specific effects of the *OCT1**2 allele (marked by Met420 deletion). For substrates for which *OCT1**2 is not active like metformin, tropisetron and tramadol, 30 % of the copies of chromosome 6 in Caucasians encode for OCT1 with strongly reduced activity (Fig. 4.1c). Therefore, 39 % of Caucasians are intermediate transporters and 9 % are poor transporters of these substrates (Fig. 4.1d). For the model substrates MPP⁺, ASP⁺ or for debrisoquine, only 15 % of the chromosomes 6 encode for *OCT1* with strongly reduced activity. Therefore, 22 % of Caucasians are intermediate transporters and 2.4 % are poor transporters of these substrates. Thus, depending on the drug tested, between 2.4 and 9 % of Caucasians are poor transporters for OCT1 substrates and will lack or have substantially reduced OCT1 activity.

Other Coding or Potentially Functional Non-coding OCT1 Polymorphisms

Several common amino acid substitutions have been reported in *OCT1* but not suggested to affect OCT1 function: Ser14Phe, Leu160Phe, Pro341Leu and Met408Val [3–5, 46]. Still, as we learned from the Met420 deletion, substrate-specific effects may be present and broader substrate screening is required before concluding that these substitutions do not affect OCT1 activity.

Recently the common Met408Val polymorphism was reported to modify the effects of the Met420 deletion [47]. However, systematic analyses showed that this modification may be of only theoretical interest, as in humans the deletion of Met420 has only been found on the Val408 and not the Met408 background [13].

Several polymorphisms that cause substantial reduction of OCT1 function were observed in Asians: Gln97Lys, Pro117Leu and Arg206Cys [6]. However, their frequencies are apparently much lower than initially assumed [27].

An eight base pair deletion at the exon 7–intron 7 border (rs36056065) is a highly common globally observed variant. Recently rs36056065 was suggested to affect exon 7 splicing, and as a consequence, transporter expression in peripheral blood [16]. However, in vitro experiments demonstrating polymorphism-dependent alternative splicing (i.e. mini gene assays) are still missing, and it remains to be clarified whether the effects are taking place in the liver where the expression of OCT1 is much higher. It should be considered that rs36056065 is not a simple 8 bp deletion, but a varying number of tandem repeat polymorphism (Fig. 4.2b). Therefore, the canonical splice site remains, even if only one copy of the repeat is present. The highly genetically linked polymorphism Met408Val (Fig. 4.2a) showed an association with mRNA levels, but not with protein levels of OCT1 in the liver [31].

Other intronic polymorphisms like rs662138 and rs622342 have been associated with changes in the metabolite profile, disease progression, or drug response in large epidemiological studies [17–21]. These studies used microarray platforms for high-throughput genotyping and the analyzed SNPs were not selected based on previous information about functionality, e.g. effects on transporter expression and activity. Therefore, the reported variants may not be the ones causing the association. This makes the interpretation of the obtained data rather difficult. Some of the reported SNPs are either completely (rs662138) or partially (rs622342) genetically linked to functional *OCT1* variants (Fig. 4.2), which may deliver a mechanistic explanation of the observed associations. Still, this approach does not completely cover the known functional variability in OCT1 and studies designed to analyze all the known functional variants are needed to validate these findings.

Effects of Genetic Polymorphisms on *OCT1* Expression

Strong variability in OCT1 expression was reported. More than a 23-fold variability in *OCT1* mRNA and more than 16-fold variability in OCT1 protein expression were reported for the liver [31, 48, 49], the organ with by far the strongest OCT1 expression in humans [50]. However, only a marginal part of this variability could be explained by genetic polymorphisms within the OCT1 locus. Nies et al. found an association of the Cys61 allele of the Arg61Cys polymorphism with decreased *OCT1* expression [31], but this observation was not consistent with the study of O'Brien et al., in which the Cys₆₁ was associated with increased *OCT1* expression [48]. Two other common polymorphisms in the *OCT1* promoter, –1795G>A (rs6935207) and –1756Tins, did not show any association with variations in *OCT1* expression [49].

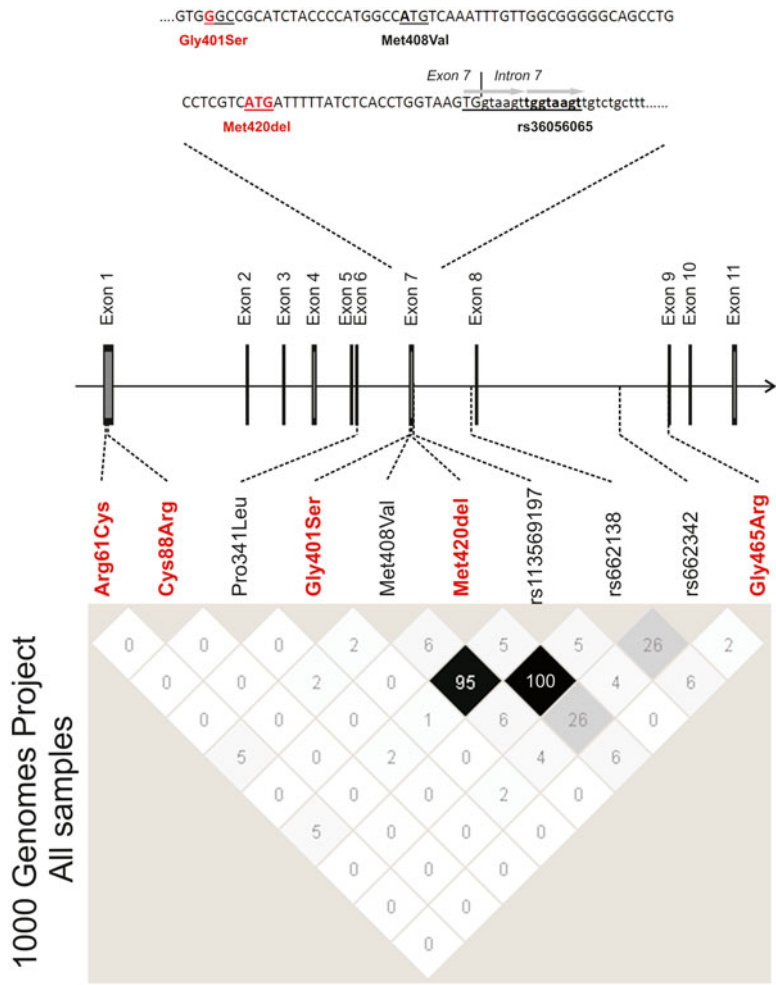


Fig. 4.2 Localization of and genetic linkage between *OCT1* variants reported to be of potential physiological, pathophysiological, and pharmacological relevance. The upper part of the figure shows the localization of the five common amino acid substitutions with clear effects on *OCT1* activity (shown in red, for details about the five substitutions see Fig. 4.1 and the text) in relation to the other coding and non-coding variants (shown in black) These variants were also suggested to affect *OCT1* expression or/and function, but clear clinical data is missing or is contradictory. Special attention was paid to variants within exon 7. Arrows indicate a tandem repeated sequence at the exon7–intron7 border. The deleted sequence of the polymorphism rs36056065 is highlighted in bold. The lower part of the figure illustrates the pairwise linkage disequilibrium between the five common amino acid substitutions and the other clinically associated variants. The numbers representing r^2 values are given as percentages

It could be speculated that *trans*-acting factors, i.e. genetic and epigenetic variations in transcriptional factors involved in the regulation of OCT1 expression, may affect OCT1 expression. The transcriptional factor HNF1alpha has been recently shown to strongly enhance OCT1 expression in the liver [48]. Polymorphisms causing amino acid exchanges in *HNF1alpha* like rs2464186 (Ser487Asn) are known to affect the expression of several downstream genes [51, 52], but systematic analyses of the effects on OCT1 expression are still missing.

Tumor-Specific OCT1 Variants

Recently Herraiz et al. reported that along with the common germline polymorphisms, a substantial number of somatic *OCT1* mutations exist in hepatocellular carcinoma [46]. Some of these mutations strongly affect splicing and therefore completely change protein structure and lead to complete inactivation of OCT1. This may be of special importance if the tumors are treated with cytotoxic drugs that are substrates of OCT1 like sorafenib.

Physiological Consequences of OCT1 Genetic Variability

Association of SNPs in OCT1 with Plasma Metabolite Concentrations at Genome-Wide Levels of Significance

A genome wide association study (GWAS) of levels of metabolites in human plasma revealed a highly significant association between SNPs in *OCT1* and plasma levels of isobutyryl-carnitine [17]. The strongest association was observed with the rs662138 SNP located in intron 7 of the *OCT1* gene. The rs662138 SNP is in a complete genetic linkage with the most common functional *OCT1* polymorphism Met420del (Fig. 4.2), but there is no data available about the ability of OCT1 to transport isobutyryl-carnitine.

Isobutyryl-carnitine is an intermediate product in the metabolism of fatty acids and branched amino acids. Increased levels of plasma isobutyryl-carnitine were observed in children carrying a very rare genetic defect in short chain acyl-CoA dehydrogenase (SCAD) [53, 54]. However, this increase is believed to be just a marker and not a part of the pathophysiological mechanism of SCAD. Therefore, no direct effects of OCT1 genetic variants on the pathophysiology of this disease should be expected.

Effects on Vitamin B1 Transport

Recently OCT1 was reported to be responsible for thiamine (vitamine B1) transport in the liver [15]. Oct1 knockout mice showed higher plasma and lower liver concentrations of vitamin B1, decreased liver weight, and decreased triglyceride

concentrations in the liver. Therefore, OCT1 may play a role in the pathophysiology of hepatic steatosis. In the same study, genetic polymorphisms in *OCT1*, including the common functional variants Arg61Cys, Gly401Ser, Gly465Arg, and Met420del, were shown to affect vitamin B1 uptake in vitro. However, studies addressing the effects of *OCT1* polymorphisms on vitamin B1 concentrations and susceptibility to hepatic steatosis in humans are still missing.

Pharmacological Implications of OCT1 Genetic Variability

The strong hepatic expression and the high genetic variability of OCT1 attracted attention to the *OCT1* polymorphisms in relation to hepatic drug uptake and metabolism. OCT1 is highly expressed at the sinusoidal membrane of the human liver, and genetic variants leading to decreased OCT1 activity will first and foremost decrease the hepatic drug uptake. Two potential consequences could be expected. For drugs substantially metabolized in the liver, loss or decrease of OCT1 activity may lead to increased plasma concentrations resulting in improved efficacy, but also increased toxicity. Such effects have been reported for the opiates morphine and tramadol and for the antiemetics tropisetron and ondansetron. For drugs acting in the liver, loss or decrease of OCT1 activity may lead to lower liver concentration and thus to decreased activity, as reported for the antidiabetic drug metformin (Fig. 4.1d).

Genetic polymorphisms in *OCT1* have been suggested to affect the pharmacokinetics and/or efficacy of a number of drugs. However, these studies differ substantially in the strength of the presented evidence, varying from a very low level of evidence such as a single association study, to a high level of evidence such multiple studies with the underlined functional mechanism clearly identified (Fig. 4.3).

Effect on Pharmacokinetics and Efficacy of Opioids

The opiate morphine is a hydrophilic weak base that is substantially metabolized in the liver. We have demonstrated that hepatic uptake of morphine depends on OCT1 and that the common functional polymorphisms in *OCT1* lead to a substantial decrease or even to complete loss of morphine uptake [8]. In the same study we also reported significantly increased plasma concentrations of morphine in poor and intermediate compared to extensive OCT1 transporters (carriers of two fully active *OCT1* alleles). There were also indications of elevated frequency of adverse effects in poor and intermediate OCT1 transporters. These results were confirmed and substantially extended by Fukuda et al. [55], who analyzed morphine clearance in 146 children receiving metformin after adenotonsillectomy. The same group extended their findings, showing that poor OCT1 transporters had also reduced formation of morphine 3-glucuronid, the main hepatic metabolite of morphine, and that these effects are independent on the genotypes of MDR3, the main efflux transporter of morphine metabolites in the liver [56]. Currently the effects on morphine

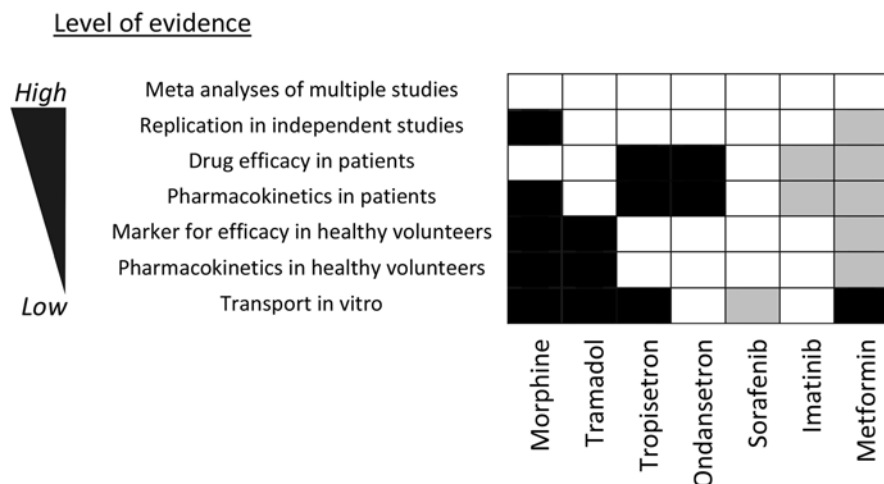


Fig. 4.3 Summary of the known effects of genetically based loss of OCT1 activity on the pharmacokinetics and efficacy of commonly used drugs. The information about the effects on different drugs is compared according to the levels of evidence available. Grey color indicates controversial literature data

pharmacokinetics are the most consistent pharmacological consequences reported for *OCT1* polymorphisms. However, these findings are still new. The pharmacokinetics findings need further validation and studies on the effects of loss of OCT1 activity on efficacy and toxicity of morphine should be performed.

The loss of OCT1 activity has been also associated with a significant increase in the plasma concentration of *O*-desmethyltramadol in healthy volunteers [10]. *O*-desmethyltramadol is the active metabolite of the synthetic opioid tramadol. The intermediate and poor OCT1 transporters showed not only increased plasma concentrations of *O*-desmethyltramadol, but more intensive and prolonged miosis, a surrogate marker for opioidnergic effect of tramadol [10]. This results were obtained in healthy volunteers and require validation in patients receiving tramadol.

Interestingly codeine, the pro-drug of morphine, and tramadol, the pro-drug of *O*-desmethyltramadol, were found to be good inhibitors, but not substrates of OCT1, and plasma concentrations of codeine and tramadol itself did not depend on *OCT1* polymorphisms [8, 10]. This suggests that the pharmacokinetics of only cationic and weak basic drugs with low passive membrane permeability may be affected by loss of OCT1 activity.

Effect on Pharmacokinetics and Efficacy of Antiemetics Tropisetron and Ondansetron

Tropisetron and ondansetron are antiemetics given to treat chemotherapy or surgery induced nausea and vomiting. Tropisetron and ondansetron are weak basic drugs that are substantially metabolized in the human liver. Poor OCT1 transporters had

significantly higher plasma concentration and respond significantly better to tropisetron and ondansetron treatment than extensive OCT1 transporters [9]. The observed association was supported by in vitro experiments showing OCT1-mediated uptake of tropisetron, but not of ondansetron [9]. Therefore, the mechanisms causing the association between the poor OCT1 transporter phenotype and increased plasma concentrations and improved efficacy of ondansetron still need to be clarified.

Effects on Pharmacokinetics and Efficacy of Metformin

Metformin is a cationic drug very broadly used in the treatment of diabetes mellitus and was one of the first drugs reported to be a substrate of OCT1. Effects of *OCT1* polymorphisms on metformin pharmacokinetics and efficacy have been of great interest and more than 15 studies have been done on this topic (Table 4.4). Unfortunately the different studies showed highly contradictory results regarding the effects of *OCT1* polymorphisms on both metformin pharmacokinetics and efficacy. In Caucasians, poor OCT1 transporters were reported to have higher [57], lower [11, 58] or no significantly different plasma concentrations of metformin compared to extensive OCT1 transporters [59]. In Asians the “canonical” loss of function OCT1 polymorphisms (Met420 del, Arg61Cys, Cys88Arg, Gly401Ser and Gly465Arg) are almost completely missing (Fig. 4.1d, Table 4.2). Analyses of the “Asian-specific” polymorphisms showed only a marginally decreased metformin clearance in carriers of the Leu341 allele of the Pro341Leu polymorphism, and this decrease was not significant after correcting for other polymorphisms like Ala270Ser in OCT2 [22, 27]. This is in agreement with in vitro data showing no effect of Pro341Leu on metformin uptake [5].

In humans OCT1 is predominantly expressed in the liver, but some (trace) OCT1 expression was reported for other pharmacokinetically relevant organs like the kidney (on the apical membrane of the proximal and distal tubules) [11] and small intestine [68, 69]. The effects of *OCT1* polymorphisms on metformin pharmacokinetics have been explained by changes in OCT1 activity in these organs [11, 57, 58].

Metformin is not metabolized, but is believed to act in the human liver [70]. Therefore, polymorphisms resulting in loss of OCT1 activity could be expected to affect metformin efficacy without strongly affecting metformin pharmacokinetics. Indeed, the initial study of Shu et al. showed significantly reduced metformin efficacy in poor OCT1 transporters as measured by a glucose tolerance test in healthy volunteers [5]. These results were in line with the reduced liver concentrations of metformin in Oct1 knockout mice [71]. However, no clear and reproducible effects of *OCT1* polymorphisms on metformin efficacy were observed in diabetic patients (Table 4.4). Some studies showed limited, but significant effects of OCT1 polymorphisms [19, 20, 58]. However, these studies analyzed only a highly limited number of individuals. The large cohort studies with long-term follow ups did not find an association between *OCT1* polymorphisms and metformin efficacy [60, 72]. Furthermore, artifacts in the analyses of some of the small studies were reported [67].

Table 4.4 Effects of genetic polymorphisms in *OCT1* on the pharmacokinetics, efficacy and toxicity of metformin

Study	Year	# of analyzed individuals	Observed associations	References
<i>Effects on metformin pharmacokinetics</i>				
Shu et al.	2008	20 healthy volunteers	Decreased clearance in heterozygous carriers of R61C, G401S, M420del and G465R alleles	[57]
Tzvetkov et al.	2009	103 healthy volunteers	Increased renal clearance in compound homozygous carriers of R61C, G401S, M420del and G465R. The increase was in a gene dosage manner.	[11]
Christensen et al.	2011	147 patients	Increased clearance in compound heterozygous carriers of R61C, G401S, M420del and G465R. The increase was in a gene dosage manner.	[58]
Christensen et al.	2013	50 healthy volunteers preselected for OCT2 Ala270Ser variants	Lack of effects of compound heterozygous carries of R61C, G401S, M420del and G465R on the renal clearance. Total clearance was not analyzed.	[59]
Yoon et al.	2013	96 healthy volunteers	Lack of effects of Pro341Leu on metformin pharmacokinetics. (The other Asian specific variants Q97K, P117L and R206C were not found in the study population.)	[27]
<i>Effects on therapy efficacy in diabetes</i>				
Shu et al.	2007	20 healthy volunteers	Decreased metformin efficacy (measured using glucose tolerance test) in heterozygous carries of R61C, G401S, M420del and G465R	[5]
Zhou et al.	2009	3450 patients	Lack of association between R61C and Met420del SNPs and metformin response (analysis of the GoDARTS study)	[60]
Becker et al.	2009	102 patients	Decreased efficacy of the homozygous G-allele carriers of the rs622342 SNP in OCT1	[19]
Becker et al.	2010	98 patients	Decreased efficacy of the homozygous G-allele carriers of the rs622342 SNP in OCT1 is independent of MATE1 polymorphisms	[20]

(continued)

Table 4.4 (continued)

Study	Year	# of analyzed individuals	Observed associations	References
Christensen et al.	2011	149 patients	No association with efficacy of metformin (measured as reduction of HbA1c) ^a	[58]
Shikata et al.	2007	33 patients: 24 responders/9 non-responders	The Met408 allele of the Met408Val polymorphism was associated with lack of metformin response	[61]
Gambineri et al.	2010	150 patients	Decreased metformin efficacy (in terms of reduction of total cholesterol, triglycerides or insulin) in carriers of R61C, G401S, M420del and G465R	[62]
Jablonski et al.	2010	990 patients	Associated with frequency of metformin usage in a diabetes prevention program with Leu160Phe and Met408Val polymorphism in OCT1	[63]
Choi et al.	2011	253 patients	Lack of effects of the major functional OCT1 polymorphisms (Arg61Cys, Gly401Ser, Met420del and Gly465Arg) on metformin efficacy measured as relative change in HbA1c	[43]
<i>Effects on adverse effects of metformin</i>				
Tarasova et al.	2012	246 patients: 53 cases/193 controls	Met408 of the Met408Val polymorphism was associated with increased risk of gastro-intestinal adverse effects	[14]
Klein et al.	2014	135 patients	Borderline increased risk of hypoglycemia in carriers of the Val408 allele	[64]
Dujic et al.	2014	2166 patient: 251 cases/1915 controls	Carriers of two reduced function OCT1 alleles were associated with significantly increased risk of metformin intolerance (mostly gastro-intestinal adverse effects; analysis of the GoDARTS study)	[65]
<i>Effects on therapy efficacy in polycystic ovarian syndrome</i>				
Gambineri et al.	2010	150 patients	Decreased metformin efficacy (in terms of reduction of total cholesterol, triglycerides or insulin) in carriers of R61C, G401S, M420del and G465R	[62]

(continued)

Table 4.4 (continued)

Study	Year	# of analyzed individuals	Observed associations	References
Schweighofer et al.	2014	676 patients: 90 controls	OCT1 variants (in combination with OCT2 variants) correlated with response to metformin in PCOS patients	[66]

^aA corrigendum of the original paper was recently published showing that the observed differences after metformin treatment were due to significant differences in the background levels of Hb1Ac [67]

This suggests that *OCT1* polymorphisms have only a limited effect on metformin activity that could not be observed in large, but highly heterogeneous patient populations. However, heterogeneous patient populations reflect better the daily praxis and therefore only effects observed in such populations may be therapeutically relevant.

Different speculations may be made to explain the lack of effects of *OCT1* polymorphisms on metformin efficacy. First, there may be another metformin uptake transporter in the human liver. There is no data about reduction of metformin hepatic concentrations in humans. This transporter may be absent in rodents, which would explain the clear decrease of metformin concentration in the liver of the Oct1 knock-out mice. Second, metformin may not need to enter the liver to execute its effects [15], or the role of the liver in the antidiabetic effects of metformin may have been generally overestimated. All these speculations, however, are yet to be addressed experimentally.

Interestingly, recent analyses of large diabetes cohort studies showed that carriers of two reduced function *OCT1* alleles have a 2.4-fold increased risk to develop intolerance to metformin [65]. It remains to be elucidated whether the observed increase can be attributed to the relatively small increase in metformin plasma concentrations in carriers of two reduced activity alleles, or whether other mechanisms are involved.

Beyond diabetes, metformin is used in the treatment of polycystic ovary syndrome (PCOS). Several studies have reported effects of *OCT1* polymorphisms on metformin efficacy in PCOS treatment [62, 66].

Effects on the Tyrosine Kinase Inhibitors Imatinib and Sorafenib

Multiple studies have reported an association between *OCT1* polymorphisms or expression levels of OCT1 and the efficacy of treatment of chronic myeloid leukemia with the tyrosine kinase inhibitor imatinib [73]. These associations, however, were not always reproducible (for overview see supplementary Table 4.1 in [74]) and there were some technical problems with the initial expression studies [47]. Most importantly, recent detailed analyses argued that imatinib is not transported by OCT1 at all [74].

Similarly, sorafenib, a tyrosine kinase inhibitor, has been suggested to depend on OCT1 for its hepatic uptake [75]. This is of special interest as sorafenib is used to treat hepatocellular carcinoma (HCC), and genetic polymorphisms and tumor-specific mutations in OCT1 have been suggested to affect sorafenib toxicity [46]. Still the in vitro data about OCT1-mediated sorafenib uptake is controversial ([76] and Tzvetkov et al., unpublished results) and clinical studies associating *OCT1* polymorphisms with sorafenib efficacy are missing. The clinical studies may be further complicated by the observed strong loss of OCT1 expression in the majority of HCC tumors [77, 78] due to promoter methylation of the gene [79].

It cannot be excluded that OCT1 may indirectly affect the efficacy of tyrosine kinase inhibitors without affecting their uptake. This hypothesis has the advantage that it will explain both the observed effects on sorafenib and imatinib cytotoxicity and the lack of strong effects on the direct transport, but it requires experimental validation.

Potential Effects on Other Drugs

Genetic polymorphisms in *OCT1* have been associated with the prescribed dosages and mortality after levodopa treatment of Parkinson's disease [18]. The functional mechanisms behind this association remain unclear: there is no data demonstrating levodopa as a substrate of OCT1 and there are no clinical studies on the effect of *OCT1* polymorphisms on levodopa pharmacokinetics.

OCT1 was suggested to mediate the transport of the anticonvulsant lamotrigine through the blood–brain barrier [80]. Therefore, polymorphisms leading to the poor OCT1 transporter phenotype may lead to reduced efficacy of lamotrigine. However, there are currently no in vivo data to support this hypothesis. Furthermore, the pharmacological relevance of the low level of expression of OCT1 at the blood–brain barrier is still controversially discussed.

Recently we reported that the psychotropic drugs amisulpride and sulpiride are substrates of OCT1 and that their uptake is strongly affected by *OCT1* polymorphisms [81]. Neither amisulpride, nor sulpiride are substantially metabolized by, or act in, the liver. Still it may be interesting to analyze the potential role of loss of OCT1 activity on the biliary excretion or uptake in the brain of both drugs. The biliary excretion may account for up to 20 % of the total clearance of amisulpride and sulpiride [82, 83]. Furthermore, both drugs act in the brain and OCT1 expression has been previously reported at the blood–brain-barrier [84], although the data is controversial.

Also very recently *OCT1* polymorphisms were shown to affect the cellular uptake of the cytostatic bendamustine and were associated with decreased bendamustine cytotoxicity ex vivo [85]. In addition, OCT1 was suggested to act as an efflux transporter of the DNMT inhibitor zebularine and the common *OCT1* polymorphisms caused a reduction of the efflux of zebularine in oocytes [86].

Interestingly, the Met408Val substitution, which typically do not show any changes in the OCT1-mediated drug uptake, caused significant decrease in the OCT1-mediated efflux of zebularine. This illustrates that genetic polymorphism may have different effects depending on the direction of the OCT1-mediated transport.

The antihypertensive drug debrisoquine has been shown to be a substrate of OCT1 and polymorphisms in *OCT1* have been shown to affect debrisoquine uptake in vitro [12]. Currently debrisoquine is not therapeutically used, but historically it played a pivotal role in revealing the effects of *CYP2D6* polymorphisms on drug pharmacokinetics and efficacy [87]. The requirement of carrier-mediated membrane transport for debrisoquine clearly illustrates the importance of regarding polymorphic drug uptake transporters (also known as phase 0 transporters) when considering the inter-individual variability in drug metabolism.

OCT2

Genetic Variability

OCT2 is less genetically polymorphic than *OCT1*. Although 23 non-synonymous polymorphisms are observed in the *OCT2* gene, they are much less frequent (average heterozygosity of 1.2 compared to 7.9 in *OCT1*; Table 4.1) and only a few of them affect OCT2 expression or function (Table 4.2; [23]).

Ala270Ser (c.808G>T)

An alanine-to-serine substitution at codon 270 (Ala270Ser) is the only common functional amino acid polymorphism in *OCT2*. This substitution leads to a moderate and substrate-dependent decrease in OCT2 activity [22–26, 30]. For some substrates like MPP⁺ and metformin, the reports about the effects of Ala270Ser on OCT2 activity are rather controversial. Ala270Ser SNP was claimed not to affect MPP⁺ uptake [23], to affect it strongly [26, 30, 88], or to cause only a 30 % reduction in v_{MAX} without affecting K_{M} [25, 30]. The data about the effects on metformin uptake is even more controversial (Fig. 4.4a).

The exact mechanism for how Ala270Ser affects OCT2 function remains unknown. Ala270Ser is localized in the sixth *trans*-membrane domain of OCT2. The polymorphism does not affect the subcellular localization of OCT2 in oocytes or in HEK293 cells [25, 30]. The effects on OCT2 function seem to depend on the model system used. The effects observed in oocytes were much stronger than those in HEK293 cells [22, 26, 30]. This may indicate an important role of membrane thickness and constitution in the mechanism of Ala270Ser effects.

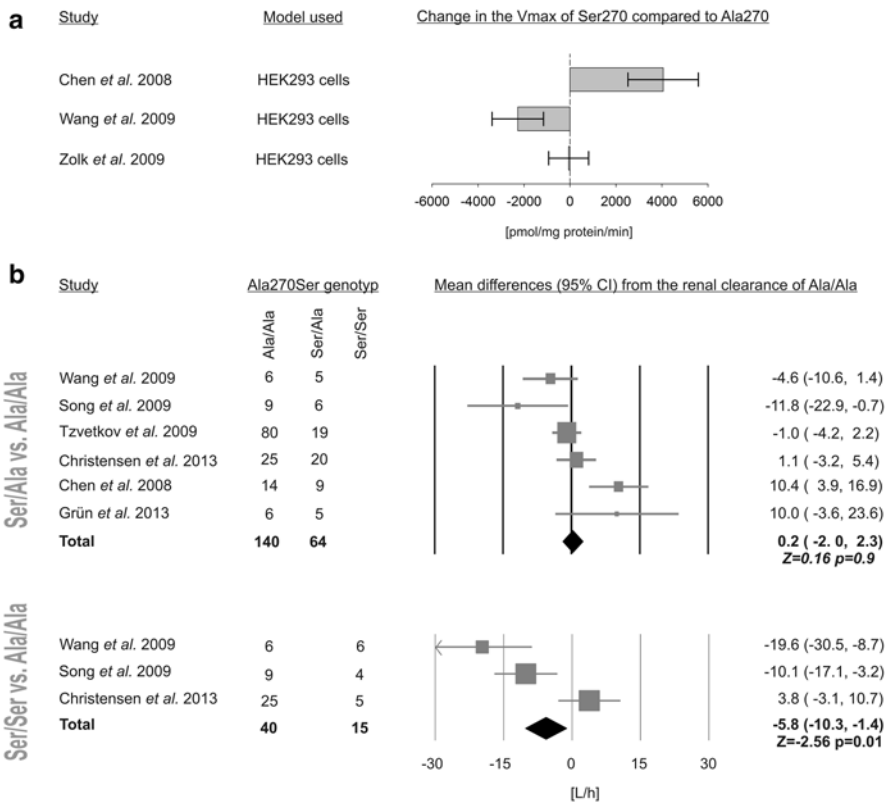


Fig. 4.4 Effects of the Ala270Ser polymorphism in OCT2 on the cellular uptake and renal clearance of metformin. (a) Summary of the available in vitro studies analyzing the effects of the Ala270Ser polymorphism on the v_{MAX} of the cellular uptake of metformin in HEK293 cells. There were no change in K_M of the metformin uptake observed. (b) Meta-analyses of the effects of the Ala270Ser polymorphism on renal clearance of metformin in humans. We compared data from all eligible studies that analyzed renal clearance of metformin (six studies totaling 219 volunteers; [11, 26, 28, 59, 89, 90]). The effects of the heterozygous (*upper part*) and the homozygous (*lower part*) Ser270 carriers were compared in separate analyses. Several transformations of the data and assumptions were made to enable the analyses. First, the renal clearance was recalculated in liters per hour (L/h) for all the studies. Second, in two of the studies only the geometric means were given [59, 90]. Therefore we assumed that the geometric means were equal to the arithmetic means in these two cases. Third, Wang *et al.* [28] did not provide the mean for body weight. We assumed from the range given in their study that participants had a mean body weight of 62 kg. Finally, the reader should be aware that Grün *et al.* [90] gave 500 mg metformin thrice daily, whereas in all other studies 500 mg metformin was given as a single dose

Other Potentially Relevant Variants

Despite systematic re-sequencing efforts only five additional coding genetic variants were found to affect OCT2 function: Met165Ile (c.495G>A), Thr199Ile (c.596C>T), Thr201Met (c.602C>T), Arg400Cys (c.1198C>T) and Lys432Gln

(c.1294A>C) [23, 30]. The Thr199Ile and Thr201Met variants were reported to strongly decrease metformin uptake in vitro [22, 26] and to associate with reduced renal clearance of metformin in vivo [23]. However, all these variants are population-specific and their maximal frequency reaches only about 1 %.

Systematic haplotype analyses in relation to the renal clearance of metformin [11], did not provide any evidence for existence of other clinically relevant polymorphisms in OCT2 beyond the coding one.

Pathophysiological Consequences of OCT2 Polymorphisms

Recently the rs316009 SNP in intron 2 of *OCT2* was reported to be associated with fractional creatinine excretion and moderately increased risk of end stage renal disease (ESRD) [91]. rs316009 is completely genetically linked to Ala270Ser. Therefore the observed association may be explained by the functional effects of Ala270Ser on OCT2-mediated tubular secretion of creatinine. This data is in agreement with the demonstrated role of OCT2 in the proximal tubular secretion of creatinine in mice and humans [92].

Analyses of urine metabolome showed significant association between the Ala270Ser polymorphism and tryptophan levels in urine [29]. The homozygous Ser270 carriers showed a 30 % reduction of tryptophan in urine, but potential pathophysiological consequences were not analyzed.

Ser270 allele carriers also showed a lower prevalence of essential hypertension [93]. The authors speculated that the underlying mechanism involved reduced renal dopamine levels, as dopamine is a known OCT2 substrate [94]. However, there is no in vitro data demonstrating that the Ala270Ser polymorphism affects dopamine uptake, nor is there data about an association of dopamine levels in urine or plasma with Ala270Ser.

Pharmacological Implications of OCT2 Polymorphisms

Metformin

Metformin is completely eliminated through the kidney, without being metabolized. Tubular secretion is responsible for about 2/3 of the elimination and OCT2 is known to mediate the initial step in this tubular secretion—the uptake into the tubular epithelia. Therefore, *OCT2* polymorphisms were a subject of interest for causing inter-individual variations of the renal clearance of metformin. Indeed, initially several studies reported an association between the Ala270Ser polymorphism and variations in metformin renal clearance [22, 28], especially for the homozygous Ser270 carriers [95]. However, our meta-analyses including recently acquired data show a lack of effects for the heterozygous carriers. The homozygous Ser270 carriers showed on average only 18 % (about 6 L/h) decrease in the renal clearance of

metformin compared to the homozygous Ala270 carriers and this decrease was not always reproducible (Fig. 4.4b). These results do not question the important role of OCT2 in the renal metformin elimination, but point to the lack of a strong functional effect of the Ala270Ser variant on metformin uptake. The lack of strong functional effects may also explain the highly contradictory results of the in vitro studies on the effects of Ala270Ser on metformin uptake (Fig. 4.4a).

Interestingly, a recent study suggested an interplay between Ala27Ser and polymorphisms in *MATE1* [59]. *MATE1* and *MATE2-K* are known to contribute to the renal secretion of metformin by transporting it out of the epithelial cells into the lumen of the proximal kidney tubule [96]. Therefore, interactions between *OCT2* and *MATE* polymorphisms are plausible. It will be interesting whether rare genetic variants in *OCT2* and *MATEs* may be related with the development of metformin-associated lactic acidosis, but systematic analyses are still missing.

Cisplatin

The chemotherapeutic drug cisplatin is a substrate of the human OCT2 [97, 98]. Experiments with OCT2 inhibitors and in knockout animals clearly showed that the nephrotoxicity and ototoxicity of cisplatin depend on cellular uptake by OCT2 [99, 100]. Therefore, there was understandably interest in assessing whether genetic polymorphisms in *OCT2* may confer protection from cisplatin toxicity. A study of Filipinski et al. suggested an association between the Ala270Ser polymorphism and cisplatin nephrotoxicity [100]. The suggestion was based on the observation of a non-significant increase in creatinine clearance after cisplatin treatment in heterozygous Ser270 carriers compared to a significant increase in the non-carriers. Similar results were reported also by Iwata et al. [101]. However, the statistical analyses used by Filipinski et al. were questionable [102] and the results could not be replicated in other studies with larger numbers of variant carriers [102]. Furthermore, creatinine clearance, which was used as a marker for nephrotoxicity in the study of Filipinski et al. and Iwata et al., may also depend on *OCT2* polymorphisms [91]. In addition, no differences in cisplatin uptake between the Ala270 and the Ser270 alleles could be measured in vitro [24]. Therefore, *OCT2* polymorphisms seem to have only very limited, if any, effects on cisplatin nephrotoxicity. A recent study suggested significant effects of Ala270Ser polymorphism on cisplatin ototoxicity [103], but the exact functional mechanism how Ala270Ser polymorphism may affect the uptake of cisplatin remains unclear.

OCT3

Genetic Variability

OCT3/SLC22A3 is apparently the least genetically variable among the organic cation transporters (Table 4.1). Systematically screening for common *OCT3* polymorphisms by analyzing the exons, the exon–intron borders and the 500 bp proximal

promoter in 100 Caucasians did not identify common amino acid changes in OCT3 [32]. The silent coding variant 1233G>A (Ala411Ala, rs2292334) was suggested to generate a cryptic acceptor splice site within exon 7 of the gene. This is supported by association of the 1233A allele with reduced OCT3 expression in the liver [31]. However, there are no functional analyses (e.g. mini-gene analyses) confirming this suggestion, and no association was observed between this polymorphism and obsessive-compulsive disorder or metformin pharmacokinetics [11, 104]. Beyond 1233G>A, several genetically linked polymorphisms, the 3'UTR SNPs rs3088442 and rs1810126 and the intronic SNP rs2048327, were associated with variations in hepatic OCT3 expression [31]. The underlying mechanisms remain unknown.

Rare amino acid variants Thr40Met, Thr400Ile (rs8187725) and Val423Phe were shown to affect OCT3 transport activity [33]. Thr40Met showed a substrate-specific increase in V_{MAX} without affecting K_M . In contrast, Thr400Ile and Val423Phe showed significantly lower affinities, but no change in capacity of OCT3 uptake. The changes were also substrate-specific, with an increase in K_M for the uptake of metformin, dopamine, adrenaline and noradrenaline, but no change in K_M values for MPP⁺, tyramine, histamine, and serotonin. Val423Phe was relatively common, but specific to Asians (Table 4.2), whereas the other two variants were observed only in single individuals.

Analyses for rare variants in patients with obsessive-compulsive disorder led to the discovery of several novel variants including an AG deletion located -106/107 bp upstream of the start codon and Met370Ile [104]. Both variants were detected only in patients, but not in control individuals. The Met370 allele co-segregated with the disease development in a family and showed significantly increased uptake of noradrenaline after overexpression in HEK293 cells.

Pathophysiological and Pharmacological Implications

Genome-wide association studies reported associations between SNPs within intron 5 of the *OCT3* gene and susceptibility to prostate cancer, colon cancer and coronary artery disease [34–36]. One of the associated SNPs, rs2048327, was also associated with changes in OCT3 expression [31]. However, there are no in vitro or animal experiments directly supporting the involvement of OCT3 in the etiology of any of the associated diseases. Alternatively, all associated SNPs are in high linkage disequilibrium with SNPs in the two adjacent genes *LPAL2* and *LPA* (Fig. 4.5). Therefore, it is probable that the SNPs in *OCT3* are only tagging causal polymorphisms within the *LPAL2* and *LPA* genes.

Although metformin is a substrate of OCT3 [31], systematic analyses of genetic variability including haplotype tagging SNPs and the potentially functional polymorphism 1233G>A did not show associations with the pharmacokinetics of metformin [11].

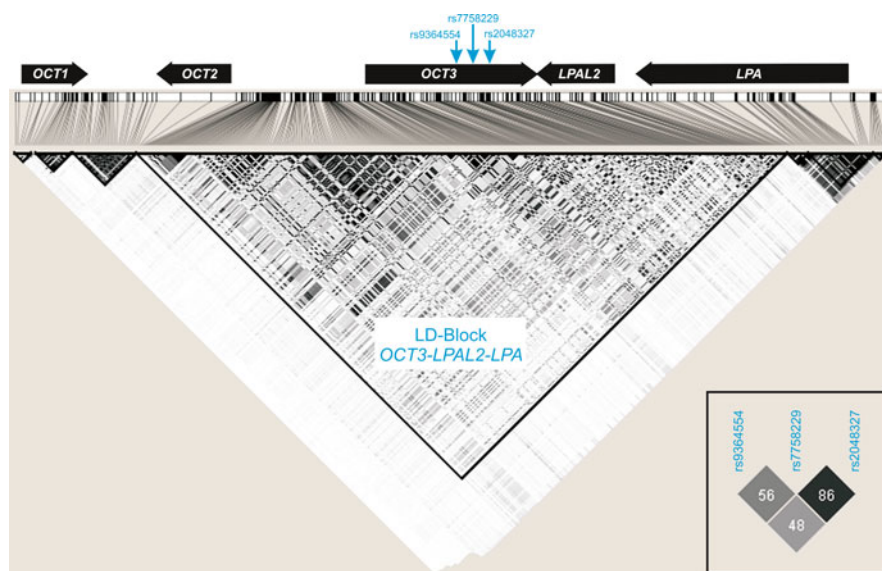


Fig. 4.5 Genetic linkage between the OCT3 gene and genes involved in lipid metabolism co-localized at chromosome 6. The strength of the pairwise linkage disequilibrium is shown as shades of grey varying from black (complete linkage) to white (no linkage). The numbers representing r^2 values are given as percentages. The three common polymorphisms in intron 5 of *OCT3*, for which clinical associations were reported, are shown in blue

OCTN1 and OCTN2

Genetic Variability

The *OCTN1/SLC22A4* and *OCTN2/SLC22A5* genes are clustered together in a tandem orientation at the long arm of chromosome 5. Due to the close proximity, the polymorphisms in the two genes are highly genetically linked. Two common functional polymorphisms were identified and dominated the association analyses with these genes—the amino acid substitution leucine to phenylalanine at codon 503 of the *OCTN1* gene (OCTN1 Leu503Phe) and a C-to-G substitution 207 bp upstream of the start codon of *OCTN2* (OCTN2 –207G>C, Table 4.2). In Caucasians, both polymorphisms are highly linked (Fig. 4.6a). Therefore it is difficult to elucidate which of the two polymorphisms is causal in clinical association studies, e.g. with susceptibility to Crohn's disease. Furthermore, it has been suggested that the presence of both polymorphisms is necessary for developing the pathophysiological phenotype [105].

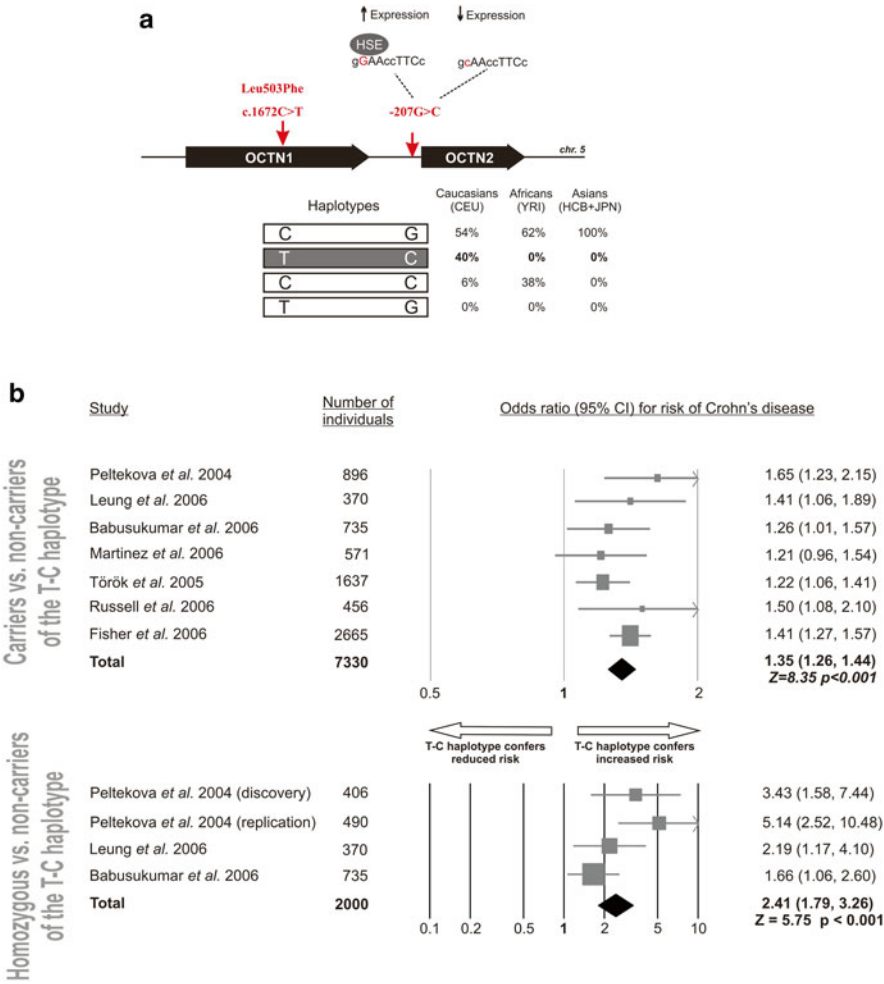


Fig. 4.6 Association between polymorphisms in OCTN1 and OCTN2 genes and susceptibility to Crohn's disease. (a) The Leu503Phe and -207G>C polymorphisms, their function and their haplotype combinations. The risk haplotype is highlighted in gray and its frequencies in Caucasian, African and Asian populations are given. **(b)** Meta analyses of the association between the risk Leu503Phe/-207G>C haplotype and Crohn's disease

OCTN1 Leu503Phe (c.1672C>T)

The Leu503Phe polymorphism is very common in Caucasians. The functional effects of Leu503Phe are highly substrate-specific. Compared to the reference Leu503 allele, the Phe503 allele showed strongly decreased transport of carnitine (both decreased v_{MAX} and increased K_M values), but strongly increased uptake of TEA⁺ and

acetylcholine (again both increased v_{MAX} and decreased K_M values; [37, 106]). The polymorphism is completely absent in Asians and Sub-Saharan Africans (Table 4.2).

The Leu503Phe substitution is localized in the 12th transmembrane domain of OCTN1. The mechanisms causing the strong substrate-specific changes in activity have not been studied in detail. There is no data showing impaired membrane localization of the transporter due to the polymorphism. This is in line with the reported substrate-specific effects on OCTN1 activity, as substrate-specific effects will exclude general impairment in the localization of OCTN1 at the cytoplasmic membrane.

OCTN2 –207G>C

The transversion –207G>C disrupts a heat shock element gGAAccTTCC (the consensus bases are shown in capital letters) localized –207 to –200 bp upstream of the start codon of OCTN2 and disables the binding of the transcriptional factor HSF-1 (Fig. 4.6a) [37]. The –207C allele has 60 % lower background promoter activity compared to the –207G allele [107] and is not inducible by heat shock or arachidonic acid [37]. The C and G alleles have equal frequencies in Caucasians; the C-allele has only a 32.3 % frequency in Sub-Saharan Africans and is completely missing in Asians (Table 4.2).

Other Polymorphisms

Several polymorphisms leading to amino acid substitutions beyond the common Leu503Phe were identified after systematic re-sequencing of *OCTN1* and *OCTN2* [108, 109]. The rare amino acid substitution Gly462Glu (rs46462019) was shown to lead to complete loss of OCTN1 activity without affecting the membrane localization of OCTN1 [108]. The rare amino acid substitutions Phe17Leu (rs11568520), Tyr449Asp (rs11568514) and Val481Phe (rs11568513) were identified and were shown to change the transport activity of OCTN2 [109]. Phe17Leu leads to reduced uptake of L-carnitine and TEA⁺ by OCTN2 due to impaired localization in the plasma membrane. Thr449Asp showed a substrate-dependent increase in OCTN2 activity, while Val481Phe showed a decrease in OCTN2 activity with all substrates tested. All four variants (the one in OCTN1 and the three in OCTN2) are very rare with minor allele frequencies of 0.5 % or below.

Systematic re-sequencing and functional analyses of the *OCTN1* and *OCTN2* promoters revealed several common polymorphisms [107]. The polymorphisms in *OCTN1* did not show any effect on the promoter activity. In contrast, in addition to –207G>C the polymorphism –319C>A (rs60978556) was shown to affect *OCTN2* promoter activity [107]. Therefore a haplotype, 319A/–207G, which is present only in 2 % of Caucasians, is expected to confer lower *OCTN2* promoter activity and lack of inducibility by arachidonic acid despite the presence of the –207G allele.

Physiological and Pathophysiological Consequences of OCTN1–OCTN2 Polymorphisms

Susceptibility to Crohn's Disease

Discovery of the involvement of OCTN1 and OCTN2 in the pathophysiology of Crohn's disease is an emblematic example how genetic analyses may help to reveal important physiological roles of organic cation transporters. Substantial steps in understanding the pathophysiology of inflammatory bowel disease were made by performing genetic linkage analyses. Six loci numbered IBD1 to IBD6 were reproducibly linked with susceptibility to Crohn's disease. The IBD5 locus represents a 250 kb haplotype block at chromosome 5 (5q31) that was associated with susceptibility to Crohn's disease in multiple studies [110, 111].

The seminal study of Peltekova et al. showed that the association of the IBD5 region with Crohn's disease is based on two functional polymorphisms, one in *OCTN1* and one in *OCTN2* gene, that are in strong linkage disequilibrium between each other [37]. The T-C haplotype representing the combination of Phe503 and the reduced *OCTN2* promoter activity allele –207C was associated with higher susceptibility for the development of Crohn's disease (Fig. 4.6a). The homozygous carriers of the T-C haplotype were estimated to have a more than fivefold higher risk of developing Crohn's disease compared to non-carriers [37].

Since then, more than 10 studies have confirmed the finding of Peltekova et al. [105, 112–121]. Although the initially reported effect sizes were not confirmed, meta analyses suggest a 2.4-fold increased risk of Crohn's disease in homozygous carriers of the T-C haplotype compared to non-carriers (Fig. 4.6). This remains the only extremely consistent reproduced finding for the involvement of genetic variants in organic cation transporters in the pathophysiology of a common disease. The associations were not restricted only to Crohn's disease, but were also reported for ulcerative colitis, the other major form of inflammatory bowel disease [122].

The significant association of the T-C haplotype with Crohn's disease was not observed in Asians [123, 124]. This could be explained by the much lower frequency of the two relevant polymorphisms (Table 4.2) and the complete lack of the risk haplotype in Asians (Fig. 4.6a). Interestingly, the prevalence of Crohn's disease in Asia is also much lower than in Europe (0.13 % in Japan compared to 1.45 % in the United Kingdom) and cases of familial Crohn's disease were rarer in Asia [123, 125, 126]. Alternative functional polymorphisms in *OCTN1* were associated with susceptibility to rheumatoid arthritis in Japanese patients [38].

Part of the associations of the IBD5 locus with Crohn's disease may be not attributable directly to the 503Phe–207C haplotype, but rather to other variations in the same locus with partial linkage disequilibrium to the Leu503Phe and –207G>C polymorphisms, so called “hitchhiking polymorphisms,” e.g. in the IRF1 gene [127].

OCTN1 and OCTN2 were shown to be expressed in the colon and other regions relevant for the pathophysiology of Crohn's disease [37]. *Octn1* knockout mice showed increased susceptibility to intestinal inflammation after ischemia, supporting the involvement of OCTN1 in the etiology of Crohn's disease [128]. Multiple mechanisms were proposed to explain these associations, including changes in the plasma and organ concentrations of ergothioneine [128], L-carnitine deficiency [129] or reduced release of non-neuronal acetylcholine [106].

Other Physiological Implications

Polymorphisms in the *OCTN1–OCTN2* locus, including the T-C haplotype, were associated with increased risk for type I diabetes, but the association was of borderline significance [130]. The Leu503Phe polymorphism in *OCTN1* was associated with ulcerative colitis and with cancer progression [131], but the association was not consistent [132].

Rare genetic variations leading to the inactivation of OCTN2 have been associated with the development of primary systemic carnitine deficiency (PSCD, OMIM 212140). PSCD is an autosomal recessive disorder that is linked in humans to defects in the 5q31 locus [133], the locus of the carnitine transporter OCTN2. The first missense and nonsense mutations in OCTN2 were described for pedigrees with PSCD in 1999 [134]. Currently hundreds of rare inactivation mutations in *OCTN2* were described in PSCD patients (for review see [135], [136] and [137]). The rare, but highly functional, missense and nonsense mutations in OCTN2 lead to an inability to reabsorb carnitine from urine, leading to systemic carnitine deficiency that may have different clinical manifestations.

Pharmacological Implications

Octn1 knockout mice showed significantly higher plasma concentrations after oral (but not after intravenous) administration of metformin, suggesting an involvement of *Octn1* in the intestinal transport of metformin [138]. The functional *OCTN1* polymorphism Leu503Phe was not associated with any changes in metformin pharmacokinetics [11], but another amino acid substitution, Thr306Ile (c.917C>T), was associated with changes in the total clearance and maximal plasma concentration of metformin [27]. The in vitro analyses showed that the Thr306Ile polymorphism had no effect on OCTN1 transport of TEA⁺ [108], but currently there is no data about effects on metformin uptake.

OCTN2 has been suggested to affect the uptake of cardiovascular drugs in the human heart, but no association of the –207G>C polymorphisms with OCTN2 mRNA expression and drug efficacy in the heart were found [139].

MATE1

Genetic Variability

The *MATE1/SLC47A1* and *MATE2K/SLC47A2* genes are co-localized approximately 100 kb apart at the short arm of chromosome 17. Several polymorphisms, especially common promoter polymorphisms, were reported to affect expression of the *MATE* genes and pharmacokinetics and efficacy of metformin.

–66T>C (rs2252281)

Luciferase reporter gene assays showed that the C-allele causes up to a twofold decrease in the promoter activity and a 34 % decrease in *MATE1* mRNA expression in the kidney [40]. This polymorphism is suggested to change the balance between the binding of the transcriptional factors AP-1 and AP-2. AP-1 apparently has a higher affinity for the T-allele and is suggested to induce *MATE1* transcription, while AP-2 has apparently higher affinity to the C-allele and is suggested to suppress *MATE1* transcription.

Other Polymorphisms

Several amino acid substitutions (including Gly69Asp, Leu128Phe, Thr159Met, Gly211Val, Ala310Val and Val338Ala) were reported [140–142] and substantial reduction of *MATE1* activity was demonstrated for some of them [140, 141]. However, the variants are very rare and therefore could not be analyzed statistically for associations with drug pharmacokinetics [27, 142]. The common intron 10 polymorphism rs2289669 G>A was associated with metformin efficacy, but there is no data for functional effects of this polymorphism and it is not genetically linked to other functional polymorphisms [42].

Pharmacological Implications

MATE1 polymorphisms were repeatedly analyzed in relation to metformin pharmacokinetics and efficacy. The intron 10 polymorphism rs2289669 was associated with the glucose-lowering efficacy of metformin in 116 patients from the Rotterdam Study [42]. The rs2289669 A-allele showed about a 30 % stronger reduction in HbA1c after metformin treatment per copy of the A-allele. The same polymorphism was also associated with lower total cholesterol levels [64]. However, no effects of the intron 10 polymorphism were observed in pharmacokinetics [11] or gastrointestinal side effects of metformin [14].

This may be explained by the dual role of MATE1 in metformin efficacy. On the one hand, MATE1 is expressed in the kidney and MATE1 polymorphisms may affect the renal clearance and, as a consequence, the plasma concentrations of metformin. On the other hand, MATE1 is expressed in the liver, and reduced MATE1 activity may lead to a substantial increase in the liver concentrations of metformin. Therefore *MATE1* polymorphisms may affect metformin efficacy without affecting its plasma concentrations. This is supported by another study reporting an association of the $-66C>T$ polymorphism with efficacy, but not with renal clearance of metformin [41]. Interestingly, the MATE1 $-66C>T$ polymorphism was reported to modulate the effects of polymorphisms in other renal transporters on renal clearance of metformin [20, 41, 59].

The *MATE1* rs2289669 G>A polymorphism was analyzed, but shows no association with cisplatin induced nephro- and hematotoxicity [101].

MATE2-K

Genetic Variability

$-130G>A$

The polymorphism $-130G>A$ was shown to affect promoter activity of MAT2-K. Luciferase reporter gene assays showed doubling of the promoter activity when the $-130A$ -allele is present [43]. The increase in the promoter activity was explained by impaired binding of the repressor myeloid zinc finger 1 (MZF1). The effect was not observed in all cell lines tested. The strongest effects were observed in HCT-116, followed by HEK293T cells. No effects were observed in ACHN and LLC-PK1 cells. The increased promoter activity allele has similar frequencies in different world populations (Table 4.2).

Other Polymorphisms

A number of functional amino acid substitutions including Pro162Leu, Gly211Val, and Gly393Arg have been reported to reduce the uptake of metformin and other substrates by MATE2-K in vitro [43, 140]. However, these variants are very rare. They were found only in a few world populations with minor allele frequencies of maximally 2 %.

Pharmacological Implications

The promoter polymorphism $-130G>A$ was associated with changes in metformin efficacy [43]. The high expression $-130A$ -allele was associated with significantly poorer response to metformin [43]. However, no association of $-130G>A$ with

AUC or $C_{\max}$ of metformin were observed [27]. Significant increases in total renal clearance and renal secretion were observed, but only in dependence also on the *MATE1* –66A>T genotype [41]. This suggests that the *MATE1* genetic background is important for the effects of *MATE2-K* polymorphisms. In addition, a second common promoter polymorphism, –396G>A (rs12943590), was recently shown to cause an increase in *MATE2-K* transcription independently from –130G>A [44]. Combined analyses of both promoter variants showed significant increases in metformin renal clearance independently of the *MATE1* genotype.

Summary

Organic cation transporters strongly differ in their genetic variability and in the resulting pathophysiological and pharmacological consequences. OCT1, OCTN1 and OCTN2 have several common genetic polymorphisms clearly affecting their activity or expression. In OCT1, five amino acid polymorphisms, Arg61Cys, Cys88Arg, Gly401Ser, Gly465Arg and Met420del, are known to cause a substantial decrease in activity. Although the pathophysiological consequences of the decreased activity are not clear, data about the potential pharmacological implications is rapidly accumulating. In OCTN1 and OCTN2, a functional haplotype between polymorphisms Leu503Phe in OCTN1 and –207G>C in OCTN2 is a valid predictive marker for risk of Crohn's disease. In this case, the genetic variants actually led to the discovery of the role of the two cation transporters in the pathophysiology of Crohn's disease.

On the other hand, OCT2 and especially OCT3 are less genetically polymorphic. The OCT2 polymorphism Ala270Ser was associated with changes in creatinine clearance, metformin pharmacokinetics and cisplatin toxicity. However, the effects are small, the results are not consistent among different studies, and even the in vitro effects of the polymorphism are not clear.

More recently the *MATE1* and *MATE2-K* transporters were genetically analyzed and polymorphisms identified in the promoter regions of the two genes were related to the pharmacokinetics and efficacy of metformin.

In conclusion, polymorphisms in organic cation transporters (especially OCTN1 and OCTN2) have physiological and pathophysiological consequences. However, it is much more common that polymorphisms in organic cation transporters affect drug pharmacokinetics, efficacy and toxicity. Initially only the commonly prescribed antidiabetic drug metformin was studied, but recently the number of drugs affected was extended to morphine, tramadol, tropisetron and cisplatin. The spectrum of affected drugs is expected to increase steadily in the future.

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Chapter 5

Physiological and Pathophysiological Regulation of Transporters for Organic Cations

Eberhard Schlatter

Abstract Transporters for organic cations (OCTs, OCTNs, MATEs) are polyspecific membrane proteins which translocate many organic and some inorganic cations across plasma membranes in a driving force dependent and saturable manner. These transporters are responsible for the cellular uptake and extrusion of many cationic drugs and toxins and therefore prerequisite for their metabolism, therapeutical actions, but also for their negative side effects. Together with the molecular identification of these transporters data were published from in vivo and in vitro experiments demonstrating that drug transport is regulated independent of changes in their driving forces. In this chapter the manyfold regulation of OCTs, OCTNs, and MATEs from various mammals including humans is summarized. These regulatory processes involve both changes in substrate affinities (K_m) as well as in transport capacity (V_{max}) or changes in expression rates. Importantly, all these regulatory processes of the various transporter proteins are highly paralog and ortholog dependent. Acute effects of various protein kinases on transport activity within minutes has been demonstrated, including effects on the transporter molecule itself as well as on transporter trafficking to and from the membrane. Kinases and several other cytosolic factors are involved in a chronic regulation of the expression of transporters for organic cations in several organs. In addition, transporter expression is also epigenetically regulated, a mechanism which apparently contributes to the organ specific expression of the transporters. It also plays a role in altered transporter expression in tumor tissues, a feature which is highly relevant for drug efficiencies in antitumor therapies. Finally, in this chapter many studies on transporter regulation in very divers pathophysiological situations including several hepatic or renal diseases are discussed

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Introduction

Members of the SLC-protein family of transporters for organic cations translocate organic cations (OCs) across the plasma membrane of cells as bidirectional uniporters (OCTs) [12, 75] or as H⁺-antiporters (MATEs or OCTNs). In various epithelia like the renal proximal tubule or the liver hepatocytes these transporters are localized to distinct membrane domains where they operate as uptake (OCTs) or efflux transporters (MATEs and OCTNs). Typically OCTs are restricted to the basolateral membrane and MATEs and OCTNs to the apical membrane [1, 18, 67, 71, 80, 82, 83, 85, 90, 104, 110, 111, 129, 130, 153, 156, 158, 171, 178]. Together these transporters mediate *trans*-cellular reabsorption or excretion of endogenous cationic metabolites and exogenous xenobiotics, among those a large number of drugs. As polyspecific transporters they translocate a wide variety of chemically very diverse cationic or zwitterionic (OCTNs) organic molecules [85]. The magnitude of the transport of the respective organic cationic substrates depends on the driving forces which are, besides the chemical gradient of the substrates, the membrane potential in the case of the electrogenic organic cation transporters (OCTs) [12, 50, 75, 81, 103] and the opposite pH-gradient for the organic cation antiporters (OCTNs and MATEs) [28, 121, 157, 170]. In the case of OCTN1 also the Na⁺ gradient can add to the driving force as this transporter can also operate in a Na⁺ dependent manner [118]. In addition, these transporters for organic cations not only work strictly temperature dependent [6, 152], as most if not all transport proteins do, but their transport activity is grossly impaired when cells are energy depleted [152]. This energy dependence, however, does not reflect a direct ATP-dependence of these types of transporters for organic cations. Thus, changes in the transmembrane pH and/or Na⁺ gradient or changes in membrane voltage, e.g. due to metabolic alterations or changes in inorganic ion gradients and transport, will modify their transport rate. Unlike ion channels these transporters typically show a saturation of transport capacity if concentrations of the substrate reach a maximum. This maximal transport capacity differs from transporter protein to transporter protein and probably also from substrate to substrate due to different binding properties. An additional important mechanism inducing changes in transport capacity and rates for organic cations by these transporter molecules is their biochemical, genetical, and epigenetical regulation. Such regulations in response to endogenous or exogenous signals involve transcriptional regulations by repressors or activators, translational regulations by variations in mRNA stability, and posttranslational modifications by changes in protein structure, function, or localization. While transcriptional modifications of transporters for organic cations influence their activity on a long-term

basis, translational modifications lead to short-term regulation of their transport rates. These regulatory modifications involve on the one hand changes in the number of transporters in the membrane ($V_{\max}$ -effect) by modifications in transporter gene transcription or translation, transporter protein insertion into or transporter retrieval from the plasma membrane. On the other hand, also the affinity of the transporter for specific substrates can be modified (K_m -effect), both mechanisms potentially resulting in marked changes in substrate/xenobiotic/drug uptake or excretion rates. The latter short-term regulatory mechanisms of transporters for organic cation involve phosphorylation/ dephosphorylation, glycolization, and ubiquitination processes. In the case of drugs (about 40 % of prescribed drugs are organic cations and thus, potential substrates of these transporters for organic cations), such regulatory changes in transporter abundance or activity are of fundamental importance for drug efficacies or drug toxicities when administered to patients.

Several excellent reviews on various aspects of regulation of the above mentioned organic cation transporters (OCTs, OCTNs, MATEs) have been published during the last 10–15 years, many of them addressing special aspects of regulation only [5, 18, 19, 34, 36, 74, 78, 82, 97, 137, 142, 154, 159, 164, 172]. In this chapter the currently available information on a wide spectrum of aspects of short term and highly reversible regulation of transporter membrane abundance or activity mediated by various cytosolic protein kinases and other cellular factors of three groups of organic cation transporters (OCTs, OCTNs and MATEs), is summarized. In addition to these acute regulatory mechanisms, various aspects of chronic regulation of the expression of these transporters e.g. by nuclear factors, during development, or by epigenetic phenomena will be discussed. Finally the growing knowledge on the involvement of regulation of these transporters for organic cations in highly diverse pathological situations and diseases is summarized.

Acute Regulation of Organic Cation Transport Systems by Protein Kinases

The first studies on acute (within minutes) regulation of organic cation transport were performed with isolated non perfused proximal tubules from rabbit kidneys and revealed a PKC-mediated stimulation of organic cation transport across the basolateral membrane of these tubules [59, 60]. Meanwhile we know, that the basolateral membrane of the proximal tubule cells of all examined mammals (rabbit, mouse, rat, man) and also reptiles [77] expresses OCTs as the only transport system for organic cations in this membrane domain. Therefore, these studies on rabbit proximal tubules suggested for the first time that OCTs can be acutely regulated by protein kinases. Such a possible regulation by protein kinases was further supported by the identification of several potential phosphorylation sites in the amino acid sequence of the large intracellular loop and the C-terminal domain of the cloned molecules [13, 19]. While in these first studies and most other studies

examining organic cation transport until that time radioactively labeled substrates of transporters for organic cations had been used, in 1994 the fluorescent organic cation 4-(4-dimethylaminostyryl)-*N*-methylpyridinium (ASP⁺) was introduced as fluorescent substrate for the analysis of organic cation transport. This was first established in the rat kidney in vivo by recording the fluorescence increase due to proximal tubular accumulation viewed from the surface of the kidney in situ [126, 133]. Thereafter this fluorescent approach was adjusted by us to the analysis of organic cation transport in vitro in cell culture systems predominantly across the apical membrane of cells grown on non-permeable supports [150, 151]. Thereafter we performed the next studies on acute regulation of organic cation transport with two renal proximal tubular cell lines, the porcine LLC-PK₁ and the human IHKE-1 cells, using ASP⁺ as substrate and recording dynamically the cellular fluorescence increase as transport parameter [62]. In this study ASP⁺ uptake across the apical membrane of IHKE1-cells grown on glass coverslips was stimulated by direct activation of PKC, PKA, or PKG. Various hormones (bradykinin, oxytocin, angiotensin II) or ATP, all leading to an increase in cellular Ca²⁺ activity in these proximal tubular cell lines, also increased ASP⁺ uptake across the apical membrane, again most likely via PKC activation. Interestingly, already in this first comparative report between cell lines which were generated from different species it became evident that acute and protein kinase mediated regulation of organic cation transport differs markedly between different species. In contrast to the human IHKE-1 cell line, in the porcine LLC-PK₁ cell line PKC activation reduced organic cation transport while PKA or PKG stimulation had no effect. This transport across the apical membrane of the two proximal tubular cell lines was probably mediated by members of the MATE and/or OCTN transporters, although in LLC-PK₁ cells Oct2, which otherwise is restricted to the basolateral membrane of the proximal tubule seems also to be expressed in the apical membrane [33, 51] and, thus, may have contributed to the regulation of apical organic cation transport in the study by Stachon et al. [151].

Acute Regulation of OCTs by Protein Kinases

As the first cloned member of the family of organic cation transporters was the rat Oct1 (rOct1) [50], acute regulation of a molecularly identified transporter for organic cations by various protein kinases was first examined for this transporter [103]. PKC, PKA or tyrosine kinase (p56^{lck}) activation all led to a stimulation of rOct1 mediated transport of the fluorescent model substrate ASP⁺. These observations suggested that, depending on the metabolic state and the protein kinase activities e.g. of proximal tubular cells, the renal excretion rate of cationic drugs is highly variable at least in this species. Of course this would also suggest a similar variable transport activity for organic cations in other OCT expressing tissues, like the liver, the intestine, or the brain.

A comparison of the pattern of acute regulation by protein kinases of the two Oct paralogs rOct1 and rOct2 of the rat, individually over expressed in HEK293-cells,

reveals that these two paralogs, both expressed in parallel e.g. in proximal tubule cells, differ slightly in this respect. While rOct1 and rOct2 mediated transport was stimulated by activation of PKA, p56^{lck}, or calmodulin (CaM), PKC activation stimulated only rOct1 and had no effect on rOct2 mediated transport [43, 103, 168] (see Table 5.1). The stimulatory effect of PI3-kinase (PI3K) activation on ASP⁺ transport was only examined for rOct2 and no such data on acute regulation of transport by protein kinases exist for rOct3 so far. However, an inhibitory effect of insulin on rOct1 and on rOct2 expressed in HEK293 cells was reported [43]. This effect was apparently mediated via cAMP.

The regulatory pattern of transport via a given OCT of one species differs significantly from that of its orthologs in other species. The regulation of ASP⁺ transport mediated by the three mouse Oct paralogs has recently been studied using mOcts individually over expressed in HEK293 cells [101, 144]. As summarized in Table 5.2 PKA, p56^{lck}, and CaM activation stimulated transport in rat and also in mouse Oct1 and Oct2. PI3K activation which stimulated rOct2 only activated mOct1 and PKC activation, which stimulated rOct1, reduced mOct1 activity and had no effect on both rOct2 and mOct2. The ubiquitously expressed mOct3 was only regulated by p56^{lck} activation. Although there are several similarities in the transporter regulation

Table 5.1 Acute regulation of rOCT1 and rOCT2 by protein kinases

	rOCT1	rOCT2
PKA	↑	↑
PKC	↑	0
p56 ^{lck}	↑	↑
PI3K		↑
CaM	↑	↑

Effects of stimulation of protein kinases on ASP⁺ uptake by HEK293 cells stably transfected with rOCT1 or rOCT2. ↑ indicates stimulation of transport, 0 indicates no effect; an empty cell indicates not tested; for references see text

Table 5.2 Acute regulation of rOct1 and 2, mOct2, 2, and 3 by protein kinases

	rOct1	rOct2	mOct1	mOct2	mOct3
PKA	↑	↑	↑	↑	0
PKC	↑	0	↓	0	0
p56 ^{lck}	↑	↑	↑	↑	↑
PI3K		↑	↑	0	0
CaM	↑	↑	↑	↑	0

Effects of stimulation of protein kinases on ASP⁺ uptake by HEK293 cells stably transfected with rOct1, rOct2, mOct1, mOct2, or mOct3. ↑ indicates stimulation, ↓ indicates reduction of transport, 0 indicates no effect; an empty cell indicates not tested; for references see text

of the Octs between the two rodents rats and mice there are also distinct differences. Thus, it is important to note that a transfer of observations on organic cation transport from one rodent model to the other is not directly possible.

Mouse models, especially genetically modified strains are more and more used in a wide variety of studies addressing drug transport, but also with the focus on many other issues. However, many drugs and pharmacological tools are substrates of transporters for organic cations and are species dependently transported, metabolized or excreted and thus, influence the efficacy of these substances in these animal models questioning the translational relevance of such results. The relevance of the findings from animal models particularly addressing drug transport, metabolism or action for a potential transfer to the situation in human patients makes the knowledge of the respective properties of the human OCTs compared to those of laboratory animals mandatory [82]. Indeed, when comparing the findings from human and rodent OCT/Octs over expressed in HEK293 or CHO cells a grossly different pattern of regulation by protein kinases is evident (see Table 5.3).

Whereas PKA activation always led to a stimulation of transport in rodent Oct1 or 2, it reduced transport activity of human OCT1 and 2 [13, 20]. Unlike rodent Octs, where PKC stimulated only rOct1 or mOct1, in humans PKC reduced only hOCT2 activity. A down regulation of transport activity by activation of PKG was reported for rOct1 and hOCT2, while hOCT1 and hOCT3 were unaffected by this kinase [20, 21]. Also the effects of PI3K were opposite in rodent and human Oct/OCT 1 and 2 [13, 20]. In contrast, p56^{lck} or CaM activation had similar stimulatory effects in rodent and human Oct/OCT 1 or 2 (with the exception of hOCT2). For human OCTs in addition to the regulation of transport by CaM also the involvement of the downstream effectors (CamMII) and myosin light chain kinase (MLCK) was demonstrated [13, 20, 98]. Both mouse and human Oct3/OCT3 were poorly regulated and only activation of CaM or CamMII led to a decrease or increase in transport activity in hOCT3-transfected cells, respectively [98, 101]. Only an activation of p56^{lck} reduced transport activity in mOct3-transfected cells [101]. While in all studies summarized in Tables 5.1, and 5.3 short term effects were examined (10 min

Table 5.3 Regulation of OCTs from rat, mouse and man by protein kinases

	rOct1	rOct2	mOct1	mOct2	mOct3	hOCT1	hOCT2	hOCT3
PKA	↑	↑	↑	↑	0	↓	↓	0
PKC	↑	0	↓	0	0	0	↓	0
PKG	↓					0	↓	0
p56 ^{lck}	↑	↑	↑	↑	↑	↑	0	
PI3K		↑	↑	0	0	0	↓	
CaM	↑	↑	↑	↑	0	↑	↑	↓
CamMII						↑	↑	↑
MLCK						0	↑	

Effects of stimulation of protein kinases on ASP⁺ uptake by HEK293 cells stably transfected with rOct1, rOct2, mOct1, mOct2, mOct3, hOCT1, hOCT2, or hOCT3. ↑ indicates stimulation, ↓ indicates reduction of transport, 0 indicates no effect; empty cell indicates not tested; for references see text

incubation with agonists or antagonists), in another study hOCT3 (also named human extra neuronal monoamine transporter—hEMT) regulation was examined after 60 min incubation with several agonists or antagonist [98]. No effects of PKA, PKC or PKG activity changes were reported while MAP-kinase-, CaM-, phosphodiesterase (PDE) or alkaline phosphatase (ALP) inhibition all reduced transport activity. For hOCT2 and rOct1 a direct inhibitory effect of cGMP on transport activity without involving PKG-dependent phosphorylation was demonstrated [143].

In the human kidney only hOCT2 and to a very small extend also hOCT3 are expressed in the basolateral membrane of proximal tubules [46, 55, 80, 110]. In the rodent kidney all three Oct paralogs are expressed [63, 80, 84, 107, 120, 144, 153, 156, 163]. In the mouse the mRNA expression of the three paralogs does not significantly differ between S1, S2, and S3 segments of proximal tubules [63]. Renal expression levels between the three mouse paralogs, however, differ significantly. The main subtype, at least on mRNA level, is mOct1, mOct2 expression level reaches about 30 % of that of mOct1 and mOct3 expression is marginal with only about 1 % of that of mOct1 [63]. These mRNA-expression levels of OCTs suggest that in the human kidney the organic cation transport across the basolateral membrane of proximal tubular cells is dominated by hOCT2, while in rat and mouse kidney Oct1 and Oct2 both contribute to this process. Thus, in rodents characteristics, kinetics, or regulation of drug transport across the basolateral membrane of proximal tubules is a composite of the properties of the two paralogs while in humans they reflect mainly the properties of one paralog. To support this assumption it was important to study organic cation transport in isolated renal proximal tubules and compare the results of those studies with findings from cloned transporters transiently or stably expressed in various expression systems. This was first done with proximal tubules isolated from rabbit kidney (see above), although no systematic characterization of transport characteristics of organic cations in these proximal tubules was provided [59–61]. Next, such studies were performed with proximal tubules isolated from rat kidney, however in those studies no regulation of organic cation transport was examined [4, 44, 45]. Such regulation studies were performed by us using freshly isolated proximal tubules from human kidneys. This tissue was obtained from patients undergoing tumor nephrectomy and tumor free tissue surrounding the tumor was used [125]. For these freshly isolated tubules acute down regulation of organic cation transport across the basolateral membrane was reported for PKA, PKC, or PKG activation. A similar down regulation was also observed by us for PI3K activation (Schlatter and Ciarimboli, unpublished observation). When this regulation pattern of organic cation transport in isolated human proximal tubules is compared to the respective pattern of regulation of hOCTs individually expressed in HEK293 cells, it is evident that it matches only with that of hOCT2 (see Tables 5.3 and 5.4).

Recently, we have reported more detailed studies on acute regulation of organic cation transport across the basolateral membrane of isolated proximal tubules of mouse kidneys [52, 63]. As summarized in Table 5.4 activation of PKA, p56^{lck}, PI3K, or CaM led to an increased mOct-mediated transport, while activation of PKC decreased this transport.

Table 5.4 Regulation of organic cation transport by protein kinases in mouse and human proximal tubules and of OCTs expressed in HEK293 cells

	Mouse PT	Human PT	mOct1	mOct2	mOct3	hOCT1	hOCT2	hOCT3
PKA	↑	↓	↑	↑	0	↓	↓	0
PKC	↓	↓	↓	0	0	0	↓	0
p56 ^{lck}	↑		↑	↑	↑	↑	0	
PI3K	↑	↓	↑	0	0	0	↓	
CaM	↑		↑	↑	0	↑	↑	↓

Effects of stimulation of protein kinases on ASP⁺ across the basolateral membrane of isolated proximal tubules (mouse or human PT) and by HEK293 cells stably transfected with mOct1, mOct2, mOct3, hOCT1, hOCT2, or hOCT3. ↑ indicates stimulation, ↓ indicates reduction of transport, 0 indicates no effect; empty cell indicates not tested; for references see text

Table 5.5 Regulation of organic cation transport by protein kinases in mouse proximal tubules isolated from wild type, OCT2^{-/-}, or OCT1/2^{-/-} mice

	WT	OCT2 ^{-/-}	OCT1/2 ^{-/-}
PKA	↑	↑	0
PKC	↓	↓	0
p56 ^{lck}	↑	↑	↑
PI3K	↑	↑	0
CaM	↑	↑	0

Effects of stimulation of protein kinases on ASP⁺ across the basolateral membrane of isolated proximal tubules from wild type (WT) or OCT2^{-/-} and OCT1/2^{-/-} mice. ↑ indicates stimulation, ↓ indicates reduction of transport, 0 indicates no effect; for references see text

The comparison of regulation of organic cation transport in mouse proximal tubules with the regulation patterns of mOct1, mOct2 and mOct3 reveals that apparently mOct1 is the dominant paralog in the mouse kidney in vivo. This conclusion is further supported by findings obtained from proximal tubules isolated from mice which are deficient of mOct2 (mOct2^{-/-}) or of mOct1 and mOct2 (mOct1/2^{-/-}) [63, 144]. Tubules from the mOct2^{-/-} mouse express mOct1 and to a very small degree mOct3, thus predominantly reflecting properties of mOct1, tubules from the mOct1/2^{-/-} mouse express only mOct3, thus reflecting properties of mOct3 [63].

As evident from this comparison (see Table 5.5), the regulation pattern of organic cation transport across the basolateral membrane of OCT2^{-/-} mice was identical to that of wild type mice, suggesting that in wild type mice mOct1 is mostly responsible for the overall uptake of organic cations [144]. Figure 5.1 summarizes the complex pattern of acute regulation of organic cation transporters (OCT type) by protein kinases, as it is evident at present. As discussed above in detail, the depicted various regulatory pathways interact differently with mouse, rat or human Oct/OCT paralogs.

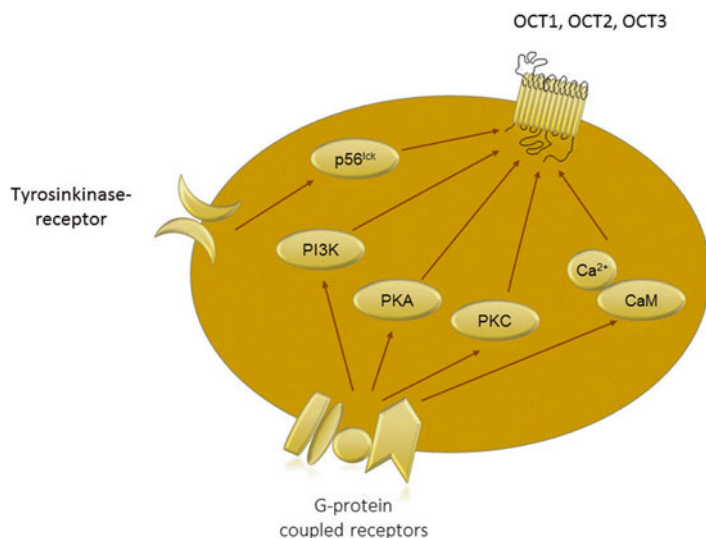


Fig. 5.1 Protein kinase mediated pathways described for acute regulation of rat, mouse or human OCT paralogs which are identified so far. For details see text

Mechanism of Acute Regulation of OCTs by Protein Kinases

Changes in transport activity of e.g. transporters of organic cations can be mediated by changes in the affinity of the binding sites of the transporter for their various substrates (changes in K_m) or in the number of active transport proteins expressed in the plasma membrane (changes in V_{max}). The latter depends on the directed trafficking of the transporter proteins to the respective membrane (the basolateral membrane in case of OCTs) and/or the retrieval and consecutive lysosomal degradation of the transporter protein from the plasma membrane. In a few studies these questions were addressed for the protein kinase dependent regulation both for individual Oct/OCTs of rat (rOct1) or man (hOCT2) expressed in HEK293 cells [6, 103] and for organic cation transport across the basolateral membrane of isolated proximal tubules of mice [52]. The stimulation of rOct1 by PKC activation directly phosphorylated the rOct1 protein [103]. This PKC-dependent phosphorylation of rOct1 was accompanied by an increase in affinities for different substrates, which results in an increased transport rate for these substrates seen under PKC-activation. The amino acid sequence of the rOct1 molecule contains five putative PKC phosphorylation sites, four of which are localized in the large intracellular loop and one in the intracellular C-terminus. Apparently all five PKC phosphorylation sites are

phosphorylated by PKC activation, as mutation of each of these sites or of all five sites together resulted in distinct affinity changes for different substrates [21].

Using isolated mouse proximal tubules, activation of the CaM led to an increased affinity for organic cation transport across the basolateral membrane [52]. So far, such a change in substrate affinity due to protein kinase activation has not yet been demonstrated for the human OCTs. However, since not all protein kinase pathways which modify transport via human OCTs have been studied in this respect, the phenomenon of modifications in substrate affinities under different metabolic states or protein kinase activities could well be relevant also in humans and thereby modify e.g. drug excretion in patients, drug uptake by target cells, or toxic side effects in non-target cells. In contrast to these affinity changes induced by alterations in protein kinase activities, a decrease in the transport capacity was reported after CaM inhibition for hOCT2 [6]. As expected, this effect was independent of the cationic substrate used for these transport studies (amiloride or ASP⁺). Similar changes in the transport capacity were also reported for the effects of several protein kinases on organic cation transport across the basolateral membrane of mouse proximal tubules [52]. In this report, changes in the $V_{\max}$ of the ASP⁺ transport were described for an inhibition of PKA, PKC, PI3K, or p56^{lck}. Furthermore, inhibition of the proteolytic pathway by bafilomycin (an inhibitor of vacuolar type H⁺-ATPase and thereby of endosomal recycling and lysosomal degradation) reduced organic cation transport, suggesting that these processes are active already in unstimulated isolated proximal tubules of these mice. In addition, bafilomycin was also able to prevent the PI3K-dependent modification of transport capacity, again supporting the hypothesis that these pathways modify the trafficking of mouse Octs from and to the basolateral membrane. A schematic summary of these trafficking effects suggested for mouse organic cation transporters in proximal tubules is depicted in Fig. 5.2. Finally, modifying the cytoskeleton by colchicine was also shown to reduce ASP⁺ transport in these isolated proximal tubules.

Acute Regulation of MATEs or OCTNs by Protein Kinases

Compared to the OCTs much less information is available about acute protein kinase dependent regulation of the apically localized organic cation/H⁺ exchange transporters. So far there exist no studies examining the acute regulation of MATEs or OCTN1. For hOCTN2 expressed in the human choriocarcinoma cell line BeWo an involvement of phosphorylation and dephosphorylation in transport activity was demonstrated [136]. Specifically, activation or inhibition of alkaline phosphatase activity led to a stimulation or reduction in hOCTN2 mediated carnitine uptake in these cells, respectively. Octn2 is a crucial uptake mechanisms for carnitine in rat skeletal muscle cells [41]. In this study in rats a muscle contraction dependent acute translocation of rOctn2 to the plasma membrane was reported.

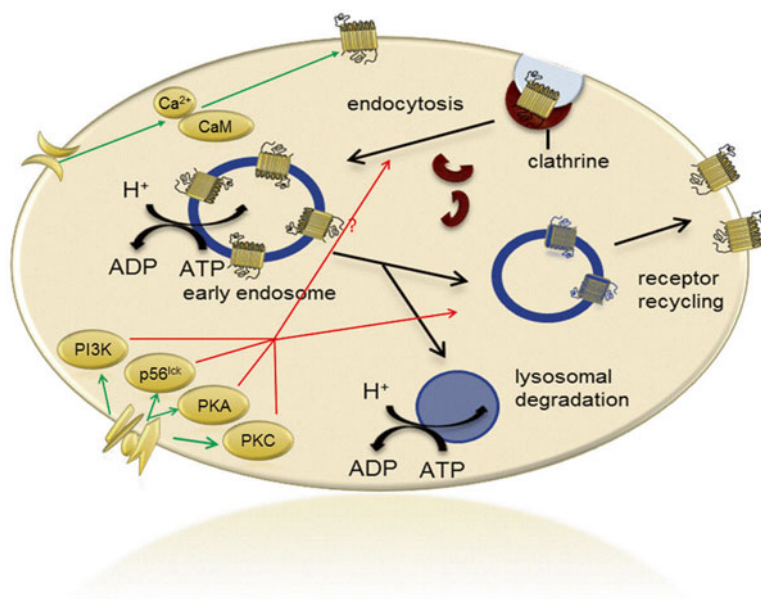


Fig. 5.2 Schematic summary of mechanisms of regulation of organic cation transporters as proposed for mouse proximal tubules. For details see text. Modified from Kikuchi et al. [76]

Chronic Regulation of Organic Cation Transport Systems

As discussed above, acute regulation of organic cation transporters mediated by alterations in protein kinase activities or metabolic conditions includes affinity changes, but more importantly modified trafficking of the transporter proteins to and from the plasma membrane. However, the abundance of drug transporter mRNA and consequently the number of transporter protein, of active transporters in the plasma membrane and thus, of transport rate for organic cations is also regulated on a long term basis by cytosolic and nuclear factors.

Chronic Regulation of Organic Cation Transporters by Cytosolic Factors

One group of such cytosolic proteins, which are involved in the trafficking of many membrane proteins, are scaffold proteins which interact with PDZ domains (post-synaptic density (PSD)-95/disc-large/zona occludens) in the transporter molecules. Such PDZ domains are also located at the C-terminus of several drug transporters including OCTNs and OCT3 (for review see [72, 74, 78]). Interaction of these scaffold proteins with PDZ domains of the transporters influences subcellular trafficking, transport activity and stability of the transporters in the plasma membrane.

OCT1 and OCT2, however, do lack PDZ binding domains. Cytosolic PDZ domain-containing proteins which interact with OCTNs are PDZK1, PDZK2, NHERF1 and NHERF2, whereas only PDZK2 interacts with OCT3. The physiological relevance of such interactions has been suggested especially for mouse Octn2 [73, 166]. In pull down assays PDZK1 interaction with mOctn1 and mOctn2 was demonstrated, which required the last four amino acids in the Octn molecules. In kidney brush-border membrane vesicles PDZK1 and mOctn2 colocalized in the membrane and increased transport capacity [73]. This effect was accompanied by an only minimal effect on cell-surface expression of mOctn2 and probably involved an increase in substrate translocation activity. Using coexpression in HEK293 cells the same group reported that PDZK2 interacts with mOctn2 and increased mOctn2 mediated carnitine uptake but did not modify transport by mOctn1 or mOct3. Most likely mOctn2 was accumulated in a sub apical compartment and transferred to the plasma membrane to account for the observed increase in transport activity. When the last four amino acids, forming the PDZ-binding domain were deleted no effect on translocation and transport was seen. The interaction of PDZK2 with mOctn2 seems to be important for the stabilization of cell surface expression of the transporter [166]. In addition, this group reported that genetic deletion of PDZK1 in mice reduced apical membrane localization, membrane protein expression and transport activity of mOctn2 in the small intestine [155].

Regulation of organic cation transporter localization and or transport activity by PDZ binding proteins appears to be included in a more complex network. Using BeWo cells, which express Octn2, PDZK1, PDZK2, NHERF1, and NHERF2, it was demonstrated that stimulation of adenylate cyclase by forskolin during syncytialization resulted in and increased Octn2 protein expression, however, no increased uptake capacity, which is probably due to the concomitant decreased expression of PDZK1, NHERF1, or NHERF2 by forskolin [65].

Tyrosine kinase inhibitors, like imatinib and others, have been identified as substrates of the organic cation transporters OCT1, OCT2, and MATE1 [106, 145, 160]. It was demonstrated that the mRNA and protein surface expression of hMATE1 were down regulated in synovial fibroblasts isolated from rheumatoid arthritis patients by stimulation with the cytokines TNF α , IL-1 β , and IL-6 [145]. A similar down regulation of rOct1 expression was observed in rat kidney and liver during inflammation induced by activation of the immune reaction by Freund's adjuvant [54].

Besides the above discussed cytosolic factors (scaffold-proteins and cytokines) other cytosolic proteins have been identified to interact especially with OCTs and thereby modify their plasma membrane targeting or membrane surface stability [47, 58]. Using a human kidney library and the mating-based split-ubiquitin-yeast-two-hybrid (mbSUS) screening assay with yeast cells expressing hOCT2 the lysosomal-associated protein trans membrane 4 alpha (LAPTM4A) was identified as interaction partner [47]. This interaction was confirmed by pull down assays, FRET-analysis, and immunofluorescence microscopy. In functional studies a down regulation of hOCT2-mediated transport activity was seen when LAPTM4A was co-expressed in hOCT2 expressing HEK293 cells. Furthermore, LAPTM4A overexpression reduced the plasma membrane expression of hOCT2 in biotinylation experiments.

The possible relevance of this interaction is suggested by the demonstration of LAPT4A expression in human kidneys by controlling hOCT2 trafficking to/from the plasma membrane and processing it via the intracellular sorting machinery.

Regulation of Gene Expression of Organic Cation Transporters

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors which regulate fatty acid β -oxidation and energy homeostasis. Interestingly, it has been shown that members of this protein family (PPAR α and PPAR β/δ) also regulate the mRNA abundance of drug transporters, suggesting their possible involvement in fatty acid metabolism [74]. It was reported that mice treated with a PPAR α agonist had a greater Oct1, Octn2, and Octn3 but not Octn1 mRNA abundance than untreated mice in liver, muscle, kidney, and small intestine [34, 79, 114]. Similarly, rOctn2 mRNA was increased in primary cultured rat hepatocytes and in liver of rats after treatment with a PPAR α agonist with an increase in hepatic carnitine uptake [95]. The same stimulatory effect of PPAR α was seen in bovine MDBK cells [180]. Consequently, it was demonstrated that a functional peroxisome proliferator response element (PPRE) in the first intron of mOctn2 is a direct target of PPAR α [167]. Besides PPAR α , PPAR γ similarly activates Octn2 mRNA abundance in mice and also in human colonic epithelial cells [29]. Recently also an effect of PPAR β/δ activation which increases Octn2 gene expression and transport at least in bovine cells was shown [179].

PPAR α is also involved in the circadian regulation of drug transporter expression [117]. Variations in expression and function of drug transporters in a daily rhythm is thought to significantly influence efficacy of drugs in patient care. Dosing time dependent therapeutic effects and toxic side effects of drugs have been attributed to such circadian expression rhythms for the detoxification of xenobiotics (for review see [117]). In mammals the circadian clock works as a transcriptional and translational regulatory loop in which the clock proteins CLOCK and BMAL1 are important regulators. Clock genes of the central oscillation loop also regulate the expression of PPAR α [119]. In mice it was demonstrated that the expression of mOct2, but not of mMate1 in the kidney was under circadian control [117]. Both transporters are responsible for the excretion of many drugs including the chemotherapeutic cisplatin and thereby also contribute significantly to their nephrotoxicity [22, 23, 39, 113]. The circadian oscillation of mOct2 could be attributed to PPAR α activity. These observations certainly prompt further detailed examination of circadian regulation of drug transporter expression and thereby should have significant influences on dosing time in pharmacotherapy of patients. For hMATE2-K, the kidney specific paralog, a regulation by the transcription factor myeloid zinc finger was reported [17].

A specific aspect of regulation of drug transporter expression is its modulation during development. The spatiotemporal expression pattern of several drug transporters including Oct1 was examined during murine embryogenesis and development [124]. First transcripts appeared at midgestation and coincided with proximal tubule differentiation and gradually increased during nephron maturation. The authors of this study suggested that these drug transporters also play a role in the

transport of morphogenetic active molecules. This developmental regulation of Oct1 in the renal proximal tubule is apparently controlled by a network of genes, among which the hepatocyte nuclear factor 4 α (Hnf4 α) plays a key role by regulating the terminal differentiation and capacity for drug transport in the nascent proximal tubule [100]. In ex vivo rat kidney organ cultures, an antagonist of Hnf4 α reduced rOct1, rOct2 and also rMate1 gene expression, while overexpression of Hnf1 α and Hnf4 α in primary mouse embryonic fibroblasts induced gene expression [42, 96], suggesting that Hnf1 α and Hnf4 α play a critical role in the tissue-specific regulation of drug handling and differentiation toward a proximal tubular like cellular identity. In more detailed studies, it was demonstrated that disruption of the HNF1 α gene in mice led to tissue-specific regulation of mRNA-expression for mOct2, mOct3, mOctn2, and mOctn3, while the mRNA level of mOct1 and mOctn1 remained unchanged [96]. Another group demonstrated that in humans the hOCT1 and hOCT2 genes are transactivated by HNF4 α [128, 138]. The transcriptional factor small heterodimer partner (SHP) was reported to down-regulate the gene expression of hOCT1 [114] and the hepatocyte growth factor (HGF) led to a down-regulation of hOCT1 mRNA expression in human hepatocytes [89].

Cellular osmotic stress induced by an increase in extracellular osmolality activates cellular mechanisms to increase intracellular osmolality to prevent cellular water loss and increases in ionic strength in many cells. One mechanism to increase cellular osmolality as regulatory and protective response of the cells is the import of L-carnitine, which is mediated by OCTN2. In epididymis cells an increase in extracellular osmolality increases the transcription of several human genes among those also hOCTN2, which protects the luminal cells and spermatozoa [26].

Another important aspect of transcriptional regulation of organic cation transporters is their gender dependent expression. This certainly highly relevant issue also for the pharmacokinetics and efficacy of drug therapies is addressed in detail by L.M. Aleksunes and I. Sabolic in Chaps. 6 and 9.

Epigenetic Regulation of Organic Cation Transport Systems

Epigenetic gene regulation is mediated by DNA methylation and leads to silencing of the respective genes and plays an important role in tumorigenesis [9, 30, 132]. DNA methylation is of central importance for the tissue-specific expression also of transporter proteins and regulates their expression in cancer cells (see below). This was first examined for the kidney and liver specific expressions of hOCT2 and hOCT1, respectively [3]. While the proximal promoter region for the hOCT2 gene was hypo methylated in the kidney, it was hyper methylated in the liver corresponding to the specific expression of hOCT2 only in kidney. The respective promoter region for the hOCT1 gene was hyper methylated in both organs. Methylation of the hOCT2 promoter was especially low in the binding site (E-box) of the basal transcription factor upstream stimulating factor (USF). These data suggest that the tissue-specific expression of at least hOCT2 is controlled by DNA methylation.

For hOCT2, unlike hOCT1 or hOCT3, an interindividually different expression in the placenta was observed, which was attributed to loss of maternal imprinting in some patients [139]. From this study a role of histone H3 acetylation for the allelic expression and of trimethylation of lysine-9 on histone H3 for interindividual placental expression of the hOCT2 gene was evident. For hOCT3 a correlation between polymorphisms in the proximal promoter region of the gene, which leads to an altered degree of methylation, in Asian and Caucasian liver samples and hOCT3 expression in the liver was observed [15].

Regulation of Organic Cation Transport Systems under Pathophysiological Conditions

Differences in the expression levels of several drug transporters including transporters for organic cations have been described for a large variety of diseases in animal models as well as in human patients. This includes liver diseases (Crohn's disease, cholestasis, drug induced hepatotoxicity, and liver regeneration), kidney diseases (acute renal failure, chronic renal failure, kidney transplantation, drug-induced nephrotoxicity), hypertension, cardiomyopathy, inflammatory bowel disease, ischemia/reperfusion injury, diabetes, neurological disorders, primary carnitine deficiency, obstructive azoospermia, and cancer (for review see: [27, 35, 78, 82, 127]). Such changes in transporter activities, which are mostly down regulated in pathophysiological conditions, have significant effects on drug uptake in target cells with decreased efficiency, decreased metabolism and/or excretion of drugs with altered pharmacokinetics and potentially increased toxic side effects in such patients.

Involvement of Organic Cation Transporter Regulation in Cancer

Drug transporters are key proteins involved in successful cancer therapy [35]. One aspect of several anticancer drugs is the activation or inhibition of transporters as e.g. many cancer cells activate substrate transporters in response to increased nutritional demand. Drug delivery to cancer cells by such influx transporters is often an important first step to efficacy of anticancer therapy, while efflux transporters lead to increased chemo-resistance of the cancer cells. The majority of studies have drug efflux transporters in their focus, as these are responsible for multidrug resistance. On the other hand influx of toxic anticancer drugs by uptake transporters in non-target cells induce specific toxicities, which are potentially aggravated when renal transporter expression and consequently renal clearance are reduced. Finally, interference of co-medication given together with anticancer medication at these transporters potentially reduce their uptake by cancer cells. Many of these diverse

functions of drug transporters in cancer therapy are mediated via other members of the SLC protein family which are not topic of this book. The transporters for organic cations (OCTs, OCTNs, MATEs) are especially involved in renal drug excretion and also in toxic side effects of several cationic chemotherapeutics [2, 10, 22–24, 32, 39, 68, 87, 94, 105, 113, 149, 175–177].

Several studies report on changes in the expression of these transporters in response to tumor genesis and/or chemotherapy (for review see also [27]). Besides epigenetic up-regulation of e.g. OCT3 methylation leading to a decreased expression of the transporter in prostate tumor cells (see above), down regulation of especially hOCT1 but also of hOCT3 mRNA and protein was also reported in hepatocellular carcinoma [57]. This down regulation was restricted to the tumor cells and absent in non-neoplastic surrounding tissue and was strictly associated with advanced hepatocellular carcinoma stages. Similarly also in cholangio-cellular carcinoma a down regulation of hOCT1 and hOCT3 mRNA and protein was seen in cancerous tissue compared to the tumor surrounding tissue and the expression level of hOCT1 significantly correlated directly with tumor progression and inversely with patient survival [88]. In another study a differential expression of hOCT3 in oral premalignant and malignant lesions was reported, which has potential implications in the antineoplastic therapy using e.g. metformin, which is a substrate of hOCT3 [123]. Down regulation of Oct1, Oct2, and Oct3 in renal tumors of Tsc1 (hamartin) deficient mice was again dependent on an increased DNA methylation [174]. Another complication in chemotherapy is a potential drug induced down regulation of uptake transporter expression. Such a phenomenon was described for hOCT1, which was down regulated in human esophageal cancer cells by long-term cisplatin therapy via a promotion of methylation of the hOCT1 gene [92].

In ulcerative colitis patients, hOCTN1 has been linked to an increased risk of colorectal cancer [99] and an involvement of hOCTN2 in breast cancer cell lines and specimens [165] was also demonstrated. In the latter study an estrogen-dependent gene regulation via a newly identified intronic estrogen-responsive element was described in breast cancer cell lines and human tissue samples. Unlike this intronic gene regulation, a promoter methylation of the hOCTN2 gene with corresponding decreased expression was seen in a hepatocellular carcinoma cell line (HepG2) and a human Caucasian colon adenocarcinoma line (LS174T) [131].

Involvement of Organic Cation Transporter Regulation in Renal Disease

The importance of drug transporters in renal clearance of drugs is well known and accepted by clinicians and also by the pharmaceutical industry. Prediction of renal clearance of specific drugs is an important issue both in drug design and development as well as in therapeutic dosage (for review see [38, 127, 164]). Renal drug

excretion for a given drug is not only significantly influenced by competition at the renal drug transporters by co-medication but also by disease dependent alterations in transporter regulation and expression.

Interestingly, most renal diseases lead to significant down regulations of transporters for organic cations with corresponding clinically relevant effects on dosage of several drugs in these patients. These facts resulted in suggestions for dosing adjustments long before all drug transporters were molecularly identified [37, 147]. The mechanisms behind these regulatory effects on drug transporters including transporters for organic cations are diverse, but not always clear. One of the earlier detailed studies on this issue demonstrated a down-regulation of Oct2 in rats after 5/6 nephrectomy as a model for chronic kidney disease [69]. The authors suggested a testosterone-dependent down-regulation of rOct2 in this animal model. In contrast, in another rat model for acute renal failure, in which uranyl nitrate was used, a testosterone-independent down-regulation of rOct2 expression in the kidney medulla was observed [148]. In a different rat model, the adenine-induced chronic renal failure, a down-regulation of the apical transporters rMate1 and rOctn2 together with several other drug transporters was reported [86]. This effect did not depend on a circulating factor as infusion of serum of chronic renal failure rats did not show any changes in transporter expression. Also in acute renal failure induced by ischemia/reperfusion injury again in rats a down-regulation of rOct1, rOct2, and of rMate1 was shown [102, 146]. One mechanism which might be responsible for this down-regulation of Octs in the rat may be the increase in NO generation after renal ischemia/reperfusion injury as inhibition of NO generation prevented this down-regulation of rOcts [146]. The effect of NO produced by the renal iNOS on down-regulation of rat Octs was also reported for endotoxemia induced in the isolated perfused rat kidney [56]. For endotoxemia in mice an activation of the ischemia/reperfusion-inducible protein (IRIP) was reported, which mediated the down regulation of mOct2 and mOct3 [70]. Despite an up-regulation of *P*-glycoprotein (Abcb1), rOct1 and rOct2 mRNA and protein expressions were down-regulated in dependence of iNOS activity. Recently it was demonstrated in cell culture systems that IRIP negatively regulates the membrane localization of rOct1 and rMate1 without changes in their transcription levels [91]. Further evidence for a circulating factor, which induces OCT down-regulation at least in rats was shown for the renal expression of rOct2 which was decreased after 60 min liver ischemia/reperfusion injury [66].

Acute renal failure can also be induced by various nephrotoxic drugs. This is clinically highly relevant for the cytostatic cisplatin, the excretion of which depends on the activity of OCT2 and MATE1 in humans (see above). In the rat a down-regulation of rMate1 and rOct2, but not of rOct1 mRNA and protein was seen after a single injection of cisplatin [109]. The authors suggested an involvement of the cisplatin-induced increase in indoxyl sulfate, a uremic toxin significantly increased in serum and kidney after cisplatin treatment. Interestingly, another group reported a down-regulation of both rOct1 and rOct2 mRNA by cisplatin treatment of rats and this down-regulation could be prevented by simultaneous treatment with the nephro-protective natural compound thymoquinone [162]. Recently, it was reported that the

aging suppressor Klotho, which also confers protection against acute ischemia/reperfusion injury, has protective effects on cisplatin-induced acute kidney injury in mice [122]. This effect of Klotho was mediated both, by a reduction in renal mOct2 expression and thus cisplatin uptake, and by a direct anti-apoptotic effect.

Another pathological situation with specific changes in the expression of OCTs at least in rats is kidney transplantation [25]. In this study we demonstrated that kidney transplantation leads to a parallel down-regulation of rOct1 and rOct2 mRNA and protein expression within 4 days. In the case of rOct1 this down regulation is most likely due to the ischemia/reperfusion injury, which is inevitable during the operation procedure as it was also seen in syngeneic kidney transplantations without any rejection. Unlike rOct1, rOct2 expression was only down-regulated after allogeneic transplantation, which leads to a marked immunological rejection within 4 days, while there was no decrease in expression of rOct2 in the syngeneic transplantation model. In the same microarray analysis [25] a down-regulation of the mRNA for the renal apical efflux organic cation transporters rOctn1 and rOctn2 was also observed after allogeneic transplantation, however, not for rMate1 and not after syngeneic kidney transplantation (Ciarimboli G, Edemir B, Schlatter E: own, unpublished observations). Such a down regulation of renal uptake transporters and possibly also of renal efflux transporters (not examined so far) has clear clinical relevance as transplanted patients mostly receive other cationic drugs (e.g. antibiotics or antihypertensives) besides their immunosuppression. Since these drugs are also substrates of these transporters their renal clearance will be reduced [25].

In rats, which were fed oxonic acid to induce hyperuricemia, a down-regulation of mRNA expression levels of rOct2, but not of rOct1 was observed [53]. In hyperuricemic mice also a down-regulation of mOct1, mOct2, mOctn1, and mOctn2 mRNA levels was reported [64].

Unfortunately all these studies on transporter regulation in renal pathologies have been performed in animal or cell models and as their relevance for human patients is likely such studies in humans are urgently needed.

Involvement of Organic Cation Transporter Regulation in Liver and Intestinal Diseases

Intestinal inflammation seems also to induce an altered regulation of drug transporters expressed in the intestinal epithelia. In human colonic epithelial cells (Caco2BBE) interferon- γ (IFN- γ) or tumor necrosis factor- α (TNF- α) increased the apical expression of hOCTN2 [40]. In the same study the expression of mOctn2 was increased in the colon of Rag-1 deficient mice (a model to study the role of adaptive immune cells) which were treated with IFN- γ or TNF- α to induce intestinal inflammation. Similarly epithelial hOCTN2 expression was increased in actively inflamed regions of Crohn's disease and ulcerative colitis. In contrast, hypoxia in human intestinal epithelial cells and duodenal biopsies obtained from individuals acutely

exposed to high altitude showed reduced levels of hOCTN2 mRNA and protein expression which was accompanied by increased mRNA levels of several pro-inflammatory proteins [169]. The discrepancy between these latter two studies indicates that apparently the effect of reduced oxygen supply by systemic hypoxia overrides the effect of cytokines in the regulation of OCTN2 expression. Similarly controversial observations were made in BeWo cells as an in vitro model for the human trophoblast where hypoxia increased transcription but not translation of Octn2, but reduced Octn2-mediated carnitine transport [135]. In another study again with BeWo cells and human placental explants a hypoxia-dependent down regulation of hOCTN2 mRNA and protein levels was reported which apparently was mediated via PPAR α [14]. A down-regulation of the mRNA for hOCT3, but not of hOCT1 and hOCT2 was also reported for pre-eclamptic patients suggesting a mechanism to prevent vasoconstriction by reducing the activity of this monoamine transporter [8].

Patients with chronic hepatitis C and progression of liver fibrosis demonstrated a reduction in hOCT1 mRNA expression [112]. In chimeric mice with humanized liver, infection with the hepatitis C virus led to a reduction in OCT1 expression, besides altered expression of some other drug transporters [76]. In a clinical study examining 150 Caucasian subjects a significantly reduced expression of hOCT1 and hOCT3 was observed in liver donors diagnosed cholestatic, which indicates consequences for the hepatic elimination of OCT substrates such as metformin in these patients [115]. Similarly in rats a down regulation of rOCT1 was observed after bilateral bile duct ligation [31]. In other studies using LPS-treated mice or rats as models for hepatic inflammation again a down regulation of Oct1 among other drug transporters was reported [7, 16], which at least in the mouse appeared to be mediated mostly by interleukin-6 [7].

Involvement of OCTN Regulation in Other Diseases

In addition to the above discussed various renal, intestinal and hepatic disorders which involve modulation of organic cation transporter regulation, the involvement of OCTN2, which provides the cells with the nutrient L-carnitine, in various additional disorders has been reported. A reduced L-carnitine uptake correlates with increased blood pressure and L-carnitine infusion leads to endothelial improvement in humans [134]. In rats a reduced L-carnitine uptake mediated via rOctn2 by the endothelium was found in aortic rings from spontaneously hypertensive rats [140]. Thus OCTN2 apparently is an important transporter for the metabolic needs also of endothelial cells and its reduced activity increases the risk for hypertension. The exact mechanism behind this blood-pressure dependent regulation of OCTN2 is still unknown.

The effects of cytokines on expression and function of organic cation transporters in various inflammations is already mentioned above. OCTNs are important transporters for L-carnitine uptake also in the mammary epithelium. In the rat

mammary gland the expression of rOcn2 increased with the duration of lactation and experimentally (LPS-) induced inflammation in the lactating rat resulted in a reduction in these rOcn2 mRNA levels [93]. Such pathological down-regulation of this and also other substrate transporters may lead to reduced mammary epithelial function and consequently reduced milk quality and yield.

Several organic cation transporters are expressed in the heart (vascular: OCT3, OCTN2, OCTN1; cardiomyocytes: OCT1, OCTN1) [49]. In this study it was also demonstrated that coxsackievirus-infected mice as model for cardiomyopathy showed a reduced mOcn2 expression. In endomyocardial biopsies of patients with dilated cardiomyopathy hOCTN2 expression was also reduced, while that of the other transporters was unaltered [49]. This study also demonstrated a significant correlation between expression of hOCTN2 and ventricular ejection fraction. Furthermore, it was shown, that therapy with oxazaphorines, cyclophosphamide or ifosfamide down regulates rOcn2 expression in rats and contributes to the development of cardiomyopathy [141].

L-carnitine uptake is essential in sperm maturation and metabolism in the epididymis. Data from a mouse model of obstructive azoospermia (JVS mice) suggest the important role of mOcn2 for male fertility as these mice are dysfunctional for mOcn2 [173].

The important function of OCTN2 as uptake mechanism for the nutrient L-carnitine in the mammalian body is also evident from a study in rats, in which an increased expression of rOcn2 in liver and kidney with a corresponding increased carnitine plasma level and carnitine biosynthesis was observed after intensive exercise for a 10 week period [11]. The increased Ocn2 expression in the kidney probably led to maximal reabsorption of carnitine from the primary urine.

Down-regulation of organic cation transporters has also been reported for diabetes type 1 or type 2 [22, 48, 108, 116, 161] in several mouse and rat models. These effects of diabetes on transporters for organic cations are more specifically addressed in Chap. 6 by Aleksunes.

Table 5.6. summarizes all pathways and pathophysiological situations involving a regulation of expression and/or function of these transporters for organic cations as discussed in this chapter.

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Chapter 6

Endocrine and Metabolic Regulation of Transporters for Organic Cations

Lauren M. Aleksunes

Abstract The disposition of organic cations in the liver and kidneys are under a dynamic regulation by the endocrine and metabolic systems of the body. Researchers have demonstrated the ability of steroids, sex hormones, and physiological states such as pregnancy to alter the expression and function of transporter proteins that mediate the uptake and efflux of organic cations. This chapter reviews the endocrine and metabolic pathways that influence the pharmacokinetics as well as the toxicity of organic cations.

Keywords Diabetes • Estradiol • Gender • Obesity • Testosterone • Glucocorticoid

Introduction

The organic cation transporters (OCTs) and multidrug and toxin extrusion transporters (MATEs) are membrane-spanning proteins that mediate the uptake and efflux of organic cations into and out of the cell, respectively. OCTs and MATEs are expressed throughout the body, although researchers have focused primarily on their regulation and activity in liver and kidneys [1–3]. Both transporter classes have an affinity for a range of substrates including endogenous compounds and xenobiotics. This chapter describes the endocrine and metabolic pathways that regulate the expression and/or function of OCT and MATE transporters. Hormones or steroids often mediate these signaling pathways. Also included in this chapter is an overview of how disease states, such as diabetes and obesity, influence the disposition of organic cations. The studies in this field have been performed primarily in rodents, cultured cells and tissue slices using probe organic cations to assess the transport activity of OCTs and MATEs. Probe cationic substrates include tetraethylammonium (TEA),

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N-methyl-nicotinamide (NMN), 1-methyl-4-phenylpyridium (MPP⁺) and the drug cisplatin (*cis*-diammine dichloroplatinum). Cisplatin is a substrate of rodent and human Oct2/OCT2 [4–6] as well as Mate1/MATE1 [7]. Because of its routine use as a chemotherapeutic drug to treat solid tumors, researchers often employ cisplatin as a relevant nephrotoxic substrate for understanding the consequences of perturbations in the disposition of cationic drugs.

Gender Differences

Early investigation into potential gender differences in organic cation transport revealed that accumulation of TEA was greater in renal cortical slices from male rats compared to female rats (Table 6.1) [8, 9]. This resulted from higher mRNA and protein expression of Oct2 in the kidneys of male rats [2, 9]. In turn, elevated Oct2 expression in male rats increased the uptake of cisplatin into the kidneys [10]. Additional studies have also determined whether species other than rats have similar gender differences in Oct2 transporter expression and function. Male mice exhibited two-fold higher expression of renal Oct2 mRNA than female mice [3]. Likewise, proximal tubules from male rabbits had higher mRNA expression of Oct2 compared to females [11]. It should be noted however that the male-predominant expression of Oct2 mRNA was not reflected in differences in Oct2 protein expression or TEA uptake into isolated tubules from rabbits [11].

Mouse kidneys express not only Oct2 but also the Oct1 transporter [3]. To determine whether there are gender differences in the Oct1-mediated basolateral uptake of cations, proximal tubules were isolated from male and female Oct2-null mice [12]. Accumulation of the fluorescent cation 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium (ASP⁺), was modestly lower (~20 %) in the tubules isolated from female mice [12]. Interestingly, no differences in Oct1 mRNA were noted between male and female Oct2-null kidneys. The authors suggest that the gender difference in ASP⁺ uptake results from post-translational modifications to the Oct1 protein rather than changes in transcriptional regulation. The intermediary signaling protein, calmodulin, has been postulated as a potential mediator responsible for this gender difference in Oct1 activity. Calmodulin binds calcium and transduces intracellular signals in coordinated cellular responses. Kidneys from male rats have 2.3-fold higher protein expression of calmodulin than females [14]. Interestingly, inhibition of calmodulin activity using a pharmacological antagonist reduced organic cation transport in renal tubules from male rats, but not from female rats [14]. Taken together, gender-dependent regulation of organic cation transport, in the absence of mRNA or protein differences, may be modulated by intracellular pathways such as Ca²⁺-calmodulin signaling in rodents. It is not known whether these gender differences in the calmodulin pathway extend to human kidneys.

There are no gender differences in the renal expression of Oct1, Oct3, Octn1, or Octn2 mRNA protein in mice and rats, while there is an increase in Mate1 mRNA in male mice [1, 3, 9, 11]. The hepatic regulation of Oct1 mRNA is similar between

Table 6.1 Gender differences in Oct/OCT, Octn/OCTN, and mate expression and function

Experimental design		Organic cation transport			References
Species	Model	Tissue	Expression	Function	
Rat	Slices	Kidneys	↑ Oct2 protein in males	↑ TEA accumulation in males	[8, 9]
			↔ Oct1, Oct3 proteins		
Rat	Tissue	Kidneys	↑ Oct2 mRNA in males	↑ Cisplatin accumulation in males	[2, 10]
			↑ Oct1 mRNA in females		
			↔ Oct3, Octn1, or Octn2 mRNA		
Rabbit	Isolated Proximal Tubules	Kidneys	↑ Oct2 mRNA in males	↔ TEA accumulation	[11]
			↔ Oct2 protein		
Mouse	Isolated Proximal Tubules	Kidneys	↔ Oct1 mRNA	↓ ASP ⁺ accumulation in females	[12]
Mouse	Tissue	Kidneys	↑ Oct2 mRNA in males	N.D.	[1, 3, 11]
			↔ Oct1, Octn1, Octn2 mRNA		
			↑ Mate1 mRNA in males		
Mouse	Tissue	Liver	↔ Oct1 mRNA	N.D.	[1, 3]
			↓ Mate1 mRNA in males		
Human	Biopsy	Liver	↔ OCT1 mRNA and protein	N.D.	[13]
			↔ OCT3 mRNA		

N.D. not determined

male and female mice [3]. Like mice, the mRNA expression of OCT1 and 3 is similar in livers from 150 Caucasian men and women [13]. Collectively, these studies point to isoform and species differences in the regulation of cationic transporters between males and females.

Hormonal Regulation

To understand the mechanisms by which Oct2 displayed male-predominant expression in rodents, efforts focused largely on sex hormones such as testosterone and 17β-estradiol. In addition, the ability of glucocorticoids to regulate cation transport in the liver and kidneys has been investigated.

Testosterone

Although the level of serum creatinine is often used to estimate glomerular filtration, a portion of its renal excretion is mediated by active secretion. As early as 1966, it was evident that the secretion of creatinine was different between male and female rats [15]. Researchers demonstrated that the urinary excretion of creatinine in female rats approximated the rate of glomerular filtration; by contrast, in male rats a portion of the urinary excretion was dependent upon secretion by the proximal tubule [15]. Moreover, administration of testosterone to female rats resulted in tubular secretion of creatinine similar to that observed in males. These data served as some of the earliest evidence that sex hormones could regulate the renal clearance of ionic chemicals. It should be noted that creatinine is a substrate of both organic cation and anion transporters. Using HEK293 cells overexpressing human OAT1-3 and OCT1-3 isoforms, it has been demonstrated that creatinine is a substrate of multiple transporters with the following rank order for uptake preference: OCT2>OCT3>OAT2>OAT3 [16]. Similar studies with mouse isoforms revealed that mouse Oct2 can also transport creatinine and that mice lacking both Oct1 and Oct2 have reduced rates of creatinine clearance [16].

Surgical removal of the testes in male mice and rats reduced the renal expression of Oct2 mRNA to levels observed in females [2, 3]. As a result, male and female rats have equal susceptibility to cisplatin nephrotoxicity following gonadectomy [10]. Treatment of male and female gonadectomized rodents with dihydrotestosterone increased Oct2 mRNA expression [3] and enhanced creatinine secretion [17]. In vitro studies using Madin-Darby canine kidney cells have tested the ability of individual hormones and steroids to alter the uptake of TEA as a model organic cation across the basolateral membrane [18]. Similar to the in vivo mouse and rat studies, a 72 h exposure of these cells to testosterone increases Oct2 mRNA expression and TEA accumulation. Likewise, testosterone treatment of male and female rats with intact gonads also increases Oct2 mRNA and protein, leading to elevated TEA accumulation in kidney slices ex vivo [19]. Analysis of the rat Oct2 promoter demonstrated that testosterone enhances expression via the androgen receptor at two androgen response elements [20]. This transactivation of gene expression by testosterone was blocked using the antiandrogen drug nilutamide [20]. The effect of testosterone on the transport of organic cations was largely restricted to changes in Oct2 expression. For example, castration and treatment of male mice with testosterone had no effect on the expression of Oct1, Oct3, Mate1, or Mate2 mRNAs. Interestingly, castration increased the mRNA levels of Octn1 in male mice, which could be attenuated by testosterone treatment. While interesting, the relevance of this finding to the overall excretion of Octn's notable substrate carnitine is unclear.

Estradiol

Studies have demonstrated that estrogens can also alter organic cation transport. Treatment of intact male and female rats with 17 β -estradiol (1 mg/kg) for 7 days induced supraphysiologic circulating levels of estradiol (50–240 times normal rat concentrations) and repressed Oct2 protein expression and TEA accumulation in renal slices [19]. In line with these findings, basolateral TEA uptake was enhanced by 50 % in proximal tubules from female mice following ovariectomy [21]. Supplementation of ovariectomized mice with estradiol restored TEA transport back to baseline levels. To understand the mechanism for changes in TEA uptake and excretion by estradiol, the mRNA expression of key transporters was quantified. Ovariectomy of mice had no effect on Oct1-3, Mate1, or Octn1-2 mRNA expression and only reduced Mate2 mRNA levels [21]. It should be noted that expression of transport proteins was not included in this study. Inclusion of these data could have provided greater clarity into the regulation of TEA renal uptake by post-transcriptional regulation following ovariectomy. Supporting this contention is the fact that 6 day exposure of opossum kidney cells to a physiological concentration of 17 β -estradiol or the xenoestrogen diethylstilbesterol decreased Oct1 protein expression which led to a decline in TEA uptake [22]. Notably, the reduction in TEA uptake following treatment with either estrogen was abolished by inclusion of an antagonist of the estrogen receptor [22].

Corticosteroids

Corticosteroids are steroid hormones secreted by the adrenal cortex and are used to treat a variety of endocrine and inflammatory disorders. Investigation into the ability of corticosteroids to regulate organic cation transport has largely focused on the expression and function of Oct1 in the liver. Exposure of primary rat hepatocytes as well as rat hepatoma RL-34 cells to dexamethasone (10 and 100 μ M) for 24 h decreased the mRNA expression of Oct1 by over 60 % [23]. This down-regulation correlated with reduced uptake of MPP⁺ in both cell types. Inhibition of the glucocorticoid receptor using the antagonist RU-486 prevented the ability of dexamethasone to decrease Oct1 mRNA. Because dexamethasone can also decrease the mRNA expression of the pregnane X receptor (Pxr) [23] and since RU-486 is a PXR activator, it is possible that RU-486 prevented the decline in Oct1 by enhancing Pxr signaling in the hepatocytes. This is a potential mechanism because a Pxr agonist has been demonstrated to increase Oct1 mRNA and MPP⁺ uptake in rat hepatocytes [24].

In contrast to the work performed in rat hepatocytes, investigation of OCT1 regulation by glucocorticoids has also been performed in human hepatocytes at a lower,

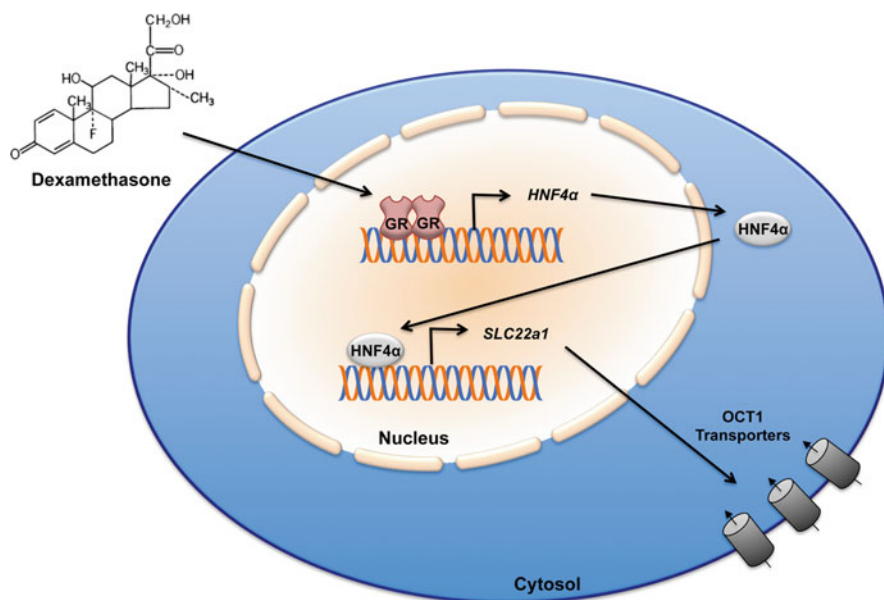


Fig. 6.1 Hepatic regulation of OCT1 transporter expression by corticosteroids. Exposure of human liver cells to dexamethasone stimulates the transcription of hepatocyte nuclear factor 4 alpha (HNF4α). In turn, HNF4α binds to the upstream region of the *SLC22A1* gene leading to the enhanced production of the OCT1 transporter in the liver. Adapted from Rulcova et al. [25]

physiologically-relevant concentration of dexamethasone (100 nM) [25]. At this concentration, dexamethasone increased OCT1 mRNA and protein expression, which could be prevented by co-exposure to RU-486. Further mechanistic studies demonstrated that dexamethasone increased expression of the hepatocyte nuclear factor 4 alpha (HNF4α) transcription factor in human hepatoma cells [25]. Given that HNF4α is a known regulator of OCT1 expression [26], it was suggested that the dexamethasone-induced increase in HNF4α lead to transactivation of the *OCT1* promoter and thus induction of OCT1 mRNA (Fig. 6.1). Together, regulation mechanisms of organic cation transporters by corticosteroids may be different between the species.

Pregnancy

There has been limited investigation of organic cation transport in the liver and kidneys during pregnancy. In part, this may be because changes in other physiological parameters during pregnancy such as increased blood volume, hepatic and renal blood flow, as well as enhanced glomerular filtration may have greater impact on pharmacokinetics. Nonetheless, characterization of the molecular changes in Oct,

Octn and Mate expression have been performed in pregnant mice. In the kidneys of pregnant mice, the mRNA and protein expression of Mate1 were reduced beginning on gestation day 11 through 17 [27]. It should be noted that gestation in a mouse is ~20 to 21 days. In addition to a decline in Mate1 gene and protein expression in the kidneys of pregnant mice, there were mild changes in the mRNA expression of other organic cation transporters. At 7 days of pregnancy, the mRNA expression of Oct1, Octn1, and Octn2 were reduced 20–40 % with no change in Oct2 or 3 levels [27]. However by gestation day 11, levels of these transporters were restored to those observed in virgin controls. In the maternal livers, expression of Oct1 mRNA was also reduced from gestation day 7 to 1 day after parturition whereas Mate1 mRNA expression was fairly similar to virgin controls throughout pregnancy [28]. Additional work is needed to determine whether these changes in the mRNA expression of Octs and Mates are relevant to the overall disposition of cationic chemicals during pregnancy.

Gender and Population Pharmacokinetics

Mechanistic studies comparing species differences in the hormonal regulation of organic cation transporters are considered important as preclinical studies are largely performed in rodents. Nonetheless, the applicability of male-predominant expression of Oct2 in rodent kidneys to human pharmacokinetics is not clear. A number of population pharmacokinetic studies have been conducted for drugs and chemicals that are OCT substrates. The *N*-methyl-D-aspartate (NMDA) receptor antagonist memantine is used to treat moderate to severe Alzheimer's disease. Memantine is a likely OCT2 substrate [29]. Interestingly, memantine clearance is affected by gender. Female patients prescribed memantine exhibited higher plasma concentrations than males suggesting reduced urinary excretion [30]. The renal clearance of another OCT substrate, amantadine also tended to be lower in women but was not statistically different between sexes [31]. The interaction of amantadine with chiral organic cations also exhibited gender differences in rat tubules [32]. In a follow-up study, the renal clearance of amantadine was in fact higher in healthy male volunteers, especially when the data were normalized to body mass index [33]. Plasma clearance of the cancer drug oxaliplatin was also higher in male patients with metastatic cancers [34]. These three examples point to a potential gender difference in the renal clearance of some cations in humans.

Given the role for OCTs in the secretion of creatinine, a number of studies have investigated gender differences in creatinine clearance. An early study comparing the renal secretion of creatinine between adult women and men revealed no differences in rates although the sample size was quite small [35]. More recently, an analysis of serum creatinine levels in 590 children revealed no differences between genders [16]. However, in a longitudinal study, it was demonstrated that urine creatinine levels were 33 % higher in males than females [36]. Given these findings, it is difficult to draw concrete conclusions regarding gender differences in creatinine secretion.

Gender does not influence the population pharmacokinetics of other cationic drugs including metformin [37], varenicline [38], or cisplatin [39]. Collectively, these data suggest that gender may impact the population pharmacokinetics of some cationic drugs. However, the contributory role of basolateral uptake by OCT2, as well as MATE1, in these differences still needs to be demonstrated.

Diabetes and Obesity

Probe Organic Cation Transport

Early studies investigated the accumulation of TEA in renal cortical slices obtained from healthy and diabetic rats [40]. Diabetes was established using a single injection of the beta islet cell toxicant, streptozotocin (STZ). Using a time course study, it was demonstrated that TEA accumulation in renal slices decreased in the diabetes model. By 2 weeks after STZ administration, TEA accumulation was reduced approximately 20 % and continued to decline out to 6 weeks of persistent hyperglycemia [40]. Administration of insulin to STZ-treated rats reduced circulating glucose levels and restored TEA accumulation in renal slices [40]. Similar to TEA, kidneys from diabetic rats also have reduced transport of another cationic probe NMN [41].

Regulation of OCT Transporters

Soon after demonstrating that diabetes impairs TEA accumulation in the kidneys, studies revealed that the mechanism for impaired transport was a down-regulation of the Oct1 and 2 proteins in diabetic rats (Table 6.2) [42]. Within 7 days after STZ injection, kidney Oct2 protein levels were 50 % of levels seen in non-diabetic rats [42]. By 14 days, expression of Oct1 protein was also reduced [42]. In line with the restoration of TEA uptake [40], insulin enhanced Oct1 and 2 mRNA and proteins in kidneys of diabetic rats [41, 42].

While the constitutive renal expression of Oct3 is lower than Oct1 and 2 in rats [2], it is still reduced in diabetic rats [41]. In addition to intervening with insulin, researchers demonstrated that inhibition of advanced glycation of proteins using aminoguanidine similarly prevented the down-regulation of Oct1-3 mRNA and protein in diabetic rats [41]. These findings suggested that the degree of glycemic control may not be the primary regulator of Oct1-3 expression, since aminoguanidine did not alter plasma glucose levels in diabetic rats (with aminoguanidine: 28.2 mmol/l and with aminoguanidine: 28.0 mmol/l compared to non-diabetic controls: 6.3 mmol/l) [41]. Nonetheless, aminoguanidine reduced accumulation of advanced glycation endproducts and restored the albumin excretion rate back to normal [41]. To further identify critical factors that led to deficits in organic cation

Table 6.2 Regulation of organic cation transporters in models of diabetes and obesity

Experimental design		Organic cation transport			References
Species	Model	Tissue	Expression	Function	
Rats	Injection of STZ (65 mg/kg i.v.) and evaluation at 1–6 weeks	Kidney slices, tissue and isolated proximal tubule cells	↔ Oct1 mRNA ↓ Oct1 protein ↓ Oct2 mRNA and protein	↓ TEA uptake in cortical slices	[40, 42]
Rats	Injection of STZ (55 mg/kg i.v.) and evaluation at 4 or 32 weeks	Kidneys	↓ Oct1-3 mRNA and protein	↓ NMN renal clearance	[41, 43]
Rats	High fat diet for 2 weeks and STZ (45 mg/kg i.p.) on day 14; evaluated on day 24	Kidneys	↓ Oct2 protein	N.D.	[44]
Mice	Db/db transgenic mice	Kidneys	↔ Oct1 and ↓ Oct2 mRNA ^a	N.D.	[45]
Mice	High fat diet for 15 weeks	Kidneys	↔ Oct1 and Oct2 mRNA	N.D.	[46]
Mice	High fat diet for 19 weeks	Liver	↑ Oct1 mRNA and protein	↑ Hepatic uptake of metformin	[47]
Human	Obese versus non-obese subjects	Adipose	↑ Oct1 mRNA and protein	N.D.	[48]

N.D. not determined

^aDown-regulation only observed in kidneys of male mice

secretion during diabetes, additional studies were performed. The renin–angiotensin system is critical for regulating vasculature pressure and sodium reabsorption by angiotensin II. Inhibition of the angiotensin-converting enzyme, which generates angiotensin II, prevented albuminuria and restored Oct1 and 2 mRNA and protein expression in diabetic rats without altering glomerular filtration rate [43]. Moreover, inhibition of the angiotensin-converting enzyme using the drug perindopril in diabetic patients enhanced NMN clearance [49]. It should be noted that non-diabetic controls were not included in this study; however, these findings generally supported the data found in prior rodent studies.

More recent studies have evaluated organic cation transport in models of type II diabetes and obesity. One common approach employed by these studies is to feed rodents a diet high in fat for 3–5 months. Evaluation of kidneys from mice fed a high fat diet for 15 weeks revealed no differences in Oct1 or 2 mRNA expression compared to mice fed a standard rodent chow [46]. However, in a separate study, an increase in Oct1 mRNA and protein was observed in the livers of mice fed a high fat diet for 19 weeks [47]. Up-regulation of Oct1 mRNA by high fat diet feeding was

associated with elevated circulating and liver metformin concentrations following i.p. or i.v. administration [47]. It is important to note that this model of obesity was not associated with changes in circulating glucose concentrations. Rather, mice fed a high fat diet exhibited elevated insulin, leptin and total cholesterol when compared to mice fed a normal diet [47]. Using human hepatoma HepG2 cells, the authors demonstrated that exposure to leptin and insulin could increase OCT1 mRNA in vitro and pointed to these two molecules as important factors for the up-regulation of Oct1 mRNA in obese mice [47].

In addition to investigating the regulation of renal and hepatic Oct transporters, a recent study demonstrated the expression of OCT1, but not OCT2, in human adipose tissue [48]. The expression of OCT1 mRNA was three- to four-fold higher in subcutaneous and visceral adipose samples from obese subjects (with and without type II diabetes) compared to non-obese subjects (sample sizes ranged from 14 to 32) [48]. A similar approximately two-fold increase in OCT1 protein was observed in adipose from obese subjects compared to lean controls [48]. The authors of this study suggest that elevated OCT1 expression from obese individuals may be a mechanism to enhance the uptake and pharmacological activity of drugs such as metformin within adipose tissue.

A one-compartment model of metformin population pharmacokinetics in obese and non-obese patients with type II diabetes was recently developed [50]. Because metformin is a hydrophilic chemical, lean body weight was determined to be the best descriptor of volume of distribution [50]. The authors noted a large between-subject variability in metformin pharmacokinetics. They mention that this may be due to differences in bioavailability or polymorphisms in OCT1 among subjects [50]. While genetic variants in *OCT1* and *MATE1* have been associated with metformin side effects [51–55], there has been little consideration of how disease severity (or lack of compliance with pharmacotherapy) may alter the transcription and subsequent function of organic cation transporters involved in metformin disposition and response.

Toxicity of Organic Cations

Alterations in the secretion of organic cations due to adaptive changes in the diabetic kidney have consequences on the pharmacokinetics and toxicity of drugs and chemicals. In fact, these data were largely generated prior to the characterization of Oct transporters. As early as 1989, it was demonstrated that STZ-treated rats were protected against the nephrotoxicity of cisplatin [56–58]. In order to understand the mechanisms underlying this protection, a number of interventions were attempted. For example, diuresis of control rats using dextrose did not alter the extent of cisplatin-induced nephrotoxicity [56]. Likewise, renal injury from cisplatin was not affected by stimulation of ketosis in control rats using acetone [56].

A role for impaired cisplatin uptake by proximal tubules was suspected to be the likely reason for renal protection from injury in diabetic rats (Fig. 6.2). Supporting

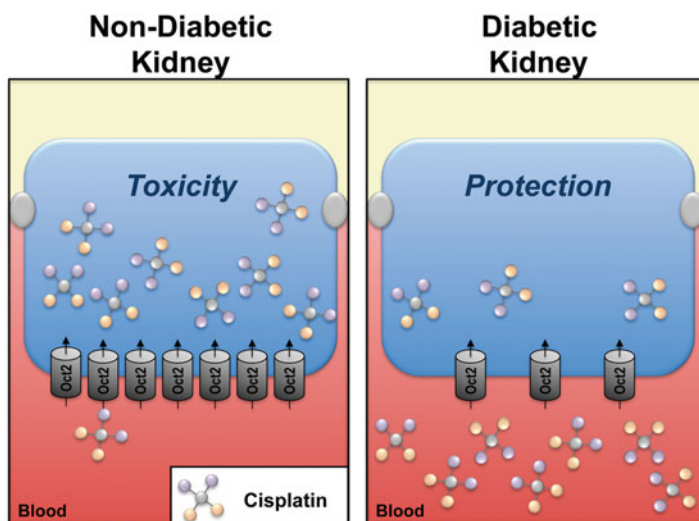


Fig. 6.2 Susceptibility of diabetic rodents to cisplatin nephrotoxicity. The expression of Oct2 is reduced in the kidneys of diabetic mice and rats leading to lower cisplatin uptake across the basolateral membrane. As a result, these animals are protected from cisplatin toxicity

this hypothesis was the fact that diabetic rats had reduced renal platinum accumulation as compared to control rats [59, 60] despite having greater total urinary excretion [59, 61]. It is not known whether this reflects a shift away from secretion and towards filtration of cisplatin during diabetes or whether Mate transporters are up-regulated in diabetic rats leading to reduced accumulation in the kidneys. Nonetheless, treatment of diabetic rats with insulin restored cisplatin accumulation in the kidneys and prevented nephroprotection [60], which paralleled the restored expression of Oct2 in other studies [41, 42]. This was evident by elevated plasma creatinine and BUN levels in diabetic rats treated with insulin and cisplatin that approached levels observed in normoglycemic rats exposed to cisplatin [60]. It is important to note that the ability of insulin to restore glucose levels to normal values was not responsible for altering cisplatin susceptibility. Treatment of diabetic rats with the insulin mimetic vanadyl sulfate similarly decreased plasma glucose levels and reduced the percent of glycosylated hemoglobin but did not alter cisplatin accumulation in kidneys nor susceptibility to nephrotoxicity [62].

Conclusions and Future Directions

The ability of endocrine and metabolic pathways to regulate the expression and function of organic cation transporters is critical to understanding how differences in physiology and pathology can influence xenobiotic disposition. While much has been learned over the past few decades, more work is needed to understand the

relevance of findings in rodents to humans. In addition, most of the work in this field has largely focused on OCT1 and 2, with little interrogation of how diabetes and obesity affect MATE1 and 2 expression and activity. Lastly, experiments testing the ability of other hormones such as the parathyroid and thyroid hormones as well as vitamin D to regulate transporters are needed to provide a more comprehensive understanding of the endocrine regulation of organic cation transport.

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Chapter 7

Organic Cation Transporters (OCTs) as Modulators of Behavior and Mood

Alejandro Orrico and Sophie Gautron

Abstract The organic cation transporters (OCTs) fulfill important functions in the absorption and excretion of endogenous compounds and xenobiotics in peripheral organs, which have been well documented. Two OCT subtypes, OCT2 and OCT3, are also expressed in the brain and predominant in aminergic projection regions. The last decade has seen substantial advances in our understanding of the implication of these transporters in a range of integrated functions of the central nervous system. Various approaches exploiting pharmacological inhibitors and mutant mice models for OCTs have disclosed that they are involved in particular in behaviors related to osmoregulation, anxiety, stress, antidepressant action and addiction. We summarize in this chapter recent developments on the roles of OCTs in central nervous system, focusing on mood-related behaviors.

Keywords Organic cation transporter • Brain • Osmoregulation • Anxiety • Stress • Antidepressants • Addiction

Abbreviations

5-HT	Serotonin
D22	Decynium 22
DA	Dopamine
DAT	Dopamine transporter
FST	Forced-swim test
NE	Norepinephrine
NET	Norepinephrine transporter
OCT	Organic cation transporter
SERT	Serotonin transporter
SFO	Subfornical organ
TST	Tail suspension test

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Introduction

Organic cation transporters (OCTs) belonging to the SLC22 family fulfill important roles in the absorption and excretion of endogenous compounds and xenobiotics in peripheral organs, which have been well documented. Three main OCT subtypes (OCT1, OCT2 and OCT3) were identified by molecular cloning, followed by their pharmacological characterization in heterologous expression systems. Studies over time showed that a large number of endogenous and exogenous substances can interact with these transporters as substrates or inhibitors. OCT ligands include various therapeutic drugs such as antivirals, cytostatics, antidiabetics and antihistaminergic agents, as well as hormones and neurotoxins [1, 2]. Interestingly, certain metabolites and neurotransmitters were also shown to be transported by OCTs, in particular the biogenic monoamines dopamine (DA), serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE), epinephrine and histamine [3–8].

Aminergic neurotransmitters modulate fundamental integrated behaviors related to mood, aggression, attention, motor function, motivation and reward. An important step in the control of the action of monoamines at the synapse is their rapid reinternalization into the presynaptic terminals by high-affinity reuptake transporters, DAT, NET and SERT, for DA, NE and 5-HT, respectively [9]. In agreement with this fundamental role in amine clearance, the high-affinity transporters are the principal targets of potent psychoactive drugs including psychostimulants and several commonly used antidepressants. The finding that OCTs can transport monoamines, albeit with lower affinity than the classical reuptake transporters, opened stimulating perspectives for the identification of novel mechanisms modulating central behaviors and therapeutic innovation.

Convincing data concerning the role of OCTs in the central nervous system was obtained only in the last decade. The study of the contribution of these transporters to monoamine clearance *in vivo* in the brain was complicated by their wide substrate specificity and their low-affinity, making difficult to distinguish their activity from that of the high-affinity transporters, much more easily detected. The creation of mouse mutants deficient for these transporters [10–12] was an essential step in the understanding of their role in neurotransmitter clearance in the brain of live animals. Since then, the number of specialized central functions implicating OCTs has been steadily expanding and will probably continue to expand in the coming years. This chapter provides an overview of the general knowledge on the role of OCTs in various behaviors and integrated functions, ranging from salt-intake regulation to mood-related behaviors and addiction.

OCTs Modulate Aminergic Neurotransmission

The OCTs display the low-affinity kinetics and overall characteristics of uptake₂, a catecholamine uptake system detected earlier in peripheral tissues with sympathetic innervation [13, 14]. In contrast with the high-affinity transporters [15], transport by

OCTs is independent of Na^+ and Cl^- ions and highly sensitive to the cyanine dye derivatives disprocynium 24 (D24) and decynium 22 (D22) [16, 17]. The properties of OCT3 were found to resemble most accurately those of uptake₂, in particular regarding sensitivity to corticosteroids and O-methylated catecholamines [5, 6, 8, 18, 19].

Although predominantly expressed in peripheral tissues, two subtypes, OCT2 and OCT3, are expressed in the central nervous system [4, 6, 8, 18, 20–26]. The first hints that these OCTs could be implicated in monoamine transport in the brain originated from studies using selective inhibitors as discriminatory pharmacological tools. By high-speed chronoamperometry, L. Daws and colleagues demonstrated the existence in rat hippocampus of a 5-HT clearance component sensitive to the local application of D22 and corticosterone [27, 28], both previously described to interact with OCTs. Remarkably, this component could be only detected in conditions of pharmacological blockade [27] or genetic [28] invalidation of SERT. In another study, P. Gasser and collaborators showed that the OCT substrate histamine, for which no high-affinity transporter has been identified, could accumulate in rat dorsomedial hypothalamus minces, and that this accumulation was sensitive to corticosterone, 5-HT, estradiol and D22 [29]. By the use of microdialysis, it was also shown that extracellular 5-HT levels in rat medial hypothalamus were notably increased by D22 perfusion [30].

Subsequently, the use of mouse mutants harboring an invalidated OCT2 or OCT3 gene allowed to tease apart the contribution of these transporters in monoamine transport in the brain and in central functions. A number of neurochemical studies confirmed the importance of both these transporters in the modulation of monoamine neurotransmission in the brain with, however, some differences in the nature of the neurotransmitters implicated. The analysis of tissue content of monoamines by high-performance liquid chromatography showed that OCT3 deletion leads to decreased intracellular levels and increased turnover of these neurotransmitters, with a preferential impact on DA content and metabolism compared to the other monoamines [24]. In rats, in agreement with a role of OCT3 in DA clearance, inhibition of OCT3 through infusion of antisense oligonucleotides into the third ventricle was shown to increase extracellular DA levels in nucleus accumbens and prefrontal cortex [31] as well as methamphetamine-induced hyperactivity, a behavioral response relying on the activation of dopaminergic pathways [32]. In contrast, OCT2 was found to be more selective of 5-HT and NE, as supported by a set of neurochemical and electrophysiological evidence. OCT2 deletion was associated with significant reductions in the content in brain tissue concentrations of 5-HT and NE [25], suggesting a role for this transporter in the preferential uptake of these two amines *in vivo*. This hypothesis was further supported by functional studies evaluating monoamine transport activity *ex vivo*, in cell suspensions from highly-expressing brain regions, in conditions where the high-affinity transporters are inhibited. These experiments showed a significant decrease in D22-sensitive 5-HT and NE uptake in OCT2 mutant mice, whereas DA uptake appeared less affected [25]. This decrease in *ex vivo* uptake could not be explained by modifications in the expression of other high or low-affinity transporters, as shown by quantification by ligand binding and immunoautoradiography. Consolidating these findings, combined

microiontophoresis and electrophysiology studies revealed dramatic consequences of OCT2 deletion on monoamine clearance *in vivo* in dorsal hippocampus [25]. These studies showed that the recovery of the firing activity of CA3 pyramidal neurons after 5-HT and NE application was significantly decreased in OCT2-deleted mice treated with the dual 5-HT/NE antidepressant venlafaxine. Besides revealing a key role of OCT2 in monoamine clearance, these findings indicate that this transporter modulates postsynaptic neuron activity *in vivo* when the SERT and NET are pharmacologically inactivated. Taken together, the above studies suggest that OCTs function as an alternate monoamine clearance mechanism in brain areas lacking the high-affinity transporters, at distance from the aminergic nerve endings, or when these high-affinity transporters are saturated or inhibited, as may be the case after antidepressants or psychostimulants administration (Fig. 7.1).

Distribution and Functional Specialization of Brain OCTs

OCT2 and OCT3 show a similar expression pattern in the brain. As expected for monoamine transporters, both are expressed in aminergic nuclei such as raphe, locus coeruleus and tuberomammillary nucleus. However, a distinguishing feature of these low-affinity transporters is their predominant expression in the major brain areas receiving aminergic innervation, like the cortex, hippocampus, thalamus, hypothalamus, amygdala and hindbrain [4, 23–26]. Contrarily to the classical reuptake transporters enriched in nerve terminals, OCT2 and OCT3 are expressed in the cells bodies and processes (of neurons and occasionally astrocytes) in these projection regions [24, 33], thus strategically positioned to internalize monoamines postsynaptically. This potential role in postsynaptic uptake is compatible with the known mode of transmission of monoamines, which may diffuse away from the site of liberation to reach remote targets [34–36] and with the high capacity of OCTs, which may compensate for low-affinity. In agreement with the participation of OCTs in aminergic clearance in physiological conditions, OCT2 and OCT3 knockout mice show diminished monoamine contents in several brain regions compared to wild-type mice, in absence of any pharmacological treatment [24, 25]. Interestingly, a similar decrease in monoamine levels was previously found in DAT, SERT and NET knockout mice brain [37–39].

In spite of their comparable general distribution, OCT2 and OCT3 also show varying expression levels and selective expression within restricted brain areas. Contrarily to OCT2, OCT3 is highly expressed in circumventricular organs (CVO) [22, 23] and in the dopaminergic nuclei substantia nigra [40]. At the cellular level, while both transporters are principally located in neurons, OCT3 was also detected in astrocytes in certain areas such as dorsomedial hypothalamus nucleus and substantia nigra. These subtle differences in expression could reflect the need for selective clearance within an area. As summarized above, OCT2 seems to prefer 5-HT and NE *in vivo* [25], while OCT3 appears more specialized for DA in several brain areas [24] except in dorsomedial hypothalamus nucleus [29]. Differential OCT expression could thus allow a precise tuning of clearance according to the

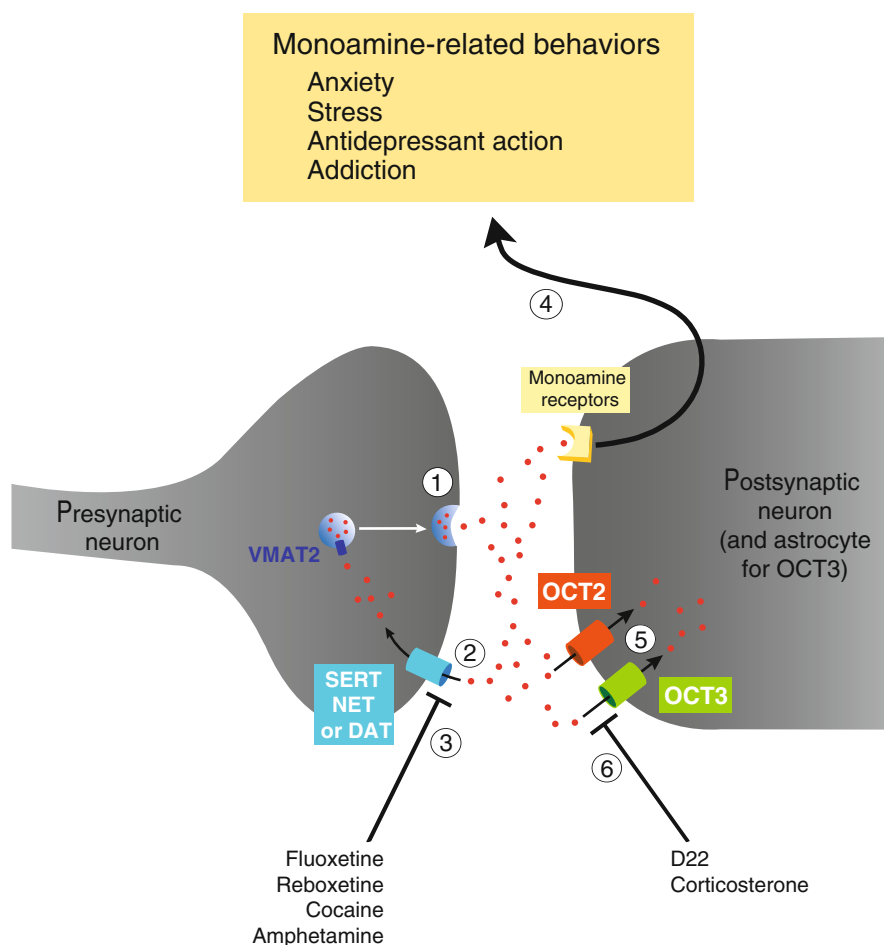
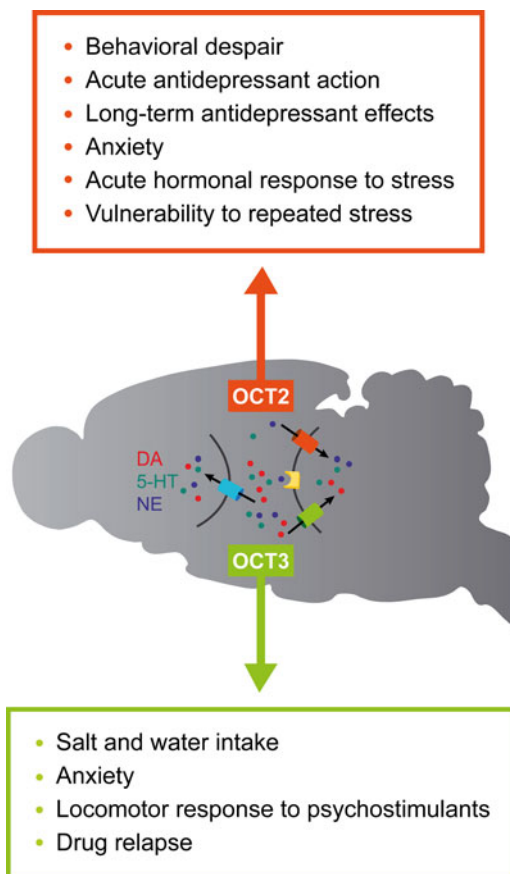


Fig. 7.1 Schematic representation of the role of OCTs in monoamine clearance in the brain. Monoamines (5-HT, NE or DA) are transported into the synaptic vesicles by the vesicular transporter 2 (VMAT2) and released in the synaptic cleft by exocytosis (1). High-affinity uptake transporters (SERT, NET and DAT) control the clearance of extracellular monoamines by ensuring their reinternalization into the pre-synaptic terminals (2). Pharmacological inhibition of these high-affinity uptake transporters by antidepressants and psychostimulants (3) increases the extracellular concentration of monoamines, modulating central behaviors (4). OCT2 and OCT3 (5) located on proximal or distal post-synaptic sites contribute to clearance when the high-affinity transporters are saturated, inhibited or absent. OCT blockers such as decynium 22 (D22) and corticosterone (6) potentiate the neurochemical and behavioral effects (4) of high-affinity transporter inhibitors (3).

nature of the aminergic afferent fibers received by the brain regions. A body of work over the last years has revealed the role of the brain OCTs in a number of integrated functions (Fig. 7.2). Although the roles of OCT2 and OCT3 are far from being fully understood, these studies implicate these two subtypes in somewhat different behaviors, which may be determined by their anatomical and functional specialization.

Fig. 7.2 Schematic summary of the roles of OCTs in central behaviors in rodents. OCT2 (in *red*) and OCT3 (in *green*) participate in monoamine (DA, 5-HT and NE) clearance in the brain in addition to the high-affinity transporters, thereby modulating their extracellular levels. OCT2 was shown to be implicated in behavioral despair, acute and long-term antidepressant effects, anxiety, and stress response and vulnerability. OCT3 was shown to be implicated in salt and water intake, anxiety, locomotor response to psychostimulants and drug abuse relapse



Role in Integrated Behaviors

Salt and Water Intake

One of the earliest demonstrations of the role of OCT3 in central functions directly proceeds from its peculiar distribution. This transporter is highly expressed in CVOs such as area postrema and subfornical organ (SFO), structures located at the blood–brain interface and controlling fluid exchange and osmotic homeostasis [23]. This initial observation led to challenge the role of OCT3 in the processing of osmotic information in the brain. Concerted sodium and water balance regulation is a complex function implicating osmoreceptors for blood-borne information located in the sensory CVOs and secondary neural circuits in forebrain and hindbrain, all interconnected. In agreement with the expression profile of this transporter, mice lacking OCT3 show an abnormal salt ingestion behavior, in particular increased preference for hypertonic saline under sodium depletion and water deprivation conditions.

Furthermore, induction of the c-Fos protein was established as a reliable indicator of neural activation in osmosensitive and relay regions. Specifically, modifications of this functional response in mice exposed to salt deprivation can be detected by quantitating the number of c-Fos expressing cells in these structures. Under conditions of salt deprivation, c-Fos induction in the SFO, where OCT3 is highly expressed, was found significantly decreased in OCT3-deficient mice in comparison with wild-type animals, reflecting a blunted neural response in this structure [23]. This study demonstrates that OCT3 modulates the neural and behavioral responses to variations in osmolarity in the environment. However, how OCT3 achieves this modulation remains to be determined. The SFO neurons themselves are sensitive to variations in extracellular osmotic pressure, presumably through the activation of ionic channels, but salt-intake is also subject to extensive control by secondary neural circuits and neuroendocrine signaling. Based on its fundamental properties, it seems unlikely that OCT3 could be implicated in osmolarity sensing per se, but rather that it could play a role in modulation of the primary neural response, either within the SFO or by afferent connections [23]. Several forebrain regions implicated in relaying osmoregulatory responses express OCT3 at a low level. Thus, OCT3 could also be required for activation of secondary neural circuits participating in the response, or for correct neurotransmission between osmosensitive structures and these relay regions [23]. The CVOs and in particular the SFO have been proposed to participate in sympathetic-mediated hypertension [41]. It will be interesting to determine in the coming years whether OCT3 function may influence blood pressure in various conditions and possibly predisposition to hypertension, as suggested for other components controlling both osmolarity sensing within CVOs and salt-intake behavior [42].

Mood-Related Behaviors

Anxiety

The role of the OCTs in mood was intensively studied, instigated by the fact that these transporters could transport biogenic amines. Alterations in anxiety-related behaviors were found in mice mutants for OCT2 [25] or OCT3 [24, 43], as expected from the involvement of aminergic neurotransmission pathways systems in these behaviors [44]. OCT2 knockout mice showed decreased anxiety-related behavior in two distinct paradigms, the elevated O-maze and the novelty-suppressed feeding test [25]. This anxiolytic phenotype was confirmed in the open-field, with the mutants showing significant increases in the time and spontaneous locomotor activity in the center of the field, and no change in activity at the periphery [25]. Contrasting with this clear phenotype, inconsistent observations were found after OCT3 genetic deletion, with decreased anxiety levels found in one mice line [43] and increased anxiety levels found in another [24]. The causes for this discrepancy are unclear but might be linked to the genetic background of the mice lines, a

parameter that may influence the expression of diverse molecules participating in aminergic signaling. In particular, the existence of eventual variations in the expression of monoamine uptake transporters was not explored in the two mice lines. The activity of the high-affinity 5-HT reuptake transporter, SERT, may for instance modulate anxiety-related behaviors [45]. In spite of these contradictions, these results point to a role of OCT3 activity in anxiety-related behavior in mice. Reinforcing this possibility, an OCT3 variant with decreased NE transport capacity was identified in patients with the anxiety-related obsessive-compulsive disorder [46, 47].

Stress Response and Vulnerability

Several studies demonstrate that the brain OCTs modulate the physiological response to stress in mammals, although the exact mechanisms of this action remain unclear. As mentioned earlier, OCTs expressed in heterologous systems can be inhibited by the stress hormone corticosterone, contrarily to the high-affinity monoamine transporters [5, 6, 19, 48, 49]. These observations laid the basis for the idea that interactions between OCTs and corticosterone could somehow play a part in the biological processes occurring during stress. One of the brain OCTs, OCT2, was detected in brain regions implicated in the response to stress, like the prelimbic and infralimbic cortices, hippocampus, amygdala, dorsomedial and arcuate nuclei of hypothalamus, paraventricular nucleus of the thalamus and pituitary, as well as in the adrenals [25, 26]. The above-mentioned circuits have been shown to modulate, through direct and indirect connections with the paraventricular nucleus (PVN) and periPVN regions, the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, which culminates in corticosteroid secretion [50, 51]. In agreement with this localization, OCT2 was found to profoundly modulate the hormonal response to acute stress. In particular, OCT2 knockout mice showed important increases in circulating corticosterone concentrations following acute stress, as well as modifications of HPA axis function at basal state [26].

Interactions between genetic and environmental factors like exposure to stress play an important role in the pathogenesis of mood-related psychiatric disorders, including major depressive disorder and post-traumatic stress disorder [52, 53]. In rodents, chronic stress paradigms such as unpredictable chronic mild stress (UCMS) can mimic these noxious effects, inducing progressive neurobiological and behavioral anomalies resembling symptoms of human depression [54, 55]. It was thus hypothesized that their increased hormonal response to acute stress could confer to OCT2 knockout mice vulnerability to repeated stressful conditions. Indeed, during UCMS, the OCT2 mutants developed more intensely than wild-type mice several depression-related phenotypes involving self-care, social interaction, spatial memory and stress-sensitive spontaneous behavior [26]. Along with these behavioral anomalies, the mutants also showed significant variations of brain glycogen synthase kinase-3 β (GSK3 β) signaling, an intracellular pathway highly sensitive to stress [26, 55]. Interestingly, GSK3 β signaling anomalies have also been associated

with major depression [56] and depressive-like behavior in rodents [57]. Taken together these data indicate that in rodents OCT2 can directly modulate the molecular and hormonal events occurring during acute stress and attenuate the harmful consequences of chronic stress. These biological processes may not be without consequences for human disorders. A prediction, which remains to be tested, is that pharmacological or genetic inhibition of OCT2 could enhance vulnerability to repeated adverse events, leading to stress-related disorders. This could occur for instance during prolonged treatment with antivirals, cytostatics and antidiabetics inhibiting OCTs [58, 59], or in individuals with genetic polymorphisms in the OCT2 gene affecting transport activity.

While the above-mentioned experiments illustrate that the OCTs exert a control over corticosterone release in the blood stream, other studies also suggest that the reverse may also hold true and that this hormone could reciprocally impair OCT-mediated clearance *in vivo*. In support of this possibility, the influx of histamine in dorsomedial hypothalamus nucleus minces was shown to be inhibited by corticosterone [29]. Since to date no high-affinity transporter for histamine has been identified and this amine is selectively transported by OCT2 and OCT3, the authors of the study suggested that during acute stress direct inhibition of OCTs by corticosterone could increase extracellular monoamine levels in this brain area [29]. A similar mechanism was proposed by Baganz and collaborators, who demonstrated by cyclic voltametry the existence of a corticosterone-sensitive 5-HT clearance component in hippocampus of SERT knockout mice [28]. Repeated swim stress was also found to attenuate 5-HT clearance in the same brain region in normal but not adrenalectomized mice, demonstrating that endogenous corticosterone release was implicated [60]. Moreover, supporting the implication of OCTs, repeated swim stress was found to decrease histamine clearance in hippocampus [60]. Blockade of the OCTs, rather than regulation of expression of this specific transporter, was proposed to be the underlying cause of this decrease in histamine transport, since it was associated with only a slight decrease in protein OCT3.

The involvement of OCT2 was not investigated in the latter study, yet recent observations suggest that monoamine uptake mediated by this transporter as well could be affected by corticosterone in physiological conditions [26]. *In vivo* microiontophoretic electrophysiology was used to assess the consequences on hippocampal monoamine clearance of the systemic administration of corticosterone. In this experimental set-up, subchronic corticosterone administration had an inhibitory effect on 5-HT and NE clearance in presence of venlafaxine, a dual SERT and NET inhibitor, in the hippocampus of wild-type mice but not OCT2 knockout mice. Altogether, these studies suggest that endogenous corticosterone in physiological conditions may modulate the activity of OCT2, OCT3 or both in certain brain areas. Such a mechanism could contribute to the biological processes underlying the response to stress, by directly impacting on aminergic signaling. In the case of OCT2, modulation of its activity by corticosterone would also provide a novel feed-forward mechanism by which this hormone could enhance its own release in the blood stream. However, direct interaction between OCT and corticosteroids *in vivo* still remains to be demonstrated. Indeed, corticosterone induces a number

of alterations in the brain [61] which could influence by diverse other mechanisms the expression or the activity of low or high-affinity transporters at the plasma membrane and account for the observations reported above.

Antidepressant Response and Efficacy

A number of antidepressants traditionally used to treat depression modulate 5-HT and NE neurotransmission, especially the activity of the high-affinity reuptake transporters. This has provided the underlying rationale for the long-lived “monoamine” hypothesis, which stipulates that toning-down of 5-HT and NE signaling occurs during depression, which may be restored by long-term antidepressant treatment [62]. Mainstream antidepressants do not however provide positive treatment outcomes for all patients. In this context, low-affinity monoamine transporters of the brain such as the OCTs represent promising novel pathways to develop alternate therapeutic approaches.

Some of the biological processes that underlie the effects of antidepressants can be investigated in detail using animal models. Behavioral despair paradigms like the tail suspension (TST) and the forced-swim (FST) tests have been used during decades to measure what is commonly called “depressive-like” behavior. These tests evaluating immobility during exposure to inescapable stress show high predictive value for evaluating antidepressants efficacy. However, they do not reflect symptoms of depression per se but the state of resignation of the animals [63]. The OCT inhibitor D22 was shown to decrease immobility in the TST when applied in the hippocampus of SERT-deficient mice [28], suggesting that brain OCTs could control this behavior. Likewise, intracerebral infusion of an OCT3 antisense RNA to reduce OCT3 function was shown to decrease immobility in the same paradigm [32]. OCT2 knockout mice, on the other hand, showed a marked increase in immobility in the TST and the FST [25], suggesting an opposite role to OCT3 for this transporter. Unraveling the neurochemical mechanisms underlying these contrasting roles may however prove to be difficult, since multiple neurotransmission pathways and components in the brain can influence performance in these rudimentary paradigms [64].

Both OCT2 and OCT3 appear to modulate the acute action of 5-HT/NE reuptake antidepressants on behavioral despair. Infusion of antisense OCT3 RNA in mice brain was found to increase the anti-immobility action of imipramine in the FST [32]. This observation implicating OCT3 in acute antidepressant action contrasts with another study, which proposed this transporter was not implicated in the effects of three antidepressants, fluvoxamine, fluoxetine and desipramine in the TST [60]. In the latter study, however, OCT3 blockade was tentatively achieved by repeated swim-stress and not monitored, and only one dose of antidepressant was tested, which may explain this discrepancy. Supporting a role of OCTs in constraining the short-term antidepressant action, normetanephrine, a NE metabolite and potent inhibitor of uptake₂, was shown to accentuate the releasing effects of venlafaxine on extracellular NE in rat cortex, as well as on immobility in the FST [65]. A similar effect was found in mice with D22, which was shown to increase the anti-immobility action of a subactive dose of fluvoxamine in the TST [66].

OCT2 as well seems to be involved in the short-term effects of 5-HT/NE antidepressants [25]. OCT2 knockout mice showed compared to wild-type mice striking modifications in the effects in the FST of the dual 5-HT/NE reuptake inhibitor venlafaxine and of the SERT- and NET-selective reuptake inhibitors, citalopram and reboxetine. In particular, genetic deletion of OCT2 increased sensitivity to the anti-immobility action of low doses of each of these antidepressants. This genetic deletion also had complex consequences on the action of higher doses of the same antidepressants, such as increasing the anti-immobility action of reboxetine and decreasing that of citalopram [25]. These observations could be related to the way each antidepressant modulates given aminergic neurotransmission pathways and how OCT2 disruption interferes with these pathways in different brain regions. Additional investigations will be needed to clarify these fine mechanisms.

As mentioned earlier, the tests evaluating resignation used in the above-mentioned studies fall far from reproducing the symptoms of depressive disorders. Furthermore, immobility in the FST or TST in rodents is influenced by the pharmacological blockade or genetic invalidation of numerous components controlling central neurotransmission [64]. Therefore, despite their complexity, well-validated long-term models of depression such as UCMS represent invaluable tools to investigate the complex neurobiological anomalies underlying this disorder. Another relevant paradigm, chronic corticosterone exposure, was used to gain insight into the role of OCT2 in antidepressant response. As for UCMS, rodents exposed to chronic corticosterone develop anhedonia, increased anxiety and social aversion [25, 67]. As human depression, these stable alterations induced by corticosterone may be reversed by long-term but not acute antidepressant treatment. In these experiments, chronic corticosterone administration induced depression-like anomalies in both wild-type and OCT2 knockout mice [25]. As expected, the administration of the dual antidepressant venlafaxine in a second phase reversed the anomalies induced by chronic corticosterone in wild-type mice. In contrast, the anomalies developed in OCT2 knockout mice remained insensitive to venlafaxine treatment, demonstrating that this transporter was essential for long-term antidepressant efficacy [25].

This long-term effect contrasts strikingly with the role of OCT2 in the FST. The explanation for this apparent contradiction may reside in the fact that the beneficial effects of antidepressants, which appear only after several weeks of treatment, involve different mechanisms than their short-term action. Specific neurotrophic and neurochemical processes occur during long-term treatments with NE and 5-HT/NE antidepressant [67, 68], for instance the desensitization of 5-HT_{1A} autoreceptors [69] and the decrease in the firing of NE neurons in the locus coeruleus [70, 71]. In addition, OCT2 knockout mice show constitutive alterations in the expression of 5-HT_{1A} receptors and of the SERT, which may also participate in long-term resistance to venlafaxine [25]. In the future, a better understanding of the neurochemical mechanisms underlying the role of OCT2 in antidepressant action may be gained by the investigation of the firing properties of raphe (5-HT) and locus coeruleus (NE) neurons and the activation state of monoamine receptors in these pathways. In any event, these findings emphasize the importance of OCT2 in the long-term antidepressant action. Since OCT2 appears to have a similar distribution in human and in rodent brain [4], it may be speculated that variations in the activity

of this transporter in humans might contribute to antidepressant resistance and, importantly, that antidepressant efficacy should be amenable to modulation by manipulation of this activity.

Addiction

DA is one of the central players in the effects of drugs of abuse. All psychoactive drugs increase DA release in the striatum and nucleus accumbens, an action mediating in part their locomotor and addictive properties [72]. In agreement with its involvement in DA signaling in the nigrostriatal and mesolimbic pathways, OCT3 has been implicated in the locomotor response to psychostimulants and in addictive behavior. In a study by Kitaichi and co-workers, intracerebral infusion of OCT3 antisense RNA in mice was found to increase the locomotor response to methamphetamine [32]. This attenuating action of OCT3 on drug-induced locomotion was confirmed in another study using OCT3-deficient mice, which showed enhanced response of the psychostimulants amphetamine and cocaine at high doses, supporting a role for this transporter when the high-affinity transporters are inhibited [24].

OCTs were also recently shown to be involved in the effects of stress on addictive behaviors. Chronic stress is a well-known risk factor for the development of addiction. In particular, craving in presence of drug-associated stimuli is perceived as increased by drug addicts during periods of stress [73], an effect that can be reproduced in rodents. In an exciting study, Graf and colleagues demonstrated that foot-shock stress could enhance cocaine-induced reinstatement of drug seeking behavior in rats [74]. This enhancing action of stress on reinstatement of drug seeking, found with a subthreshold dose of cocaine, was dependent upon corticosterone secretion, as shown by adrenalectomy, and mimicked by intra-accumbens infusion of either corticosterone or the uptake₂ blocker normetanephrine. A set of complementary experiments indicates that these effects of stress involve blockade of DA clearance in the nucleus accumbens. These findings identify a novel mechanism by which corticosterone enhances the effects of cocaine on DA transmission by decreasing OCT-mediated clearance in the nucleus accumbens. Consistent with the implication of this mechanism in drug relapse in humans, a previous study reported a correlation between single nucleotide polymorphisms in the OCT3 gene and the development of polysubstance use in patients with methamphetamine dependence [75].

Drug Action at Brain OCTs

A large proportion of therapeutic drugs is composed of organic cations, a number of which can interact with OCTs, at least in heterologous expression systems [2]. Molecules acting on central nervous system with the potential to inhibit OCTs include antidepressants [8, 21, 76–79], psychostimulants like cocaine, amphetamine

and 3,4-methylenedioxy-methamphetamine (MDMA) [8, 80] and NMDA receptor antagonists, like phencyclidine, MK-801, ketamine, memantine and amantadine [4, 80, 81]. Several classes of adrenergic ligands also functionally interact with both OCTs and α -adrenoceptors, two families that share close anatomical and functional relationships. The potent cyanine-related OCT inhibitors behave as α -adrenoceptor antagonists and, reciprocally, a number of other α - and β -adrenoceptor agonists and antagonists inhibit diverse OCT subtypes [2, 19, 82]. This promiscuity between pharmacophores recognizing α -adrenoceptors and OCTs raises the possibility that some adrenergic ligands could act concomitantly on OCT-mediated monoamine uptake and at α -adrenoceptors in sympathetically-innervated tissues or the CNS, either in a cooperative or opposite manner. Finally, various neurotoxins such as *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) [82], 1-methyl-4-phenylpyridinium (MPP⁺) [33, 83] and paraquat [84] have also been shown to interact with the OCTs. For the two latter, it was shown that these interactions underlie in part their neurotoxic effects in the brain [33, 84].

Besides compounds intended to act on CNS, various drugs aimed at peripheral targets could also influence central OCT activity. In this line we can mention the antacid cimetidine [85], the antimalarial drug quinine [86], the antiviral drugs acyclovir, lamivudine, abacavir and azidothymidine (AZT) [58, 87], antidiabetics like metformin [59], cytostatics like cisplatin [88] and the antispasmodic trospium [89]. Whether these different categories of therapeutic drugs inhibit OCTs in the brain remains uncertain, considering their low affinity and the high first-pass effect of organic cations [90]. Several lines of data support however this possibility. First, higher concentrations of some of these drugs in the brain compared to circulation could allow local interaction with the brain OCTs [77]. Second, high-affinity binding sites for some of these compounds may have gone undetected in OCTs, as documented for abacavir and AZT [87]. Third, most inhibition constants at OCTs were defined previously using artificial and not endogenous substrates, but these constants seem to vary depending on the substrate tested [91]. Finally, the affinity of these ligands for OCTs could be influenced differentially by the environment *in vivo* than *in vitro* and thus differ *in vivo* from those established in heterologous expression systems. The identification of interactions between the above-mentioned drugs and OCTs *in vivo* is an arduous task, complicated by the multiplicity of their targets and actions. If these interactions do occur in the brain, the administration of these molecules could on the long-term affect central neurotransmission, leading to unexpected and potentially undesirable secondary effects.

Conclusion and Perspectives

In recent years, a substantial body of evidence has accumulated suggesting that OCTs function as an alternate monoamine clearance system in the brain, in addition to the high-affinity monoamine transporters. These studies have disclosed that this transporter family participates in a variety of integrated central functions in rodents,

ranging from salt-intake regulatory behavior to stress response and antidepressant efficacy. It can be hypothesized that genetic and epigenetic-based variations in the activity of the brain OCTs in humans could influence individual predisposition to various disorders such as hypertension, anxiety and depression as well as the response to antidepressant therapies. Another indirect consequence of these findings might be that prolonged treatments with routinely used therapeutics could increase vulnerability to the above-mentioned disorders, through interaction with these low-affinity transporters. In the future, the design of molecules capable of modulating selectively the activity of OCTs may lead to the development of novel therapeutic strategies in several clinical fields, in particular for the treatment of mood disorders.

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Chapter 8

Organic Cation Transporters as Modulators of Neurodegeneration and Neuroprotection in the Brain

Kim Tieu

Abstract The organic cation transporters have emerged as important regulators in human health and diseases. This chapter will discuss the latest studies demonstrating how a sub-group of these transporters, the organic cation transporter-3 (OCT3), would modulate neurodegeneration and neuroprotection in the brain. These roles of OCT3 have been documented in the dopaminergic nigrostriatal system, a pathway that is affected in Parkinson's disease (PD). Three experimental models of nigrostriatal neurotoxicity will be discussed to illustrate how OCT3 modulate cell viability. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, previously discovered to cause parkinsonism in a group of drug addicts) mouse model, blocking OCT3 function is neuroprotective. However, OCT3 deletion enhances neurotoxicity in the paraquat (a herbicide that increases the risk of developing PD) and methamphetamine mouse models. These observations are consistent with the ability of OCT3 to bi-directionally transport these toxins and consistent with the expression pattern of OCT3 in the brain. Polymorphisms of OCT3 have been reported in humans and this transporter is suggested to be a susceptibility gene contributing to PD.

Keywords Parkinson's disease • Neurodegeneration • Neurotoxicity • Neuroprotection • Organic cation transporter-3 • Astrocytes • Paraquat • MPTP • Methamphetamine • Dopamine

Introduction

Neurodegeneration is a common feature in neurodegenerative disorders, which encompass a large group of diseases with heterogeneous clinical and pathological features. Hundreds of different neurodegenerative disorders have been identified.

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However, the most prevalent and publicized ones are Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis. These diseases are characterized by a relatively selective loss of a population of neurons in specific brain regions. Although mutated genes have been discovered in subsets of patients with these diseases, the mechanisms of neurodegeneration leading to specific cell loss in these disorders still remain largely elusive. Understanding the mechanisms by which neurons die is critical to developing effective therapeutic strategies for neurodegenerative diseases.

Major efforts have been made over the past decades to understand the precise mechanisms of neurodegeneration. From these intensive investigations, emphasis has been placed on pathogenic mechanisms such as bioenergetic defects, neuroinflammation, impaired autophagic or proteasomal protein degradation, and oxidative stress. However, against the backdrop of these studies are the observations that transporters also play an important role in modulating neurodegeneration. Recent studies highlight such an emerging function of the organic cation transporters (OCTs) [1–3]. So far, studies of neurodegenerative diseases have documented primarily the involvement of the organic cation transporter 3 (OCT3) in experimental models of Parkinson's disease (PD). However, it is worth noting that in the peripheral nervous system, OCT2 has been demonstrated to play a critical role in oxaliplatin-induced neurotoxicity in the dorsal root ganglia. Mice with OCT2 deletion are protected from acute onset of peripheral neuropathy caused by this anticancer drug [1]. To keep the discussion focused, the content of this chapter will be confined largely in the context of OCT3 and PD. First, some major features of PD will be briefly reviewed, followed by an overview of proposed transporters-related mechanisms of neurodegeneration in PD. Lastly, the evidence for the involvement of the OCT-3 in PD will be presented. Where relevant, how these transporters might be targeted for neuroprotection will also be discussed.

Parkinson's Disease and Transporters-Mediated Neurodegeneration

PD is the second most common chronic neurodegenerative disorder, after Alzheimer's disease. It has been estimated that up to ten million people worldwide are affected by PD. This movement disorder is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta, leading to a reduction of dopamine (DA) in the putamen [4]. When this putamenal DA is depleted by about 80 %, the symptoms of PD emerge [4]. Abnormal movements such as resting tremor, rigidity, and postural instability are common in PD patients.

The cause(s) of the majority of PD cases remains unknown. Currently about 10 % of PD cases are directly linked to monogenic mutations, resulting in autosomal dominant (*SNCA*, *LRRK2*) and recessive (*Parkin*, *DJ1*, *PINK1* and *ATP13A2*) PD. Environmental toxicants have been hypothesized to play a role based on the observations that parkinsonism can be caused by postencephalitic infection [5, 6],

by accidental injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [7], and by chronic manganese exposure [8]. Supporting this view are epidemiological studies reporting that rural living, exposure to herbicides (such as paraquat), pesticides, and heavy metals increase the risk of PD [9–14]. To study neurodegeneration in PD, both genetic and toxin-induced models have been created. Potential pathogenic mechanisms of selective vulnerability of the nigral dopaminergic neurons in PD have been proposed—including transporters mediated neurotoxicity.

An early indication that transporters might be involved in the neurodegenerative process of PD is demonstrated in the MPTP model. This model originates from the discovery in the early 1980s when several Californian intravenous drug users were admitted to hospitals exhibiting severe symptoms similar to PD [7, 15]. Further investigations revealed that these patients had self-administered synthetic meperidine contaminated with MPTP [7]. Since then, the mechanism of MPTP toxicity has been well characterized [4, 16]. Briefly, because MPTP is lipophilic, it can rapidly cross the blood–brain barrier. In astrocytes, MPTP is metabolized by monoamine oxidase-B and subsequently converted to the active toxic cation 1-methyl-4-phenylpyridinium (MPP⁺). As further discussed below, MPP⁺ is released from astrocytes into the extracellular space [2, 17] where it is taken up by the neighboring dopaminergic neurons and terminals through the dopamine transporter (DAT, SLC6A3) to subsequently induce neurotoxicity. It is well established that DAT-mediated transport is an obligatory step for MPTP toxicity in the nigral dopaminergic neurons. Inactivation of this transporter, either pharmacologically or genetically, prevents dopaminergic death induced by MPTP [18, 19].

Another transporter that has been demonstrated to modulate MPTP toxicity is the vesicular monoamine transporter 3 (VMAT2, SLC18A2). VMAT2 transports neurotransmitters such as DA, serotonin and norepinephrine from the cytosol into synaptic vesicles. Relevant to MPTP toxicity, VMAT2 confers neuroprotection by removing MPP⁺ from the cytosol through vesicular sequestration of this toxic molecule [20]. Thus, differential expression of VMAT2 in different cell types is also suggested to contribute to the selectivity of cell death in the MPTP model. Indeed, the ratio of DAT to VMAT2 seems to correlate with the magnitude of neurodegeneration. Consistent with this, the putamen, the brain region most severely affected by both MPTP and PD [20], has a higher ratio of DAT to VMAT2 than the less affected caudate region.

Although the ratio of DAT/VMATM2 has been demonstrated to modulate neurodegeneration in the nigrostriatal pathway, it does not fully address the lack of toxicity in other brain regions that can uptake MPP⁺ through other transporters. Perhaps, there is another variable that has not been adequately assessed. That is, what is the proportion of MPP⁺ available to other brain regions that are resistant to MPP⁺ toxicity? In other words, is MPP⁺ distributed evenly between these brain regions and the substantia nigra? A lack of or a low level of MPP⁺ in these less affected brain areas could contribute to their resistance. Consistent with this argument, when rats are injected with an equal dose of MPP⁺ directly into the substantia nigra, locus coeruleus, and raphe nuclei, significant damages are reported in all these monoaminergic nuclei [21]. Although much has been learned about MPTP since its discovery, some

basic but crucial questions about its mechanism of neurotoxicity still remain unanswered. For example, by what mechanism does this positively charged and membrane impermeant MPP⁺ metabolite leave astrocytes? Why are astrocytes, the originating and supplying source of MPP⁺, resistant to this toxicity but nigral dopaminergic neurons are not? How does MPP⁺ confine its toxicity primarily to the nigrostriatal system when other catecholaminergic systems also have affinity for this cation? As discussed below, OCT3 provides further insights into these questions.

OCT3 Expression in the Brain

There are two types of re-uptake transporters: neuronal and non-neuronal. Neuronal uptake transporters, also referred to as “uptake 1”, are expressed predominantly on nerve endings and they are Na⁺ and Cl⁻ dependent. This type of transporter has a high affinity for monoamines. DAT, for example, belongs to this group. “Uptake 2” transporters, on the other hand, are Na⁺ and Cl⁻ independent. These are low affinity, high capacity transporters for monoamines. The OCTs belong to the “uptake 2” group. Currently, there are three major OCTs: OCT1, OCT2 and OCT3. Excellent reviews of these transporters have been discussed elsewhere [22, 23] and in other chapters of this book and therefore, will not be covered here. Assessment of regional distributions of OCTs shows that these transporters, in general, are expressed most abundantly in the peripheral organs, especially in the kidney and liver [24–26], supporting their role in the bi-directional transport of endogenous and exogenous cationic compounds in and out of these organs. In the brain, OCT3 is expressed in most neurons [2, 27, 28]. Interesting, various *in vitro* and *in vivo* studies indicate that OCT3 is expressed quite selectively in rodent [2, 27–30] and human [2, 31–33] astrocytes.

Substrates of OCT3

OCT3 is termed “polyspecific” because of a wide range of both endogenous and exogenous cationic molecules that it recognizes. In addition to catecholamines, OCT3 also transports other endogenous substrates such as DA, adrenaline, nor-adrenaline, serotonin, histamine, choline and guanidine [25, 34, 35]. At physiological pH, the nitrogen moiety of these endogenous bioactive amines bears a net positive charge. A wide range of exogenous compounds, with a positive charge at physiological pH, are also substrates for this transporter. One compound of particular interest is MPP⁺ [25, 34, 35]. MPP⁺ is an excellent substrate for OCT3, with K_m values in the micromolar range and V_{max} values in the nanomole to picomole (per mg protein per min) range, depending on cell type [25, 31, 32, 36, 37]. This uptake is saturable and it follows first order kinetics. Interestingly, OCT3 is also capable of transporting MPP⁺ out of the cell. This bi-directional transport capability of OCT3 plays significant role in modulating neurodegeneration as discussed below.

MPTP-Induced Neurodegeneration via OCT3 on Astrocytes

Astrocytes represent a major group of cells in the mammalian brain and they are in close contact with neuronal structures. Astrocytes have increasingly been recognized to actively play critical roles in maintaining normal physiological functions as well as in aggravating the pathological state of neurons. The neuroprotective and neurodegenerative roles of astrocytes depend largely on the molecules that they release into and uptake from the extracellular space—the microenvironment that astrocytes and neurons share. For example, it is well documented that astrocytes can release and supply neurons with neurotrophic factors and metabolic substrates such as lactate for the survival and proper function of neurons. Astrocytes also confer neuroprotection by removing excess extracellular excitotoxic agents such as glutamate, potassium and calcium. On the other hand, when astrocytes undergo a state of gliosis in response to neuronal injury or toxic insults, together with microglia, they release cytokines and chemokines that are deleterious to neurons.

As discussed above, various *in vitro* and *in vivo* studies indicate that OCT3 is expressed quite selectively in rodent and human astrocytes. These observations suggest that astrocytes may play a dual role in modulating neurodegeneration by regulating the levels of toxic cations available to neurons. Indeed, the expression of OCT3 in nigrostriatal astrocytes has been demonstrated to modulate neurodegeneration in the MPTP model using mice with *OCT3* deletion (*OCT3*^{-/-}) [2]. This study demonstrates that, first, the bi-directional transport of MPP⁺ mediated by OCT3 was validated in primary astrocytes cultured from *OCT3*^{+/+} and *OCT3*^{-/-} mice. Second, to evaluate the role of OCT3 in releasing MPP⁺ to induce dopaminergic neurodegeneration, adult *OCT3*^{+/+} and *OCT3*^{-/-} littermates were injected with MPTP. As discussed above, in astrocytes, MPTP is converted to MPP⁺ by monoamine oxidase B. The MPTP mouse model therefore allowed a good opportunity to evaluate the significance of the release of toxic MPP⁺ through OCT3. The results clearly demonstrated that dopaminergic neurotoxicity was significantly attenuated in MPTP-treated *OCT3*^{-/-} mice as compared to their littermate counterparts. *In vivo* microdialysis data confirmed that the extracellular levels of striatal MPP⁺ was indeed lower in the *OCT3*^{-/-} mice, suggesting the release of MPP⁺ from astrocytes was reduced in these animals. Third, regular mice treated with decynium 22 (an OCTs inhibitor) also exhibited less cell death in a dose-dependent manner. All together, this study shows that blocking the release of a toxic molecule released by astrocytes through OCT3 can prevent neurodegeneration in the nigrostriatal pathway.

Paraquat-Induced Neurodegeneration Is Modulated by OCT3

The potent herbicide paraquat (PQ, *N,N'*-dimethyl-4-4'-bipyridinium) is a divalent cation but can undergo redox cycling with cellular diaphorase and NAD(P)H-dependent oxidoreductase such as NADPH oxidase and nitric oxide synthase [38–40] to yield the monovalent cation PQ^{•+}. From this redox cycle, superoxide is

generated, leading to oxidative stress-related cytotoxicity. Since its introduction to agriculture, there have been several cases of lethal poisoning resulting from ingestion or even from dermal exposure [41]. Until recently, most experimental studies of PQ have been related to its effects on lung, liver, and kidney—as the toxicity induced by this herbicide in these organs is responsible for death after acute exposure. However, the structural similarity of PQ to MPP⁺ (Fig. 8.1), has sparked major interest in this herbicide as a potential environmental parkinsonian toxicant. Consistent with this view are the observations that significant damage to the brain is seen in individuals who died from PQ intoxication [42, 43]. Furthermore, epidemiological studies have suggested an increased risk for PD after PQ exposure [12, 14]. When injected into mice, PQ induces motor deficits and loss of nigral dopaminergic neurons in a dose [44, 45] and age [45, 46] dependent manner. Aside from killing dopaminergic neurons, PQ induces α -synuclein up-regulation and aggregation [47], a neuropathological feature detected in PD patients. This suggests that the PQ model could be useful for some of the key neuropathological features of PD.

In contrast to MPP⁺, PQ has been reported to penetrate the blood–brain-barrier through the neutral amino acid transporter [48, 49]. Consistent with this, PQ has been detected in the brain after intraperitoneal injection [3, 48, 50]. However, once it is inside the brain, it is questionable whether PQ can enter dopaminergic neurons through DAT [51]. Such a study raises the questions regarding the mechanisms by which PQ induces selective dopaminergic neurodegeneration, whether PQ actually needs to enter dopaminergic neurons to induce toxicity, and more importantly, the link between PQ and PD in the absence of this mechanism [52–54]. Interestingly, through the initial investigations of the role of OCT3 in transporting PQ, it was discovered that although in its native divalent cation state, PQ²⁺ could not be transported by OCT3 or DAT, its metabolite monovalent cation PQ⁺ is indeed a good substrate for both of these transporters [3]. This result was generated by two key sets of experiments: Stable cells overexpressing either OCT3 or DAT were incubated with PQ²⁺. After being converted to PQ⁺ extracellularly by either a strong reducing agent (sodium dithionite) or NADPH oxidase on co-cultured microglia, the intracellular accumulation of PQ mediated by OCT3 or DAT was significantly increased. This higher intracellular content of PQ also resulted in higher oxidative stress and cytotoxicity. In *OCT3*^{−/−} mice, PQ injection induced more striatal dopaminergic damage than their wild type littermates. Microdialysis data showed higher extracellular level of PQ in these mutant mice, suggesting a reduced buffering capacity by non-dopaminergic cells that express OCT3 and therefore, more PQ was available for inducing dopaminergic neurons. In contrast to the MPTP model, where the active metabolite MPP⁺ is release from astrocytes for inducing toxicity in

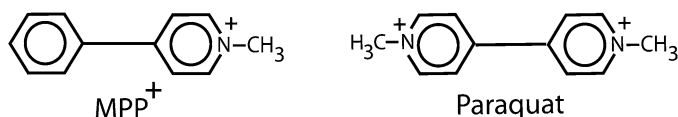


Fig. 8.1 Structural similarity between MPP⁺ and PQ

dopaminergic neurons, PQ^+ is produced extracellularly. Because of this difference, blocking OCT3 function would reduce extracellular levels of MPP^+ and increase PQ^+ levels, respectively. OCT3 therefore can modulate neurodegeneration by either releasing to or removing neurotoxic cation from the target cells. This role of OCT3 is further supported in the methamphetamine model described below.

Methamphetamine-Induced Neurodegeneration Is Modulated by OCT3

Methamphetamine can enter dopaminergic neurons through active uptake by the DAT [55–57]. Once inside dopaminergic neurons, methamphetamine induces DA release from the synaptic vesicles into the cytosol [58] where it is reversely transported by DAT into the synaptic cleft [59]. Combined with its effect on blocking DA degradation by monoamine oxidase [60], methamphetamine induces dramatic release of DA into extracellular space. In vivo microdialysis experiments demonstrate that this release peaks within 1 h after systemic injection [2, 61, 62], leading to acute behavioral changes such as increased locomotor activity [63, 64]. Another negative effect of extracellular dopamine is that such an excess of non-vesicular DA, by inducing oxidative stress through DA-related reactive products [65–67], is instrumental in methamphetamine-induced damage to dopaminergic structures. Consistent with the role of OCT3 in uptaking dopamine and methamphetamine [25, 68, 69], mice with no OCT3 function injected with methamphetamine (or amphetamine) had higher striatal extracellular dopamine level, hyperlocomotor activity [28, 69], and neurotoxicity [2].

Conclusion

The identification of OCTs such as the OCT3 in the brain has provided some novel and significant insights into the process of neurodegeneration: First, they offer additional mechanisms of how toxic organic cations are compartmentalized and distributed to their cell targets, how their concentrations are regulated in the brain, and thereby how they exert their neurotoxic effects. Second, as illustrated in the MPTP and PQ animal models using OCT3-null mice, they highlight the complexity of how different types of transporters (OCT3 and DAT, for example) in different cell types work in a concerted manner to mediate cell viability. Third, because of their bi-directional capability to transport organic cations, OCT3 can regulate the levels of both endogenous and exogenous cations intracellularly and extracellularly, thereby modulating cytotoxicity. Fourth, OCT3 provides additional insights into why some cells are resistant to certain toxic molecules. For example, it has been a topic of debate why astrocytes, the originating and supplying source of MPP^+ , are resistant to this toxicity but the nigral dopaminergic neurons are not. It is possible that this

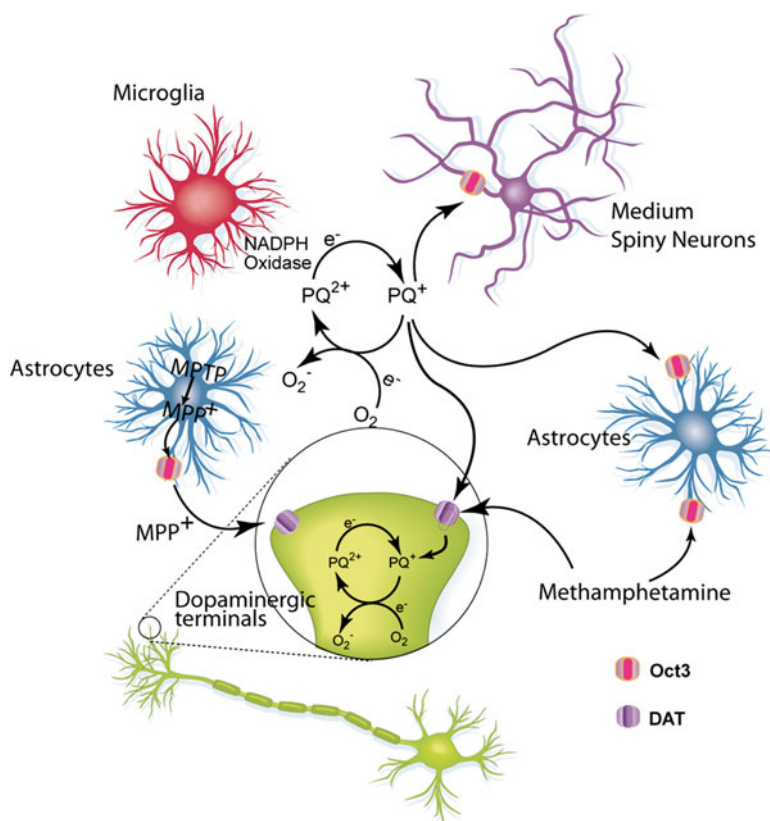


Fig. 8.2 Proposed mechanisms by which OCT3 modulates dopaminergic neurodegeneration and neuroprotection in the brain. As discussed in the main text, because of the differences in the mechanisms of toxicity of various toxins and the expression profile of OCT3, blocking OCT3 function reduces MPTP induced toxicity but increases paraquat (PQ) and methamphetamine neurotoxicity (this figure is reproduced from Rappold et al. [3])

ability of astrocytes to extrude MPP⁺ contributes to the resistance of this cell type to the toxicity of this cationic neurotoxin. Conversely, the lack of OCT3 in nigral dopaminergic neurons partly contributes to the vulnerability of these cells to MPP⁺. Consistent with this hypothesis, in cell lines with overexpression of various mutants of DAT, less cell death induced by MPP⁺ is observed in those with mutant forms of DAT, despite a higher rate of MPP⁺ uptake in these cells. This resistance is attributed to the higher rate of reverse transport of MPP⁺ through these mutant DATs, as compared to that of wild type DAT [70, 71]. Thus, the expression of the bi-directional OCT3 cells, whether on astrocytes or neurons, would likely affect the cell resistance (or sensitivity) to toxicity induced by cations. This ability of OCT3 to modulate cell viability based on the mechanisms of the toxic molecules is illustrated in Fig. 8.2, which summarizes that blocking OCT3 is neuroprotective against MPTP toxicity, but enhances cell death in the PQ and methamphetamine models.

Polymorphisms of OCT3 have been reported in humans [72, 73]. These polymorphisms may affect the function of OCT3 and therefore modulate cell susceptibility to neurotoxins. Importantly, a link between OCT3 and PD has been reported in a genome wide association study [74]. Although modest, significant association between OCT3 (SLC22A3) variants and PD (Odd ratio: 1.21, $p=0.008$) has been detected, suggesting OCT3 belongs to the group of susceptibility genes contributing to PD. Future studies of how these variants affect OCT3 function and modulate neurodegeneration may provide insights into mechanisms of cell death and potential therapeutic targets.

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Chapter 9

Translational Relevance of Animal Models for the Study of Organic Cation Transporter Function

Ivan Sabolić, Davorka Breljak, and Tvrtko Smital

Abstract In the mammalian organs, the members of SLC22 family of organic cation transporters (OCTs) mediate distribution, absorption, reabsorption, and secretion of various endogenous and xenobiotic organic cations. OCTs are also responsible for drug–drug interactions and drug-induced organ toxicity. In translational studies, various animal models have been used to study the OCTs-related pathogenesis of human diseases at molecular and cellular level, and to develop new therapeutic drugs and strategies. Particularly useful in this research proved to be mice with the inactivated genes (knockout mice) for specific OCTs. However, some findings in animal models have an uncertain significance for human conditions and diseases, because specific OCTs in their organs exhibit sex differences in protein and/or mRNA expression and species differences in cellular distribution, substrate selectivity and affinity, levels of mRNA and/or protein expression, sensitivity to inhibitors, and regulation. In comparison with animal models, in the human organs some OCTs are absent, others exhibit different localization in the cell membrane domains and different levels of expression, sensitivity to inhibitors, and rates and regulation of the transport of substrates, and none of the thus far tested exhibited the sex-dependent expression. The data from animal models initiated genetic studies in humans, which revealed that several wellknown conditions and diseases are associated with disfunctional OCTs due to gene polymorphism.

Keywords *Drosophila melanogaster* • Gene polymorphism • Human diseases • Humanized mice • Knockout mice • Organic cation transporters • *Caenorhabditis elegans* • Malpighian tubules • SLC22 family of transporters • Species differences • Sex differences • Translational research • Zebrafish

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Introduction

Translational Research and Animal Models

The basic concept of translational research is indicated by the simple expression “from bench to bedside” (or “from molecule to public health”), which in more complex form means “the multidirectional and multidisciplinary integration of basic, patient-oriented and population-based research, with the long-term aim of improving the health of the public [83]”. To understand the pathogenesis of human diseases at molecular and cellular level, and to provide systems for developing new drugs and testing new therapies, biomedical research is performed using various experimental models. Historically, basic (including preclinical) research performed in various experimental models *in vitro* and *in vivo* has generated a myriad of invaluable information about the cellular, organ-specific, and whole animal physiology, pathophysiology, pathology, toxicology, and pharmacology that can be, with more or less relevance, extrapolated to the situations in humans. In preclinical studies, rodents (mice, rats, hamsters, guinea pigs) and rabbits have been traditionally used as the models for various human health conditions and specific diseases. These models have provided an invaluable source of information about various health- and diseases-related phenomena in humans, which cannot be obtained in experimental models *in silico* or *in vitro*; the overall understanding of human biology in health and disease is largely based on results obtained from experimentation on animals.

Animal Models in Translational Research of Organic Cation Transporters

Since the identification of the first organic cation transporter OCT1 (Slc22a1) by expression cloning from the rat kidney [32], a number of other, ATP-independent, secondary- and tertiary-active polyspecific organic cation transporters (OCTs) have been cloned from animal and human tissues. In humans, these transporters are represented by: (a) secondary-active, electrogenic cation facilitators (organic cation transporters OCT1-3/SLC22A1-3) and OCT6 (SLC22A16), (b) tertiary-active cotransporters of zwitterion L-carnitine and monovalent cations Na^+ or H^+ (organic cation novel transporters OCTN1-2/SLC22A4-5 and OCTN3/SLC22A21), and (c) tertiary-active organic cation/ H^+ exchangers, multidrug and toxin extruders MATE1-2 (SLC47A1-2) and two splice variants (isoforms) of MATE2 including MATE2-K and MATE2-B. The related orthologs of these transporters have been detected in rodents, rabbits and in few other mammals. The organ and cellular distribution, as well as the biochemical and functional characteristics of these transporters have been extensively studied in experiments *in vitro* (transfected cell

lines of the animal or human origin, transfected *Xenopus laevis* oocytes, primary cultures, freshly isolated renal proximal tubules, isolated membrane vesicles from various organs) and in vivo (various animals, wild type (WT) and knockout (KO) mice, humans). These studies revealed that OCTs play an important role in: (a) absorption, distribution, secretion and reabsorption of various nutritional organic cations (OCs), (b) distribution and excretion of various endogenous cationic compounds generated by intermediary metabolism, (c) absorption, distribution, and elimination of xenobiotics, including pharmaceuticals used in human and veterinary medicine, and (d) drug-drug interactions and drug-induced organ toxicity, whereas dysfunctional transporters due to truncated isoforms or gene polymorphism can cause genetic diseases [11, 12, 15, 24, 33, 38, 45, 55, 56, 58, 60, 61, 69, 71, 89, 92, 93, 99, 131, 132, 155].

Various animal models have been developed and applied to study specific human diseases and other conditions related to physiology and malfunctions of OCTs. Most relevant information have been generated by comparing the data in WT mice and in mice with the inactivated specific OCT genes (KO mice). As elaborated previously [18, 92], KO mouse models are valuable tools in transporter research to investigate tissue distribution of endogenous compounds and drugs and determine the relevance of transporters in vivo. Data from KO mouse models may also help to predict pharmacokinetics in humans, particularly in those carrying genetic variants encoding the non-functional transporters. Various animal models used to study functional, pharmacological, and toxicological OCTs-dependent phenomena, and major findings in these studies with their possible relevance to the situations and diseases in humans have been selected from the available literature and listed in Table 9.1.

Although many findings in these models have only a weak or “possible relevance” to the situations and diseases in humans, these findings, particularly in KO mice, initiated numerous studies of OCT genes polymorphisms and their roles in humans. Importantly, the studies in KO mice revealed that none of the transporters from OCT family is life-threatening; even double KO (*Oct1/2*) mice, which exhibited a significant reabsorptive and secretory disturbances in handling various OCs, were found viable and fertile, and exhibited no visible phenotypic abnormalities [47]. Such an extreme condition of a complete absence of one or more OCT genes and their products in humans has not been reported. However, altered expression levels of some OCTs, and a single nucleotide polymorphism (SNP) in some OCTs genes were found to be associated with a number of pathophysiological conditions and diseases in human population. The polymorphisms of specific OCT genes have been recognized as: (a) risk loci for some cancers [8, 67] and other diseases [66, 68, 94, 105, 133], (b) diagnostic and prognostic parameters [35, 36, 106, 135], (c) mediators of drug–drug interactions, side-effects, and organ toxicity [12, 13, 17, 57, 61, 101, 134, 166], (d) mediators of variability in responses to drug therapy [3, 34, 57, 93, 121, 130, 143, 144, 152, 153, 166], and (e) mediators of variability in drug pharmacokinetics [14, 57, 79, 87, 92, 93, 143, 147].

Table 9.1 Animal models used in testing various organic cation transporters-related phenomena; possible relevance to human conditions or diseases

Organic cation transporter (principal substrates)	Animal model	Treatment	Effects	Possible relevance to human condition/disease	References
OCT1/Oct1 (SLC22A1/Slc22a1) Principal substrates:	<i>Oct1</i> KO vs. WT mice	TEA	Increased urine excretion of TEA, strongly reduced accumulation of TEA in the liver and excretion of TEA in the intestine	Polymorphism of OCT1 gene may cause variations in liver handling and renal secretion of organic cations	[46]
		CDPCP	Strongly diminished accumulation of hepatic and intestinal CDPCP, decreased volume distribution and clearance of CDPCP, lower hepatotoxicity	Pharmacokinetics and toxicity in chemotherapy with CDPCP	[74]
Metformin		Metformin	Strongly diminished accumulation of metformin in the liver and small intestine, abolished glucose-lowering effects of metformin, lower blood lactate concentration	Variations in response to metformin in people with polymorphic OCT1 gene	[93, 122, 123, 150, 151]
Thiamine		Thiamine	Reduced hepatic steatosis and energy production, reduced hepatic levels of thiamine	Thiamine deficiency due to OCT1 gene polymorphism may affect distribution and pharmacologic action of metformin	[9]
Various OC	Mice	Potassium oxonate	Hyperuricemia: increased serum urate and creatinine levels, decreased urate and creatinine excretion, downregulated mRNA and protein expression of mOct1, mOct2, mOctn1, and mOctn2. Extract of <i>Ramulus mori</i> (a mixture of plant antioxidants) or quercetin ameliorated these abnormalities	Anti-oxidants as potential therapeutic remedies to improve kidney functions in hyperuricemia	[41, 119]
	Rats	Bile duct ligation	Obstructive cholestasis: diminished rOct1 protein and mRNA expression in liver, diminished hepatic accumulation of TEA	Impaired handling of cationic drugs in acute cholestatic liver injury	[19]
	Rats and mice	Ovalbumin	Acute allergic airway inflammation; deranged expression of <i>rOct1</i> (and <i>rOct2</i>) mRNAs in airway epithelial cells	Animal models of allergic airway disease	[78]

OCT2/Oct2 (SLC22A2/ Slc22a2)	Oct2 KO vs. WT mice	Untreated	Reduction in brain tissue concentrations of serotonin, altered sensitivity to acute treatments with blockers of norepinephrine and /or serotoninine transporters in the forced swim test	Gene polymorphism may cause mood-related disorders, and may be a potential target for mood disorders therapy	[4]
		Oxaliplatin	KO mice were protected from cold- or mechanical-induced hypersensitivity (allodynia)	Oxaliplatin-induced neurotoxicity	[127]
Principal substrates: Creatinine Metformin	Mice	Cisplatin	Reduced renal expression of <i>mOct2</i> mRNA	Cisplatin-induced nephrotoxicity	[1, 167]
	Rats	5/6-Nephrectomy	Increased plasma concentration of cimetidine; diminished tissue uptake and renal secretion of cimetidine; diminished renal expression of rOct2 (but not rOct1) protein, testosterone treatment ameliorated these data	Diminished secretion of OC in chronic renal failure	[44, 95, 128]
Oxaliplatin		Metformin	The expression level of renal rOct2 is the key factor in controlling accumulation and secretion of metformin in the kidney	Renal OCT2 may play a dominant role for metformin pharmacokinetics	[54]
		Cisplatin	Downregulation of <i>rOct2</i> mRNA and protein expression. Administration of AST-120 partially reversed the effect	Cisplatin-induced nephrotoxicity	[88]
Various OC					(continued)

Table 9.1 (continued)

Organic cation transporter (principal substrates)	Animal model	Treatment	Effects	Possible relevance to human condition/disease	References
Oct1 + Oct2 (Oct1/2)	<i>Oct1/2</i> KO vs. WT mice	Untreated	Elevated content of acetylcholine (ACh) in airway epithelium, Oct1/2 mediate release of ACh from the respiratory epithelium.	Contribution to bronchoconstriction	[62]
		Ethidium	Accumulation of ethidium in renal proximal tubules was strong in WT and almost abolished in KO mice	Danger of nephrotoxicity and general toxicity in humans	[70]
		Creatinine	Significantly impaired creatinine clearance	Polymorphism of the OCT2 gene in humans may affect the creatinine levels and clearance	[14]
		TEA	Abolished renal secretion of TEA; increased concentration of plasma TEA	Increased drug sensitivity and toxicity	[47]
		Pralidoxime +/- TEA	Significant increase in plasma pralidoxime concentrations in the presence of TEA	Possible increase in efficiency of pralidoxime in organophosphate poisoning	[52]
		Cisplatin	Impaired urinary excretion of cisplatin; absence of cisplatin-induced renal tubular damage	In humans, some SNPs are associated with reduced cisplatin-induced nephrotoxicity in some patients; provides a rationale for the explanation of heterogeneous effects of cisplatin treatment	[25]
		Cisplatin +/- cimetidine	In KO mice, cisplatin induced only a mild nephrotoxicity without ototoxicity; in WT mice, cimetidine protected from ototoxicity and partially from nephrotoxicity; mOct2 expression was demonstrated in cochlear hair cells	OCT2 is a possible target for protective interventions in cisplatin ototoxicity and nephrotoxicity	[13]
Rats		Cisplatin +/- lycopene	Nephrotoxicity; elevated serum urea and creatinine, decreased expression of the renal rOct1 and rOct2 proteins; treatment with lycopene reversed the expression of rOcts	Possible nephroprotective effect of lycopene in cisplatin nephrotoxicity	[23]
		Streptozotocin	Experimental diabetes: progressive reduction of rOct1 protein (but not <i>rOct1</i> mRNA) expression, and reduction of both rOct2 protein and mRNA expression in proximal tubule cells; treatment with insulin prevented the reductions in transporter levels	Role of OCT1 and OCT2 in diabetes	[30]

OCT3/Oct3; Alias Oct3 in mice and EMT in humans (SLC22A3/ Slc22a3)	Oct3 KO vs. WT mice	Untreated	Disrupted sensitivity to hypertonic saline, increased intracellular levels of histamine in bone marrow basophils, altered aminergic neurotransmission in the brain, decreased anxiety	Possible target for the development of novel antidepressant medications, impaired OCT3-mediated hepatic biliary excretion and placental passage of cationic drugs, potential pharmacological target for the treatment of depression by increasing monoamine levels in the central nervous system	[40, 56, 57, 93, 94, 114, 116, 148, 149, 156]
Principal substrates		MPP+	Strong reduction of MPP ⁺ accumulation in hearts of males and females, strongly diminished accumulation of MPP ⁺ in <i>mOct3</i> KO fetuses	OCT3 gene polymorphism may result in deranged peripheral elimination of monoamine neurotransmitters and increased sensitivity to psychostimulants	[161]
Adrenaline		Brain ischemia	Diminished brain damage, increased histamine content in the brain	Increased anxiety and stress, mood disorders, regulation of fear and anxiety	[160]
Noradrenaline		LPS	Increased content of histamine in the spleen, increased morbidity	Possible role in the functional changes of immune cells	[97]
Oxaliplatin					
Serotonin					
Some toxins					
Various drugs					
OCT6/Oct6 (SLC22A16/ Slc22a16)	Animal models have not been reported	Unknown	Unknown	In humans expressed mainly in the testis. Possibly important for sperm maturation and capacitation. Polymorphism of the OCT6 gene may influence fertility and regulation of cell proliferation. Linked to bipolar disorders. Expressed in acute myeloid and lymphocytic leukemia. It mediates transport of a few chemotherapeutics that can be used to treat leukemias	[33, 56, 57]
Principal substrate L-carnitine					

(continued)

Table 9.1 (continued)

Organic cation transporter (principal substrates)	Animal model	Treatment	Effects	Possible relevance to human condition/disease	References
OCTN1/Octn1; Alias: ETT (SLC22A4/Slc22a4)	<i>Octn1</i> KO vs. WT mice	None or [³ H]-ERGO	Complete deficiency of ERGO in organs, strongly reduced intestinal absorption and renal reabsorption of ERGO, greater susceptibility to intestinal inflammation due to loss of ERGO antioxidant functions	Inflammatory bowel disease (Chron's disease, ulcerative colitis), rheumatoid arthritis, psoriatic arthritis, and mushroom intolerance due to OCTN1 gene polymorphism	[6, 12, 37, 51, 57, 103]
Principal substrates:		Metformin	Abolished hepatic and diminished intestinal uptake of metformin	Perturbed handling of metformin due to OCTN1 gene polymorphism	[56, 57, 150]
Ergothioneine (ERGO)		[³ H]-ERGO	Lower distribution of ERGO in brain regions, enhanced oxidative stress	Neuronal differentiation and proliferation	[90]
Acetylcholine		Phenformin	Diminished uptake of phenformin in mitochondria, weak lactic acidosis	Lactic acidosis in phenformin-treated diabetic patients	[120]
Zwitterions Metformin	Mice	Collagen	Collagen-induced arthritis: inflamed joints, mOctn1 expressed in inflamed but not in healthy joints	A model for rheumatoid arthritis in humans	[140]
OCTN2/Octn2 (SLC22A5/Slc22a5)	JVS mice	Untreated	Juvenile Visceral Steatosis (JVS) — autosomal recessive disorder of fatty acid oxidation due to dysfunctional Octn2 gene (spontaneous mutation): low serum and cellular carnitine, reduced renal reabsorption of carnitine, fatty liver, growth retardation, hyperammonemia, hypoglycemia; cardiac hypertrophy, muscular weakness; obstructive azoospermia	Human primary carnitine deficiency due to impaired function of OCTN2 gene; single nucleotide polymorphism of OCTN2 gene may be associated with increased incidence of rheumatoid arthritis, Chron's disease and asthma; possible model of human male infertility	[26, 57, 63, 131, 133, 137, 162, 163,164]
Principal substrates:	Octn2 KO vs. WT mice	Cephaloridine	Diminished renal clearance of the antibiotic, increased plasma levels of cephaloridine	OCTN2-mediated transport of beta-lactam antibiotic cephaloridine	[49]

L-carnitine	Rats	Untreated	Reduced carnitine in fetuses and placentas, upregulated activity of SCHAD	Effects of carnitine deficiency	[118]
Choline		N-trimethylhydrazine-3-propionate	Decreased reabsorption and increased renal excretion of carnitine, decreased plasma and tissue carnitine concentrations	Rat model for human carnitine deficiency	[126]
	Pigs	Clofibrate ^a	Upregulation of <i>pOctm2</i> mRNA in liver, muscle and enterocytes, enhanced accumulation of carnitine in tissues	Animal model for the PPAR α -stimulated transport of carnitine in animal and human organs	[108, 109]
		Fasting	Increased expression of <i>pOctm2</i> mRNA in liver, kidneys, skeletal muscle, and small intestinal mucosa, higher concentrations of carnitine in liver and kidney tissues	Animal model to increase the Octn2-mediated transport of carnitine in the cells	[110]
OCTN3/ Octn3 (SLC22A21/ SLC22a1)	Animal models have not been reported	Unknown	Unknown	A partial or complete loss of OCTN3 was detected in Zellweger (cerebrohepatorenal) syndrome due to defect genes PEX1 and/or PEX19	[5, 22, 43, 65, 132]
Principal substrate:					
L-carnitine					

(continued)

Table 9.1 (continued)

Organic cation transporter (principal substrates)	Animal model	Treatment	Effects	Possible relevance to human condition/disease	References
MATE1/Mate1 (SLC47A1/SLC47a1) Principal substrates:	<i>Mate1</i> KO vs. WT mice	Metformin	Reduced renal secretion of metformin, increased blood lactate and hepatic concentration of metformin	Lactic acidosis in diabetic, metformin-treated patients	[141, 142]
		Cisplatin	Higher plasma concentration and renal accumulation of cisplatin, diminished renal excretion of cisplatin	Cisplatin-induced nephrotoxicity	[91, 167]
		Paraquat (PQ)	Higher PQ plasma concentration, enhanced kidney injury	Intoxication and nephrotoxicity with PQ	[73]
Creatinine					
Cisplatin		Cephalexin	Higher plasma concentration of cephalexin, decreased renal concentration and excretion of cephalexin	Diminished renal secretion of zwitterionic antibiotic cephalexin	[154]
Oxaliplatin		Pyrimethamine ^b	Inhibited hepatobiliary secretion of metformin due to Mate1 inhibition	Potential diagnostic purposes in PET scan	[42]
Metformin	Mice	Ondansetron ^c + metformin or cisplatin	Increased tissue accumulation of metformin, enhanced renal accumulation of cisplatin, enhanced cisplatin-induced nephrotoxicity	Cisplatin-induced nephrotoxicity, drug–drug interactions	[72]
Various OC	Rats	Cisplatin	Nephrotoxicity: downregulation of <i>rMate1</i> mRNA and protein; renal accumulation of cisplatin was much greater than that of carboplatin, oxliplatin or nedaplatin	Cisplatin-induced nephrotoxicity	[88, 165]
		5/6-Nephrectomy	Markedly decreased expression of rMate1, but not of rOct2, following nephrectomy in males and females; good correlation with the tubular secretion of cimetidine in the female rats	Chronic renal failure	[95]
	Rats and monkeys	Cimetidine + PNU-288034 ^d	Decreased renal excretion and increased plasma concentration of PNU-288034 due to cimetidine inhibition of Mate1	Drug–drug interactions	[64]

Mate2 (Slc47a2)	Animal models have not been reported	Unknown	Unknown	Not present in humans	[82, 100]
Principal substrates:	Various OC				
MATE2-K/Mate2-K (SLC47A2/Slc47a2)	Animal models have not been reported	Unknown	Unknown	Drug–drug interactions	[12, 93]
Principal substrates:					
Creatinine					
Oxaliplatin					
Various OC					

AST-120 oral adsorbent of intestinal indol, *CDPCP cis*-(diammine(pyridine)chloroplatinum(II), *EMT* extraneuronal monoamine transporter, *ETT* ergothioneine transporter, *KO* knockout mice, *LPS* lipopolysaccharide (in LPS-induced endotoxemia model), *MPP* methyl-phenyl-pyridinium (neurotoxin), *OC* organic cations, *Oct3* organic cation transporter 3, *PET positron emission tomography*, *PEX* peroxisomal biogenesis factor, *PPAR-α* peroxisome proliferator-activated receptor α; *pralidoxime* an antidot to treat organophosphate poisoning, *SCHAD* short chain hydroxy acyl-CoA dehydrogenase, an enzyme in beta-oxidation of fatty acids in mitochondria, *SNP* single nucleotide polymorphism, *TEA* tetraethylammonium, model organic cation, *WT* wild type mice

^aAgonist of PPARα

^bAntimalarial; inhibitor of Mate1

^cAntiemetic

^dOxazolidinone antibiotic

Table 9.2 Sex differences in, and effect of gonadectomy and treatment with sex hormones on the expression and function of various OCTs in some experimental animals

Organic cation transporter	Animal species	Treatment	Sex differences; effects of gonadectomy and treatment with sex hormones	References
OCT1/Oct1 (SLC22A1/Slc22a1)	Rats	Untreated	In adult rats, the expression of renal <i>rOct1</i> mRNA was higher in females than in males	[2, 31, 84, 85, 112, 125, 145, 146]
			Sex differences and/or effects of sex hormones on <i>Oct1/OCT1</i> mRNA and/or protein expression were not found in other studies of the renal Oct1/OCT1 in rats, mice, rabbits, and humans	
OCT2/Oct2 (SLC22A2/Slc22a2)	Mice	Gonadectomy +/- treatment with sex hormones	The renal expression of <i>mOct2</i> mRNA and protein in adult animals was stronger in males than in females, castration downregulated, androgens upregulated, whereas ovariectomy and estrogen treatment had no effect on the expression of <i>mOct2</i> mRNA and/or protein	[2, 31, 84, 85, 112]
	Rats	Gonadectomy +/- treatment with sex hormones	The renal expression of <i>rOct2</i> mRNA and protein in adult animals was stronger in males than in females; castration and estrogen treatment downregulated, while testosterone treatment upregulated the expression of <i>rOct2</i> mRNA and protein. The expression of renal <i>rOct3</i> , <i>rOctn1</i> and <i>rOctn2</i> mRNAs, as well as of <i>OCT2</i> mRNA in human kidneys was sex-independent	[95, 112, 125]
	Rabbits	Untreated	The renal expression of <i>rbOct2</i> mRNA was stronger in males than in females, but the expression of <i>rbOct2</i> protein and the renal TEA uptake exhibited no sex differences	[31]
OCTN1/Octn1 (SLC22A4/Slc22a4)	Mice	Castration +/- testosterone treatment	TEA uptake in renal proximal tubules was decreased by castration and restored by testosterone treatment; the <i>mOctn1</i> mRNA expression in proximal tubules was upregulated by castration and restored by testosterone treatment	[84]
			The relative expression of <i>mOct1</i> , <i>mOct3</i> , <i>mOctn2</i> , <i>mMate1</i> and <i>mMate2</i> mRNAs were not significantly affected by castration and testosterone treatment	

(continued)

Table 9.2 (continued)

Organic cation transporter	Animal species	Treatment	Sex differences; effects of gonadectomy and treatment with sex hormones	References
MATE1/ Mate1 (SLC47A1/ Slc47a1)	Mice	Untreated	In adult mice, the expression of <i>mMate1</i> mRNA was found female-dominant in the liver and male-dominant in the kidneys. Sex differences appear at the age of 30 days	[75]
MATE2/ Mate2 (SLC47A2/ Slc47a2)	Mice	Ovariectomy +/- estradiol treatment	TEA uptake and secretion in renal proximal tubules was increased by ovariectomy and restored by estradiol treatment; the renal <i>mMate2</i> mRNA expression was downregulated by ovariectomy and restored by estradiol treatment	[85]
			The relative expression of renal <i>mOct1–3</i> , <i>mMate1</i> , and <i>mOct1–2</i> mRNAs were not affected by ovariectomy and estradiol treatment	

TEA tetraethylammonium, model organic cation

Species Differences in Distribution, Expression and Function of Organic Cation Transporters

Recent studies have shown that many parameters and phenomena, including adverse reactions to pharmaceuticals, are in better concordance between humans and nonrodents than between humans and rodents. Many preclinical studies in experimental animals show only a limited relevance to human pathology and therapy mainly due to basic genomic, phenotypic, and pathophysiological differences among species that are largely genetically determined. The pharmacokinetic and pharmacodynamic responses to drugs and drug-induced adverse reactions including drug toxicity are often species dependent, and rarely the animal model, developed and used thus far, resembles humans completely [80, 98]. An example of a positive correlation between the data in an animal model and the respective human disease is primary carnitine deficiency syndrome; the JVS (Juvenile Visceral Steatosis) mice are homozygous mutant animals exhibiting the nonfunctional carnitine transporter *Ocn2* (*Slc22a5*) and the associated syndrome of carnitine deficiency (low absorption and plasma and tissue concentration of carnitine, increased loss of carnitine in urine, fatty liver, hyperammonemia, hypoglycemia, progressive cardiomyopathy, encephalopathy, mitochondrial abnormalities, etc....) [102, 164]. Similar symptoms exist in primary carnitine deficiency syndrome in humans, which is a rare autosomal recessive disorder due to mutations in *OCTN2* (*SLC22A5*) gene and loss of the protein function. In both cases, the syndrome could be ameliorated with parenteral carnitine treatment

[26, 63, 131, 133, 137]. In most of the other cases, however, a good correlation of the data in the animal models and in corresponding human diseases is questionable due to presence of species and sex differences in various OCTs characteristics.

Comparison of biochemical and functional characteristics of the specific OCTs in transfected cell lines and *Xenopus* oocytes, as well as in tissue samples of the mouse, rat, rabbit, monkey, and human organs revealed the presence of species differences in tissue distribution and in a variety of other parameters in the kidney, liver, and gastrointestinal tract [11, 21, 39, 45, 53, 59, 69, 81, 111–113, 115, 129, 131, 138, 158]. For example, in rodent and rabbit kidneys specific OCTs exhibit species differences in distribution in the cells along the nephron, substrate selectivity and affinity, levels of mRNA and/or protein expression, and sensitivity to inhibitors, and regulation. In comparison with the data in rodents and rabbits, in the human nephron some transporters are absent, others exhibit different localization in the cell membrane domains, different sensitivity to inhibitors, different substrate specificity, and different rates and regulation of the transport of substrates [21, 59, 111, 112]. An example of species differences in organ distribution is OCT1/Oct1; in rodents, Oct1 is expressed in the liver, kidneys, and small intestine [32], whereas in humans, OCT1 is primarily expressed in the liver [29] and only weakly in the kidneys [158]. Moreover, as shown in Fig. 9.1, species-specific localization of the OCT1/Oct1 protein is present in the renal proximal tubule cells; in mice and rats the transporter is located in the cell contraluminal membrane, where it mediates secretion of OCs, whereas in humans, it is located in the cell luminal membrane, where it mediates reabsorption of OCs.

Another species-specific distribution was observed for the mRNA and/or protein expression of MATE transporters: (a) in mice, *Mate1* mRNA was detected in the liver, kidneys heart, and none-to-weak in the skeletal muscle, whereas *Mate2* mRNA was detected predominantly in the testis in males and colon in females [75, 100], (b) in rats, *Mate1* mRNA was detected in the kidneys and few other organs, but not in the liver and skeletal muscle; the presence of *Mate2* and *Mate2-K* mRNAs has not been reported [139], (c) in rabbits, *Mate1* and *Mate2-K* mRNAs were expressed in their kidneys [159], whereas (d) in humans, *MATE1* mRNA and protein were detected in the liver, kidney, skeletal and heart muscles, and in a few other organs, *MATE2* mRNA was not detected, *MATE2-K* mRNA and protein were found only in the kidneys, whereas the nonfunctional isoform *MATE2-B* mRNA was found in the brain [82, 100].

Sex Differences in Expression and Function of Organic Cation Transporters

The rodents and rabbits as the most common animal models exhibit in their secretory and/or reabsorptive organs (liver, kidneys) sex differences in the expression of some OCT proteins and/or their mRNAs, which affect the related functions in these organs.

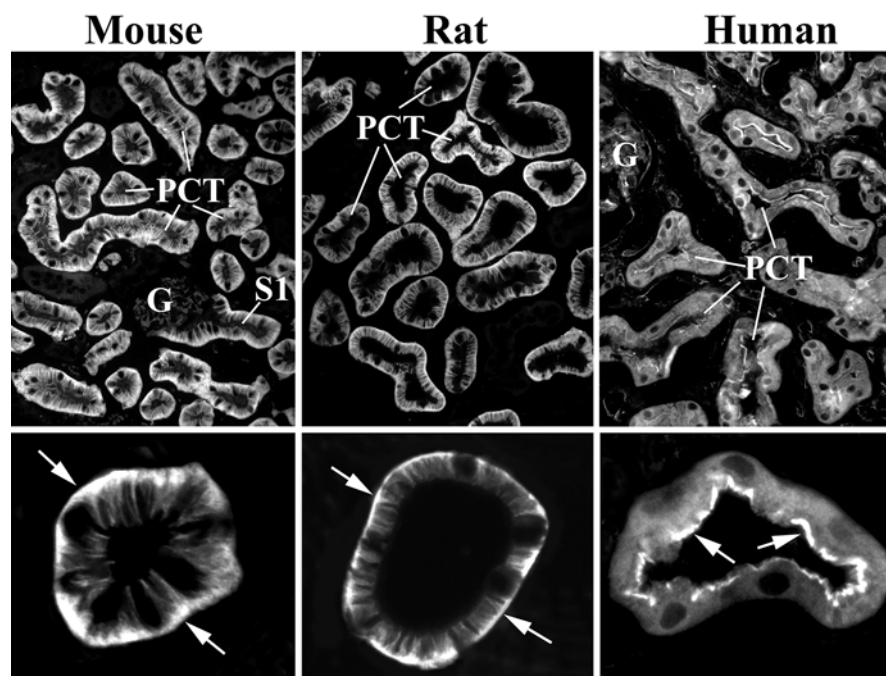


Fig. 9.1 Species differences in localization of OCT1 protein in proximal tubules of the renal cortex, as shown by immunocytochemical staining in tissue cryosections using species-specific polyclonal anti-OCT1 antibodies and fluorophore-labeled secondary antibody. *Arrows* indicate localization of the staining. In rats and mice, the protein is localized to the basolateral membrane of proximal convoluted tubules (PCT), whereas in humans, the protein is localized to the luminal (brush-border) membrane of PCT. In humans, but not rodents, a weak OCT1-related immunostaining was detected also in the peritubular capillaries and luminal membrane and intracellular organelles of the distal tubules, where it colocalized with the arginine vasopressin receptor 2 [144]. *G* glomerulus, *SI* the initial proximal tubule segment

As listed in Table 9.2, sex differences in, and the effects of sex hormones/gonadectomy on the expression and function of specific OCTs have been described for the renal Oct1 protein and/or mRNA in rats (females>males), renal Oct2 in mice, rats, and rabbits (males>females), and *Mate1* mRNA in the mouse liver (females>males) and kidneys (males>females). The expression of renal *Octn1* mRNA and the related transport of model organic cation tetraethylammonim (TEA) in mice seems to be regulated by androgens, whereas the renal *Mate2* in mice may be regulated by estrogens. Regarding the expression of OCTs in other species and their organs, sex differences were either not found [84, 85, 112, 159] or not searched for. However, none of the thus far tested OCTs exhibited the sex-dependent expression in human kidneys ([112]; Breljak et al., unpublished). Since a number of sex and species differences are known also for organic anion transporters in the mammalian organs, we have concluded that the data on the membrane transporters-related properties and functions in one species can not simply be regarded as relevant

for other species, and that various biochemical, physiological, pharmacological, and toxicological findings related to organic ion transporters in rodents and rabbits do not reflect the situation in humans [112]. However, some studies in monkeys indicated that this species, being closer relative to humans, may be better animal model for the transporters-related evaluations of the renal functions [11, 129].

Emerging Animal Models to Study Translational Roles of OCTs

An animal model which may be highly useful in translational studies of membrane transporters is the “knockin” or “humanized” mouse model, representing a mouse in which a mouse gene is replaced with its human counterpart or in which various types of human cells or tissues are engrafted and function as they would in humans. The humanized mouse model is considered very useful in basic and applied human disease research; it has been applied in research of human hematopoiesis, innate and adaptive immunity, autoimmunity, infectious diseases, regenerative medicine, and cancer biology [124], but the use of this model in studying the mammalian OCTs has not been reported thus far. However, as pointed out by Lin [77], in spite of high expectations, humanized mice still express murine transporters and drug metabolizing enzymes, and multiple transporters and enzymes contribute to the handling and elimination of drugs. It is, therefore, critical to assess if a specific drug has similar elimination pathways in such animals as in humans, and for now it is impossible to extrapolate the contribution of transporters to humans quantitatively based on humanized animal models alone.

The zebrafish (*Danio rerio*) represents an animal model with a great potential in various aspects of translational research including human diseases and drug discovery and development. Zebrafish offers several advantages that make it an important complement to mouse models of disease including low costs of the maintenance, fast breeding that gives numerous offsprings, fast growth, easy genetic manipulation, and an easy in vivo visualization of cell-biological events. In the past 30 years, zebrafish proved to be a mainstream model in developmental biology and useful model for various genetic and acquired human conditions and diseases (renal regeneration, mitochondrial, metabolic, infectious and gastrointestinal diseases, aging, mutagenesis, brain disorders), for drug discovery, testing and screening, for studying toxicity and environmental pollutions, etc... [16, 27, 28, 48, 50, 76, 104, 117].

Although zebrafish has been adopted as an important model species in life sciences, OCTs have been poorly studied in its organs. Our recent quantitative mRNA studies have detected in zebrafish organs the orthologs of Slc22a1 and Slc22a2 (drOct1 and drOCT2), Slc22a16 (drOct6), and Slc22a4 and Slc22a5 (drOctn1 and drOctn2), with a distinct tissue expression pattern; the highest expression of drOct1 was observed in the liver (males > females) and kidneys (females > males), whereas drOct2 expression was highest in the kidney (males > females) and gonads (males > females) ([157]; Smital et al., unpublished). However, genetic manipulations of these genes that would model relevant human diseases have not been performed so far.

A common fruit fly *Drosophila melanogaster* represents a rapid model of renal development and function, and an emerging translational model of various human diseases including calcium-oxalate nephrolithiasis and several metabolic conditions [10, 20, 36, 86]. This fly is one of the most widely used animal in experimental research; it is a short lived species that breeds quickly in large numbers, it is easy to care for, and its genes are relatively easy to manipulate. It is genetically best characterized eukariote with ~15,600 genes located on four pairs of chromosomes. Its organ with secretory and osmoregulatory functions, Malpighian tubules, transports OCs by an organic cation transporter-like protein Orct, an orthologue of the basolateral OCTs in vertebrate kidneys. Orct and OCTs exhibit some similarities in the transport mechanism, selectivity of substrates, and sensitivity to inhibitors [96, 107, 136]. Having also some other excretory transporters such as multidrug resistance protein MDR1/P-glycoprotein and several multidrug resistance-associated proteins, the *Drosophila* Malpighian tubules are useful model for studying the excretion of various toxins [96]. However, the use of *Drosophila* as a translational model for diseases related to OC transport and transporters in humans has not been reported.

The soil-dwelling nematode *Caenorhabditis elegans* is an increasingly popular model for antioxidant and ageing studies. Recently, in this worm an Oct1 gene product homologous to the mammalian OCTN1 has been functionally characterized as the ergothioneine transporter. Ergothioneine is a fungal thiol compound with antioxidant and cytoprotective properties [6]. The studies by Cheah et al. [7] revealed that deleting the Oct1 function in *C. elegans* results in elevated oxidative damage and a shortening of lifespan. A comparable phenomena, related to either OCT1 or OCTN1 functions in other experimental animals and/or humans, have not been reported.

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Chapter 10

Role of Transporters for Organic Cations in Plants for Environmental Cycling of Pharmaceutical Residues

Trine Eggen and Cathrine Lillo

Abstract Pharmaceuticals, designed to be biologically active compounds, are widely spread in the environment and now considered as ‘pseudo-persistent’ substances. Plant uptake experiments have shown that the antidiabetic 2 pharmaceutical metformin can accumulate in oily seeds of rape, and that the metformin concentration is 60–70 times higher than in seeds of wheat. The mechanisms for uptake, translocation and accumulation of metformin in plants are not known. However, organic cation transporters (OCTs) actively transport metformin in mammals, and orthologues of OCTs are present in plants. Metformin has structural similarities with natural plant secondary compounds guanylurea and biguanide, which are present in medicinal plants historically known to be used against symptoms possibly caused by diabetes. It is therefore not unlikely that OCTs might be involved in active uptake of metformin in plants. Due to the large number of pharmaceuticals present in the environment, it is important to understand the uptake and translocation mechanisms involved that can lead to accumulation of biological active compounds in feed and food.

Keywords Arabidopsis • OCT • Bioaccumulation • Metformin • Medicines • Drugs • Nitrogen reallocation

Introduction

Pharmaceuticals, represented by thousands of different synthetically produced chemicals, are designed to be biologically active with the purpose to interact with living cells. After administration, pharmaceuticals are excreted as unreacted still

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active parent compound or as metabolites thereof. Residues of pharmaceuticals have been measured in sewage sludge, surface water, and sediments, even in ground-water and drinking water [1–3]. Due to the high- and widely spread usage of human and veterinary pharmaceuticals it is now commonly accepted that pharmaceuticals are ‘pseudo-persistent’ compounds. After several studies related to uptake of pharmaceuticals into plants and aquatic organisms during the last years, there is an increased focus on the potential adverse effects on non-target organisms in the environment [4–6].

Uptake of pharmaceuticals by non-target organisms is assumed to follow the same mechanisms and processes as other organic xenobiotics. However, recent experiments have demonstrated unexpected high uptake of the antidiabetic II pharmaceutical metformin in oily seeds of rape (*Brassica napus* cv. Sheik and *Brassica rapa* cv. Valo) [7, 8]. As illustrated in Fig. 10.1 the bioaccumulation factor, BCF (concentration in plant over soil, both in dry weight), in rape seeds is higher than 40 when the metformin soil concentration at the end of the experiment is used for calculating BCF, and BCF higher than 20 when initial soil concentration is used. In the same studies, the BCFs in rape seeds were 60–70 times higher than BCFs in cereals of wheat (*Triticum aestivum* cv. Bjarne) (Fig. 10.1).

Although other studies show high translocation for certain xenobiotics, including pharmaceuticals, to leaves [5, 9–11], the translocation to generative tissues such as seeds and fruits is low—commonly below the analytical detection limit of the chemical or BCF far below 1 [5, 12, 13]. Since metformin has structural similarities to arginine, canavanine and galegine, which are likely to serve as storage compounds for nitrogen (Fig. 10.2), it is possible that metformin can be involved in reactions by mimicking these substances. In addition, metformin has structural similarities with natural plant compounds guanylurea and biguanide (Fig. 10.2), which are present in medicinal plants historically known to be used against symptoms possibly caused by diabetes. In mammals, metformin is actively transported into cells by organic cation transporters (OCTs) [14], and the cationic amines such as choline, guanidine, and metformin are identified as substrates for mammalian OCTs [15].

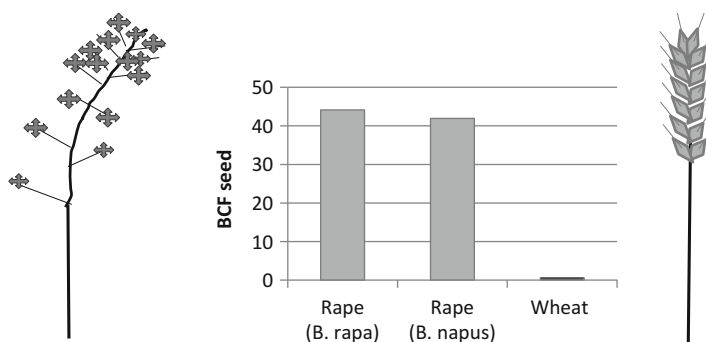


Fig. 10.1 Illustrating plant species dependent uptake and translocation pattern for metformin in seeds of rape (*Brassica rapa* spp. and *B. napus* spp.) and wheat (*Triticum aestivum* spp.) (from [7]). The bioaccumulation factor, BCF, is presented as measured concentration in seeds divided by measured concentration in soil at the time of seed harvest, both in dry weigh

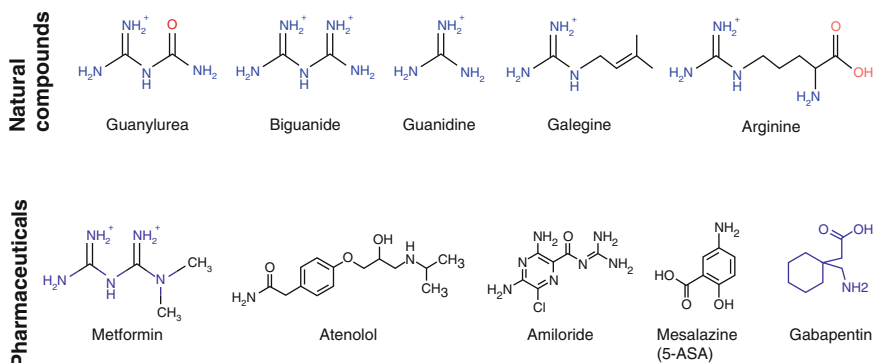


Fig. 10.2 Structures of selected natural plant compounds and the pharmaceuticals metformin, atenolol, amiloride, mezalasin, and gabapentin

Uptake of organic nitrogen compounds into plants has been scarcely studied, but plants have a wide range of transporters for various compounds, including secondary compounds for defense. In addition to uptake from the environment into root cells, transporters are engaged in loading of the vascular tissue (xylem and phloem), and mobilization of compounds during later stages of growth from source tissue, for example leaves, into sink tissue like seeds or storage organs. Furthermore, a process specifically important to plants is sequestration in vacuoles as a means of storage or detoxification. In contrast to animals, plants do not have a system to drain and excrete toxic compounds from the organism. An important function of the plant leaf cell vacuoles is to be a sink for compounds that may be detrimental if reaching a too high concentration in the cytosol. About half of the medical drugs are organic cations [16] and are candidates for being transported by organic cation transporter (OCTs), but also many other transporters may be relevant, and we are not able to cover all of them in this chapter. Some drugs are zwitterionic amino acids (non-proteinous) and may be transported by amino acid transporters.

In this chapter we address the possibilities for active uptake of pharmaceuticals via OCTs and other transporters potentially involved in uptake of nitrogen-containing molecules which strengthens the hypothesis of active uptake of metformin. The chapter also discusses the known plant uptake processes of organic xenobiotics that so far only have been based on passive processes. The occurrence of pharmaceuticals and their entrance pathways into the environment are included and emphasize the importance of more knowledge about this topic.

Pharmaceuticals as Environmental Xenobiotics

Xenobiotics are compounds that are “foreign” to nature. Many have structures that are not recognized as substrates for enzymes in microorganisms commonly present in the environment and thus are metabolized very slowly. Such slowly degradable

organic compounds are classified as persistent organic compounds. Even though a great number of pharmaceuticals are not necessarily persistent by definition, they are characterized as pseudo-persistent since the discharge into the environment is constant and higher than their degradation [17].

The three main pathways or sources for transfer of pharmaceuticals to the environment are: excreted intact or metabolized substances from humans and animals, unused medicine via solid waste handling or water from households, and via effluent water from medicine production factories. It is well known that wastewater is the main pathway for transport of pharmaceuticals to the environment. Municipal wastewater treatment plants (WWTPs) are designed and optimized for removal of suspended solids, easily degradable organic matter, and nitrogen and phosphorous nutrients, hence not surprisingly, ineffective for removal of a wide range of xenobiotics including pharmaceuticals [1, 18]. There are many variations of wastewater treatment plants, however, most contain a preliminary-, primary-, secondary and tertiary treatment, and a separate sludge treatment step. Some pharmaceuticals are transferred to the sludge while the dominating group of the commonly measured pharmaceuticals have low to medium transfer to sludge and if not degraded, transferred by effluent water to the recipient [19, 20]. The removal of pharmaceuticals during different treatment processes in a WWTP depend on their intrinsic chemical and physiological properties [20, 21]. The high numbers of pharmaceuticals found in surface water such as rivers, fjords and sea water, even ground water and drinking water [22], clearly demonstrate a continuous transfer to the environment and explain why they are characterized as pseudo-persistence emerging contaminants.

Measured environmental residue levels of the antidiabetic 2 pharmaceutical metformin and four other pharmaceuticals with interesting structures (Fig. 10.2), amiloride, atenolol, mesalazine and gabapentin, are summarized in Table 10.1. In addition to reported concentrations in influent and effluent wastewater and sewage sludge from WWTPs, measured residue levels in recipients such as rivers, lakes, seawater and ground water are included. Metformin was for instance found in concentrations up to 34.2 $\mu\text{g/L}$ [29] and 7.8 mg/kg dry weight [27] in effluent wastewater and sludge from WWTP. Residue concentrations measured in effluents from hospitals or pharmaceutical factories are not presented but these sources might have different concentration levels of pharmaceuticals [34] than typical for sewage water. Residue levels of veterinary pharmaceuticals in manure from treated livestock or after use of the coccidiostat narasin as feed additive for poultry, have been reported as high as mg/kg [35–37]. However, compared to human pharmaceuticals, the veterinary pharmaceuticals have fewer sources for spreading into the environment and the transfer is more periodic.

Plants will potentially be exposed to pharmaceuticals via two main pathways; (1) use of sludge or manure as fertilizer and soil improver either directly applied at agricultural fields, or via amendments to commercial compost products applied in kitchen gardens and similar small-scale crop and vegetable production [38], or (2) use of treated wastewater from WWTPs for irrigation of crops in regions with scarce access to water [39]. It is important to bear in mind that medium to slow degradable pharmaceuticals with a high removal efficiency in WWTPs—and thus considered successfully treated—will via reuse of sludge as fertilizer and soil improver become accessible to plants, and further potentially transferred to the food chain.

Table 10.1 Measured residue levels of metformin, atenolol, amiloride, mesalazine, and gabapentin in wastewater (presented as µg/L), sewage sludge (presented as mg/kg dw) and recipient water (presented as ng/L)

Name CAS#	Wastewater (µg/L)		Sludge (mg/kg dw)	Recipient water (ng/L)
	Influent	Effluent		River, Lake, Sea, Groundwater
Metformin <i>Antidiabetic</i> CAS# 657-24-9	Avg 73.7/84.4 [23]; >10 [24]; (101–129) [25]; (86–142) [26]; (1.8–59.0) [27]	Avg 1.8/1.2 [23]; >4 [24]; (2.2–21) [3]; (3.4–5.1) [26]; max. 10.6 [28]; max. 34.2 [29]; (0.2–8.0) [27]	(0.15–7.81) [27]	River: max 270; max 220 [25]; 143 and 216, (114–643), (176–471) [26]; Lake: (35–150), Sea: (5–10), (8–31) [26]
Atenolol <i>Beta-blockers</i> CAS# 29122-68-7	(0.1–33.1) [1]; (5.1–11.2) avg 7.8 [30]; (8.1–25.1), avg 14.2 [31]; max 12.7 [27]	(0.13–7.6) [1]; (0.26–5.9), avg 2.8 [30]; (1.3–3.2), avg 2.1 [31]; max 1.7 [27]	max 1.65 [27]	River: (5–258), avg 51; (42–560), avg 314, (47–488), avg 203, (20–193), avg 118 [32]; Groundwater: 18–106, (avg 60.8) [1]
Amiloride <i>Diuretic</i> CAS#2016-88-8	(0.02–1.26) [27]	Max 0.217 [27]	(0.001–0.093) [27]	Fjord: max 552 [27]
Mesalazine ^a <i>Anti-inflammatory</i> CAS# 89-57-6	(3.16–27.9) [31]	(<0.17–1.21) [31]		River: (<15–52), avg 28 [31]
Gabapentin <i>Anticonvulsant</i> CAS# 60142-96-3	Max. 25.0, avg 13 [18]; avg 1.0 [33]; (10.7–25.1), avg 17.9 [31]	Avg 2.6 [18]; (1.8–3.5), avg 2.6 [31]		River: (11–19), avg 15, (39–210), avg 99, (91–1879), avg 968, (87–1529), avg 685, (44–1008), avg 478 [32]; (27–565), avg 182, (184–1269), avg 517 [31]

Values given as average, range or maximum concentration

[23] Oosterhuis 2013, [24] Margot et al. 2013, [25] Scheurer et al. 2009, [3] Boxall et al. 2004, [26] Trautwein et al. 2014, [29] Al-Odaini et al. 2013, [27] Huber et al. 2013, [28] Ghoshdastidar et al. 2015, [1] Luo et al. 2014, [30] Behera et al. 2011, [32] Kasprzyk-Hordern et al. 2008, [31] Kasprzyk-Hordern et al. 2009, [18] Verlicchi et al. 2012, [33] Writer et al. 2013

^aMesalazine = 5-aminosalicylic acid (5-ASA)

Plant Uptake of Organic Xenobiotics

General Uptake and Translocation Processes

Organic compounds taken up by roots in plants need to pass the Casparian strip in the endodermis in order to enter the root xylem and to be translocated to above-ground tissues [40]. The cells of the endodermis are impregnated with the wax-like,

hydrophobic substance suberin and act as a barrier for uncontrolled transport of water and solutes into the plant xylem. Passive transport of organic xenobiotics might take place by simple diffusion through the hydrophobic phospholipid layer in the cell membrane or by help of membrane proteins such as channels or carriers, which can be either passive or active processes.

While hydrophobic xenobiotics bypass the Casparian strip through diffusion, the more water soluble compounds need to be transported via membrane proteins. Pesticide uptake and translocation related to chemical properties have been studied during the 1980s–1990s [41–45]. Passive diffusion through membranes was assumed to be the mechanisms for all uptake models regarding xenobiotics. The ‘ion trap’ process for weak electrolytes—a process trapping substances that after diffusion through the membrane as neutral compounds become charged inside the cell due to pH change and dissociation—was considered already at that time [45].

The driving forces for the passive diffusion processes are concentration—and charge differences across membranes, and the chemical properties recognized as important fate parameters and included in plant uptake models such as hydrophobicity (parameters for partition between water and hydrophobic phases) and ionizing (the dissociation coefficients pK_a) [41, 44, 46–49]. It is important to notice that these models are all based on passive and not active uptake processes. Generally, charged substances have a lower uptake rate over membranes than neutral substances.

In order to quantify plant uptake of xenobiotics in a comparable way, bioconcentration factors, BCF, were introduced. This expression is used both in plant uptake models, already early 1980s [43], as well as when presenting results from experimental plant uptake studies. The influence of compounds’ lipophilicity was well known and the partition octanol-water coefficient (expressed as P , P_{ow} or K_{ow}) was introduced as the main driving parameter. It was assumed that highly water soluble compounds (e.g. $\log K_{ow} < 0$) had a general low uptake and translocation to aboveground tissues. This was illustrated as a bell-shaped curve for the relationship between the $\log K_{ow}$ and the transpiration stream concentration factor (xylem concentration over surrounding solution, TSCF), and with a maximum TSCF value around $K_{ow} 2$ [43]. This hypothesis was reevaluated when it was demonstrated that also highly water soluble compounds had a high potential for uptake and translocation [50], and a more sigmoidal than bell-shaped $\log K_{ow}$ —TSCF relationship was established. It must be mentioned that differences in experimental design may lead to diverging observed accumulation in plants, and thus this must be evaluated carefully when comparing different studies [51].

For dissociating compounds, pK_a and the surrounding pH are important parameters for uptake and translocation processes in plants [47, 49]. Recently it has also been demonstrated how pK_a and pH influence bioavailability and toxicity of ionizing organic compounds in plants [52]. Since 70 % of the pharmaceuticals are ionizing compounds [53] and many are highly water-soluble, recognition of the influence of pH, pK_a , and high uptake of such compounds is important for environmental risk assessment.

Plant Uptake and Translocation of Metformin

Metformin is a highly water soluble strong base (pK_a 12.25) and the pH adjusted distribution coefficient $\log D$ (or $\log D_{ow}$) would be around -8 in environmental relevant pH range. The average BCFs for metformin in leaves of barley, carrot and meadow fescue (range 0.1–1.3) were clearly lower than for roots (average BCF range 2–8) [8]. The low BCFs for barley and wheat seeds (BCF 0.037 and 0.025, respectively) followed established knowledge that translocation to generative tissues are much lower than vegetative tissues [5, 54]. The translocation of metformin to rape seeds, average BCF 1.55, was between 40 to 60 times higher than BCFs for wheat and barley, and unexpected high [8]. The same result with a much higher BCF for rape seeds (both *B. napus* and *B. rapa*) than for barley and wheat, was obtained in a second experiment [7]. The BCF in rape seeds calculated based on soil concentration at harvesting was 40 while calculated based on initial soil concentration BCF was 20. The BCFs for cereals followed the order oat>barley>wheat and was 15–70 lower than for rape seeds [7]. The general higher BCFs for all seeds (11–24 times higher) in the second uptake study performed in spring-summertime [7] compared to the first study performed during wintertime [8] (both in greenhouse), is very likely related to the higher temperature and higher transpiration in the summer experiment.

The pK_a of metformin is too high that lets an ion trap occur, and a partition transfer which is typical for transfer of hydrophobic compounds into oily and fatty matrixes is neither not likely for the highly water soluble metformin into the oily rape seeds. Based on the properties of metformin and established knowledge of processes involved in uptake and translocation of organic xenobiotics in plants, it must be considered that other mechanisms, e.g. active uptake, are involved in the metformin accumulation to rape seeds.

It is well known that plant species are different regarding uptake and translocation of xenobiotics [6–9, 12, 55, 56]. Factors which might influence plant species uptake and translocation differences are for instance transpiration, lipid content, root anatomy, growth rate, life time (annual versus perennial), and metabolism/detoxification systems. However, no general high uptake of organic xenobiotics or pharmaceuticals in the *Brassica* family is so far recorded [13, 57, 58].

High remobilization of N-storage molecules from leaves to pods in *Brassica napus* [59, 60] is known, and mechanisms for long distance transport of organic N to sink organs, like seeds which is a major sink during reproductive growth, is presented by Rentsch et al. [61]. Whether the high translocation of metformin to rape seeds is in some way linked to remobilization/reallocation of N-storage molecules from leaves to seed is an interesting question.

Nitrogen Sources for Plants

The mineral most commonly being limiting for plant growth is nitrogen. Many medical compounds contain nitrogen and are potential nitrogen sources for living cells if taken up and metabolised. Especially for agricultural plants, nitrate is

generally the most important nitrogen source. Following world war II, research on nitrogen uptake in plants was almost entirely concerned with inorganic nitrogen. However, already a 100 years ago it was recognized that plants would grow better in the presence of for example peptone, casein, creatine or nucleic acids, indicating that plants may take up a wide range of organic compounds [62]. In natural ecosystems where nitrogen is often limiting, the utilization of organic nitrogen may have been underestimated in recent times. The most common form of organic nitrogen in soil is usually peptides, and both organic and inorganic nitrogen sources are generally present in μM concentration when the soil is not fertilized [63, 64]. In some boreal forests the concentrations of dissolved organic nitrogen may be up to an order of magnitude greater than those of NH_4^+ and NO_3^- [64]. Furthermore, organic nitrogen compounds can be present at high levels in soils with organically grown crops and in colder climates where degradation of biological compounds and nitrification is slowed down.

Organic nutrients are a source of essential elements, and additionally a source of signaling compounds [62]. Extensive research has been done to show that amino acids and peptides are used by plants, and all plants tested have the capacity to take up amino acids and metabolize them [62]. Uptake into plants of organic compounds other than amino acids has been scarcely studied, but plants have a wide range of transporters for uptake or distribution of various compounds.

Transporter Proteins Putatively Involved in Uptake and Translocation of Drugs

Organic Cation Transporters

In animals, recycling of carnitine from the mitochondria to the cytosol is one of the important functions of OCTs and necessary for continuously transport of acyl groups into mitochondria. In plants, the function of carnitine is not well established, and the concentration of free carnitine /acetylcarnitine is about 100 /1000 times lower than in animals [65]. Furthermore carnitine is not essential in the process of fatty acid β -oxydation because fatty acids are completely degraded inside the peroxisomes in plants [66]. Plants do, however, tolerate high carnitine concentrations. They take up carnitine when provided exogenously, and accumulate mM concentrations while still growing normally [67].

In mammalian cells, metformin has large effects in the liver, which can be explained by the high tissue concentration of metformin caused by elevated expression of *OCT1* in the plasmalemma of liver cells [68–70]. In *Arabidopsis*, orthologues to human OCTs exist, and the *Arabidopsis* OCT family contains six members, OCT1–6 [71]. The OCTs are differentially expressed in roots, stems, leaves, and flowers (Table 10.2). Promotor GUS staining showed that OCT1, 3 and 6 were expressed in the vascular tissue [71, 76]. Based on the expression

patterns, OCTs may therefore be involved not only in uptake into roots and stems, but are important also for relocalization from leaves to seeds. On the cellular level plant OCT1 has been found to be localized in the cell membrane, and therefore potentially transport substrates in and out of cells. Other OCTs (OCT2-6) were detected in the tonoplast when tagged to green fluorescent protein at their C-terminal end and transiently expressed in protoplasts [76]. Possibly metformin is translocated to the vacuole for storage or to be rendered harmless. Metformin is known to influence the protein kinase AMPK in human cells and thereby affects metabolism and is beneficial in treatment of diabetes. Although humans and plants of course differ concerning growth and development many signaling components, like kinases or phosphatases are conserved between these species and have the potential to amplify signals leading to impact on cellular processes. The diabetes drugs AICAR and A-769662 are examples of compounds that clearly have effects on signal transduction in plants as well as mammals [68, 77].

Amino Acid and Peptide Transporters

Plants have a wide range of peptide transporters, tenfold more than non-plant species [78]. They carry di, tri, tetra and penta peptides and may have especially important functions for mobilization of nitrogen reservoirs in the seeds during germination, and for mobilizing nitrogen from source organs (for example leaves) to sink organs (seeds, storage tubers) during development. Some peptide transporters may be candidates for transport also of xenobiotic compounds.

Plants express several different amino acid transporters that belong to at least five different gene families [64]. LHT1 (Lysine histidine transporter 1) was found to take up neutral and acidic amino acids, and AAP5 (Amino acid permease 5) mediated uptake of cationic amino acids. The presence of different amino acid transporters imply that cationic amino acids can be taken up separately from neutral and acidic amino acids, and hence their regulation may be independent. The LHT1 and AAP5 genes appear to be of special importance because a double mutant, *lht1* × *aap5*, showed overall reduced uptake of amino acids by 78 % (in the 2–50 μM range) [64, 79]. Some drugs are amino acids and zwitterions with a net neutral charge at physiological pH, for example gabapentin and mesalazine possess a carboxyl group and amino group and an aliphatic or aromatic ring, respectively (Fig. 10.2). These compounds may possibly be taken up and transported by amino acid transporters in plants.

NPF (NRT1/PTR) Family of Transporters

NRT1/PTR is a protein family known as nitrate and peptide transporters in plants. This family was recently renamed NPF (Nitrate transporter 1/Peptide transporter family) [80]. Arabidopsis has 53 NPF genes [81], while humans have only six.

Table 10.2 Organic cation/carnitine transporters (OCTs)

Gene acronym and AGI gene code	Tissues showing high expression levels as tested with ATH1 arrays. Highest number recorded is given in brackets for each gene	Tissues showing high expression of genes as tested by the GUS reporter linked with the OCT promoter [71, 72]	Cellular localization according to experimental data [71, 72]
	Data are collected from the TAIR database [73, 74]		Localization predictions [75] are given in brackets
OCT1 At1g73220	Developing seed (late stages), developing stamen, imbibed seed (1671)	Imbibed seeds, flowers, young leaves, cotyledons, root vascular tissue (not meristem)	Cell membrane (extracellular, plasma membrane)
OCT2 At1g79360	Developing seed (late stages), developing stamen/carpel, imbibed seed, mature pollen (374)	No data	Vacuole (extracellular, plasma membrane)
OCT3 At1g16390	Developing seed (early stages), hypocotyl (191)	Basal part of stem epidermis, seeds in young siliques, leaf epidermis	Vacuole (plastid, extracellular, plasma membrane)
OCT4 At3g20660	Developing seed (late stages), dry seeds, pollen (660)	Vascular tissue of stem, leaves and siliques	Vacuole, (cytosol, plasma membrane)
OCT5 At1g79410	Cauline leaf, sepal (113)	No data	Vacuole (extracellular, plasma membrane)
OCT6 At1g16370	Root, stamen (175)	Phloem of stem, major vein of leaf (no data for roots)	Vacuole (extracellular, plasma membrane)

A family of six genes in the model plant *Arabidopsis thaliana*—their tissue expression and cellular localization

The human NPFs belong to the SLC15 group of solute carriers [82]. It has been suggested that in plants NPF nitrate transporters have evolved from peptide transporters. While NRT1.1 import nitrate from the soil into plant roots, the mammalian PepT1 is involved in absorbing di/tripeptides from the intestine in mammals, both are proton-coupled transporters [81]. In contrast to mammalian NPFs (SLC15 group), plant NRT1.1 has been found to transport a wide range of substrates in addition to the primary nitrogen source, i.e. histidine, malate, the plant hormones auxin (indol-3-acetic acid), plant hormones abscisic acid and gibberelin (terpenoids), and glucosinolates [80, 81, 83–85]. Due to this diversity in substrates and involvement in nitrogen sensing, this family of transporters is of interest also regarding transport of xenobiotics like medical drugs.

MATE (Multidrug and Toxic Compound Extrusion) Transporters

MATE transporters are widely distributed and found in all kingdoms. Plants have a large number of MATE genes, for example *Arabidopsis* has 58 different genes encoding MATE transporters [86]. These proteins are involved in transport of secondary compounds in plants (alkaloids, flavonoids) and various detoxification processes. To regulate uptake and homeostasis of iron and aluminum they are probably involved in transport of citrate and other small organic anions [87]. Not many of the MATE proteins have been characterized, but one MATE transporter has so far been found to transport an organic cation, e.g. expression of the *Arabidopsis* ALF5 cDNA in yeast conferred resistance to tetramethylammonium [88]. In *Arabidopsis*, ALF5 was expressed in the epidermis of roots and probably has a function in extrusion of xenobiotics to protect the roots from toxic compounds [88]. However MATE proteins do not only transport compounds across the cell membrane, but also into the conductive tissue (xylem) for transport, and into leaf and seed vacuoles for storage [87]. The MATE transporters are good candidates to be involved in relocalization of xenobiotics, including organic cations, from leaves to seeds and other eatable plants parts, but this still needs to be explored.

RMV1 (Resistant to Methyl Viologen 1) Transporter

The *RMV1* gene (At5g05630) was identified in *Arabidopsis* based on variations among natural *Arabidopsis* lines to paraquat, a quaternary ammonium compound (methylviologen) which inhibits photosynthesis and is widely used as a herbicide [89]. Differences in only one amino acid in the *RMV1* protein resulted in resistance to paraquat, *RMV1* was also shown to be a transporter of natural polyamines (putrescine, spermidine, spermine). Based on the ability to transport quaternary ammonium compounds and polyamines this transporter appear to be a candidate also for metformin, and possibly some other compounds listed in Fig. 10.1. This gene is also a “proof of concept” example that variations in transporters can influence uptake of synthetic compounds, and points to the possibility that suitable plants can be selected for increased or decreased uptake when responsible transporters are known.

Concluding Remarks

A large number of pharmaceuticals are present in the environment. There is a growing awareness that these biological active compounds might have adverse effects on non-target organisms or even accumulate in forage and food. It is important to understand the uptake and translocation mechanisms involved. A successive investigation of possible involved transporters is an interesting approach for identifying potential active uptake processes and an important topic for further research.

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