Title

Characterization of Influx Transporters in the Blood-Brain Barrier: Implications for Drug Delivery

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Characterization of Influx Transporters in the Blood-Brain Barrier:
Implications for Drug Delivery

by

Lawrence Lin

DISSERTATION

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ABSTRACT

Characterization of Influx Transporters in the Blood-Brain Barrier: Implications for Drug Delivery

Lawrence Lin

Membrane transport proteins of the solute carrier (SLC) and ATP-binding cassette (ABC) superfamilies have been found to play essential roles in the absorption, distribution and elimination of drugs. However, much of the pharmacologic research on transporters has focused primarily on the intestine, liver and kidney. Here we focus on transporters in the blood-brain barrier (BBB), a complex barrier that limits penetration of most molecules from the blood into the brain. Research on transporters in the BBB has historically been centered on the ABC transporters that prevent drug entry into the brain, but recent advances suggest that SLC transporters may play an important role in mediating the uptake of many pharmacologic agents into the central nervous system (CNS). The goal of this dissertation research was to understand the role and function of several SLC transporters in the BBB, including the amino acid transporter LAT1, the organic cation transporter MATE1, the amine transporters OCT1, OCT3 and SERT, and the organic anion transporters OATP1A2 and OATP2B1. We performed inhibition and substrate screens using stably-transfected cell lines against a library of CNS-active drugs. We were able to identify four novel, structurally-diverse inhibitors of LAT1 and developed a rat perfusion model to test LAT1 substrates for *in vivo* relevance. For MATE1, we found that the majority of compounds tested from our library were inhibitors of MATE1, and identified 15 novel substrates, suggesting that MATE1 may be involved in the disposition of these drugs. Since organic anion transporting polypeptides are known to play a role in the influx

of molecules into the brain, we performed substrate screens and identified 24 novel substrates of OATP1A2, most of which are cationic drugs. About one-third of the OATP1A2 substrates were also substrates of its rodent ortholog, rat Oatp2. Finally, we found that at high concentrations, metformin is able to inhibit the uptake of two CNS active monoamines, histamine and serotonin, by OCT1, OCT3 and SERT. Though further studies are clearly needed, we posit that reduced absorption of the two monoamines as a result of metformin's effects on OCT1, OCT3 and SERT may contribute to the gastrointestinal side effects associated with metformin use. The research presented here has important implications for CNS drug delivery, as our results have expanded the chemical space, particularly the known substrates, of several transporters expressed in the BBB.

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CHAPTER ONE

Membrane Transporters in Drug Absorption, Disposition and Targeting

Introduction to Membrane Transporters

Hydrophilic compounds, including charged molecules, cannot readily diffuse across membranes, and thus rely on channels, pumps or transporters to move in and out of cells and subcellular organelles. Transporters are membrane-bound proteins that mediate the translocation of small molecules across biological membranes. Given their essential role, it is not surprising that about five percent of all human genes are thought to encode for proteins with transport function[1]. Membrane transporters are widely expressed throughout the body, most notably in the epithelia of major organs such as the liver, intestine, kidney, and in endothelia of organs with barrier functions, such as the brain, testes and placenta. Different transporters are localized to the plasma membrane, as well as to membranes that comprise various subcellular organelles, thus ensuring regulated delivery of required substrates and thereby cellular homeostasis. Many transporters are also expressed in an organ-specific manner, and facilitate the entry and elimination of endogenous and xenobiotic compounds.

The two main transporter superfamilies are the ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies. ABC transporters harness energy from ATP hydrolysis and function as efflux transporters to move solutes against the concentration gradient. SLC transporters, meanwhile, are predominantly facilitative or secondarily-active, and use an electrochemical gradient to facilitate the movement of substrates across membranes or ion gradients generated by ATP-dependent pumps to transport substrates against the concentration gradient.

SLC Transporters Play Important Roles in the Human Body

In humans, there are 395 membrane-spanning SLC transporters that are organized into 52 families. SLC transporters interact with a diverse array of substrates, including inorganic and organic ions[1,2] and range in specificity — from highly selective transporters that interact with a narrow group of substrates, such as amino acid transporters in the SLC7 family[3,4], to transporters that accept a broad range of chemically diverse substrates such as organic anion transporters in the SLCO family[5,6]. Even within a family, such as SLC22, various transporters may differ in their specificity. For example, urate transporter 1 (URAT1, SLC22A12) is selective for uric acid whereas organic anion transporter 1 (OAT1, SLC22A6) tolerates a wide range of organic anions, including various antibiotics, antiviral drugs and endogenous molecules[7]. Both are distinct from organic cation transporter 1 (OCT1, SLC22A1), which prefers organic cations.

Members of the SLC transporter families are important in human physiology. For example, amino acids, which are needed for protein synthesis, are absorbed and reabsorbed by transporters of the SLC1, SLC3, SLC6, SLC7, SLC25 and SLC36 families, many members of which are expressed in the intestine and kidney. In the intestine, amino acids are absorbed from the lumen into the body; in the kidney, amino acids that are filtered out of the bloodstream by the glomerulus are reabsorbed in the proximal tubule by many of the same transporters that are involved in intestinal absorption[8]. Glucose requires members of the SLC2, SLC5 and SLC50 families for intestinal absorption, renal reabsorption and uptake into neurons, erythrocytes, hepatocytes and other cell types[9,10]. Metals often serve as essential cofactors for important enzymes, but toxicities may occur when they are present at too high concentrations. Zinc transporters of the SLC30 and SLC39 families and iron transporters of the SLC11 and SLC40 families regulate zinc and iron levels in the body, respectively. Similarly, water-soluble vitamins

are essential for various processes but require transporters for cellular uptake; for example, SLC19 family members transport folate and thiamine, SLC46 family members transport folate and the SLC52 family transports riboflavin. In the brain, neurotransmitters released into the synapse are taken back into presynaptic neurons through SLC1 and SLC6 transporters.

Transporters in Drug Development

In drug development, there is great interest in transporters from both families. Some drugs taken orally rely on transporters to be taken up into enterocytes for absorption into the systemic circulation. Many drugs interact with uptake and efflux transporters in hepatocytes and proximal tubule cells that are involved in their elimination. Further, transporters are involved in targeting drugs to various tissues that play a role in drug efficacy and toxicity. Transporters often work together to regulate the transport of compounds throughout the body, thus playing an important role in the absorption, distribution and elimination (ADME) of structurally and pharmacologically diverse drugs. As transporters are especially abundant in barrier tissues and organs involved in the absorption and elimination of drugs, drug-drug interactions may occur at these sites, resulting in changes in ADME that may change the efficacy and/or safety profile of a given drug. For example, the package label for cidofovir warns that it can cause severe nephrotoxicity, and advises prescribers to administer probenecid concurrently to block its uptake into the renal cells through organic anion transporters (OATs). As the endogenous role of SLC transporters are often still not yet recognized, one must also consider drug interactions with nutrients and other molecules found in the body through diet. In one such example, it has been reported that grapefruit juice is able to potently inhibit fexofenadine uptake mediated by the organic anion transporting polypeptide 1A2 (OATP1A2, SLCO1A2)[11].

The U.S. Food and Drug Administration has developed guidance documents for the pharmaceutical industry listing transporters thought to be clinically important in drug absorption and disposition, as well as a framework for the studies needed to determine if a drug-drug interaction may occur that can underlie drug toxicities. The 2010 white paper from the International Transporter Consortium lists P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2), organic cation transporters (OCTs) and organic anion transporters (OATs), and organic anion transporting polypeptides (OATPs) as transporters against which new molecular entities should be screened[12]. In 2012, the bile salt export pump (BSEP, ABCB11) and multidrug and toxin extrusion transporters (MATEs, SLC47 family) were suggested to be added to the FDA guidance based on additional scientific data[13]. As our knowledge of drug transporters grows, it is likely that even more transporters will be found to be clinically important in inducing drug-drug interactions and thus added to this list.

The Blood-Brain Barrier

The central nervous system (CNS) is protected against many chemical insults by two diffusion barriers that restrict the entrance of molecules in the blood into the brain, the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). These barriers help to regulate and maintain the extracellular environment in the brain for optimal neuronal function, and also help to protect the brain from chemical toxins and other unwanted compounds. This interface between brain and systemic circulation is crucial not only for regulating brain exposure to xenobiotics in the blood, but also to facilitate the influx of nutrients and other endogenous metabolites into the brain to sustain the high metabolic demands of the CNS, controlling their concentrations and effects in the CNS. Defects in the BBB have been associated with human

diseases such as stroke, septic encephalopathy, HIV-induced dementia, multiple sclerosis and Alzheimer's disease[14].

The BCSFB is primarily located in the choroid plexus, which produces cerebrospinal fluid. However, in most regions of the brain, extracellular fluid is separated from circulating blood by the BBB, which is comprised of specialized endothelial cells that are joined by tight junctions and characterized by high transendothelial electrical resistance, minimal pinocytosis and transcytosis, lack of fenestrations and polarized cells with distinct luminal and abluminal components (Figure 1.1). While small hydrophobic molecules may be able to freely diffuse across the membrane of cells that form the BBB, most compounds require the activity of influx transporters in order to enter the brain. This primarily includes transporters of the SLC superfamily family that bring in glucose, organic acids, hormones, ions and amino acids such as tyrosine, tryptophan and histidine that are precursors of neurotransmitters. Over 60% of known SLC transporters have been found at the blood-brain barrier and blood-CSF barriers[15]. In addition, many ABC efflux transporters are preferentially expressed at the BBB at high levels[16]. In order for compounds to fully cross the blood-brain barrier, they must cross both the luminal and abluminal membranes of brain endothelial cells, and transporter expression can be different on either membrane.
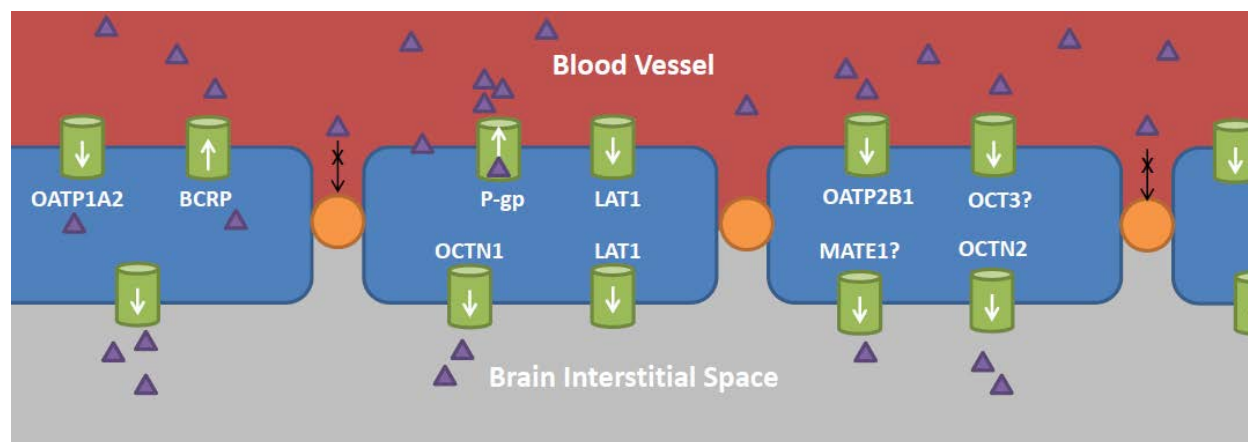
Figure 1.1. Transport of molecules at the blood-brain barrier. Tight junctions (orange circles) and efflux transporters (green cylinders with arrows pointing toward blood vessel) limit paracellular movement. Molecules (purple triangles) can either diffuse across the membrane if they are small and lipophilic, or require transporters (green cylinders) for entry into the brain. Arrows indicate the direction of transport.

Efflux Transporters and Drug-Drug Interactions at the Blood-Brain Barrier

While many SLC transporters help to bring compounds into the brain, ABC transporters primarily restrict entry by pumping compounds out of the brain, particularly hydrophobic molecules that may have diffused in. Of prime importance is P-glycoprotein (P-gp, ABCB1), which is involved in the efflux transport of xenobiotics at the blood-brain barrier on both the luminal and abluminal membranes, and elsewhere in the body, primarily in excretory organs such as the intestine, liver and kidney. In addition, it has been previously reported that P-gp expression is upregulated in a variety of tumor types. Hundreds of substrates have been reported to be a substrate of P-gp, including anticancer agents, beta-blockers, immunosuppressants, among others[17]. As P-gp can be a major barrier to drug delivery to the brain, specific inhibitors

that block P-gp have been developed that can be co-administered with another drug in order to enhance its bioavailability and access to the site of action. For example, administration of elacridar, a potent P-gp inhibitor, in mice resulted in a five-fold increase in brain paclitaxel uptake[18]. The brain/plasma concentration ratio of nelfinavir increased 26-fold in rats with concurrent administration of another P-gp inhibitor, zosuquidar[19].

Other ABC transporters such as the multidrug resistance-associated protein (MRP family) transporters and breast cancer resistance protein (BCRP, ABCG2) perform a similar function in the efflux of xenobiotics from the brain. Given that many SLC transporters are capable of transporting xenobiotics, it is not surprising that some SLC transporters also function as efflux transporters in the brain. For example, organic anion transporter 1 (OAT1, SLC22A6) and organic anion transporter 3 (OAT3, SLC22A8) are known to transport valacyclovir, zidovudine, mercaptopurine, methotrexate and valproic acid[20], and have been detected in the human choroid plexus or BCSFB[21]. The brain/plasma concentration ratio of 6-mercaptopurine increased 4.4-fold in rats with concurrent administration of probenecid and p-aminohippuric acid, known OAT inhibitors[22].

These results suggest the possibility of transporter mediated drug-drug interactions at the blood-brain barrier. However, due to the difficulty of obtaining brain drug concentration levels in humans, most of these studies have been performed in rodent models. One non-invasive method to study drug levels in human patients is through the use of PET imaging. In a 2005 study, it was reported that the AUC_{brain}/AUC_{blood} ratio of ^{11}C-verapamil increased by 88 percent in the presence of cyclosporine, a P-gp inhibitor[23]. However, whether clinically-relevant pharmacological modulation of efflux transport occurs at the human blood-brain barrier is still up for debate[24]. Nevertheless, development of a P-gp inhibitor remains a priority of the

pharmaceutical industry; a search for the term "P-gp inhibitor" on the ClinicalTrials.gov website returned 82 studies as of May 1, 2015.

Influx Transporters at the Blood-Brain Barrier

Previous research on transporters expressed at the blood-brain barrier has primarily focused on P-glycoprotein, as it has been well characterized in other tissues such as the liver and kidney. Meanwhile, not as much is known about SLC transporters and the endogenous role that they play in the body, let alone their function with xenobiotics. One potential challenge is the isolation of the BBB from other cell types that are present in the brain, such as neurons, astrocytes and pericytes. Methods involving mechanical homogenization, density gradient centrifugation with dextran, and sieves have been successfully used for specific cell isolation[25,26]. More recently, laser capture microdissection using an antibody specific to endothelial cells has been used to enhance microvessel isolation by limiting contamination from other cell types[27].

There have been several studies aimed at identifying genes expressed at the rodent BBB using a variety of approaches, including suppression subtractive hybridization[28], serial analysis of gene expression[29], and microarray approaches[30]. Purification and analysis of endothelial cells from the murine brain, liver and lung have shown a BBB-specific transcriptome[30]. Our laboratory used real-time PCR to profile the expression of 359 SLC and 49 ABC transporters from two human brain samples, and we were able to detect the expression of 244 SLC and 42 ABC transporters in isolated brain microvessels[3]. Furthermore, a proteomic profiling approach using liquid chromatography-tandem mass spectrometry has also been used to quantify protein expression of transporters and receptors at the BBB[31]. While efforts to develop a census of

transporters at the BBB are underway, a more in-depth look at specific SLC transporters at the BBB and their function in bringing drugs into the CNS is lacking.

Uptake of Drugs into the Brain

Development of CNS drugs has faced considerable challenges that have reduced success rates relative to the development of drugs for non-CNS indications. The challenges that have limited the successful development of CNS active drugs include an incomplete understanding of the BBB that restricts entry of many small- and large-molecule drugs, and in particular, an incomplete understanding of the transporters that populate the BBB and are involved in moving molecules in and out of the brain. Many CNS-active drugs have been on the market for many years, yet there is a paucity of understanding how these drugs cross the blood-brain barrier into the brain. It is generally accepted that the majority of drugs simply diffuse across the BBB; for example, Lipinski's "Rule of Five" generally focuses on lipophilicity and hydrogen-bond formation, properties focused on determining if a compound is subject to passive diffusion across lipid bilayers[32]. However, analyses of their physicochemical properties suggest that this is unlikely to be the case due to their high molecular weights, hydrophilicity or charge at pH 7.4. Furthermore, highly lipophilic drugs are often substrates of ABC efflux transporters, which can dramatically limit their brain penetration.

Wu and Benet developed the Biopharmaceutics Drug Disposition Classification System (BDDCS) in order to simplify classification of drugs to predict *in vivo* pharmacokinetic behavior from *in vitro* studies, as well as increase the number of Class 1 (high solubility, extensively metabolized) drugs eligible for bioequivalence study waivers[33,34]. A follow-up study confirmed the utility of this classification system as applied to 927 drugs[35], and it has been suggested that

new molecular entities be classified using BDDCS in order to easily predict potential drug-drug interactions, especially as BDDCS classes may be predicted computationally using molecular structure information alone[36]. Under this classification system, transporters are likely to be essential for a compound getting to a site of action for class 3 (high solubility and low metabolism) and class 4 (low solubility and low metabolism) compounds[37]. Broccatelli *et al*. found that the use of BDDCS was able to accurately predict CNS drug disposition for more than 90% of drugs in their dataset[38].

In vitro **and** *in vivo* **Models of Blood-Brain Barrier Transport**

There exist several *in vitro* models that can be used for studying transporters at the BBB, but each suffers from significant limitations. Stably-transfected cell lines are useful in studying the function and characteristics of a particular transporter, but do not represent the actual expression of a transporter at the BBB or allow for the study of multiple transporters simultaneously. Transporters are typically transfected into easy to grow cell lines that are not of brain origin, which limits their accuracy in modeling the BBB. Furthermore, depending on the mechanism of transport, additional experimental consideration may be required for good performance in an uptake study; the study of pH-dependent transporters such as MATE1 may require lowering of intracellular pH using ammonium chloride pretreatment. Brain capillary endothelial cells from primary cells or derivative cell lines may be used to study multiple transporters at once, but often lose transporter expression and activity over time and can be difficult to work with. These cells also may not efficiently form tight junctions once removed from the brain or polarize correctly. One promising model uses embryonic stem cells that are

differentiated into endothelial cells and cocultured with astrocytes, and show high transendothelial electrical resistance and appropriate expression of transporters[39].

Animal models can also be used to study transporters at the BBB. For instance, they are often used to calculate the drug concentration ratio between brain interstitial fluid and plasma at steady state ($K_{p,uu}$). If passive diffusion processes dominate, the ratio will be close to 1, whereas if the drug is actively pumped out of the brain by efflux transporters, it will be less than 1. The converse is also true, if a drug accumulates in the brain through uptake transporters, then the $K_{p,uu}$ value will be greater than 1. There are a few transporter-knockout rat and mouse models currently available, but again this only allows for the study of one transporter at a time, and systemic effects resulting from a transporter's absence from other tissues may be a confounding factor. Perfusion models have also been developed to study brain uptake directly in animals, but generally necessitates the use of chemical inhibitors that may or may not be specific to a transporter or interest and have undesired off-target effects.

Focus of Dissertation and Key Questions

This dissertation primarily focuses on influx transporters at the BBB. The BBB remains a significant challenge to CNS drug development. Membrane transporters are known to be present at the blood-brain barrier, and likely play a role in the distribution, efficacy and toxicity of drugs in the CNS. Though considerable information is available about the role of efflux transporters in the BBB, notably BCRP and P-gp, little is known about influx transporters. Our laboratory recently quantified mRNA expression levels of influx and efflux transporters at the blood-brain barrier using quantitative real-time polymerase chain reaction. We were able to compare expression of 359 SLC transporters in isolated human brain microvessels with whole

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brain as well as kidney and liver tissue. We identified several transporters previously not known to be in the BBB (*e.g*., OCT3), at both the mRNA and protein levels. We also identified a number of SLC transporters that had significantly greater expression levels in microvessel preparations from human brain samples relative to the whole brain homogenates. These transporters included LAT1 (SLC7A5), OCTN2 (SLC22A5), and OATP1A2 (SLCO1A2). It was determined that drug transporters with known important roles in renal and hepatic drug disposition were expressed at similar or higher levels in the human BBB[16].

In this dissertation, we considered the role of transporters in drug development and discuss how current drugs target SLC transporters and how future drugs can be developed that target other SLC transporters for the treatment of human disease. We performed experiments aimed at understanding the role of the following transporters in the BBB: the amino acid transporter LAT1 (SLC7A5), the organic cation transporters OCT3 (SLC22A3) and MATE1 (SLC47A1), and the organic anion transporters OATP1A2 and OATP2B1 (Figure 1.2). The pharmacologic role of these transporters in the BBB has not been well characterized. In Table 1.1 below, we provide a summary of the substrate specificity and tissue distribution of each of these transporters. In this dissertation, we primarily used a library of CNS active drugs from a variety of therapeutic classes, as shown in Table 1.2.
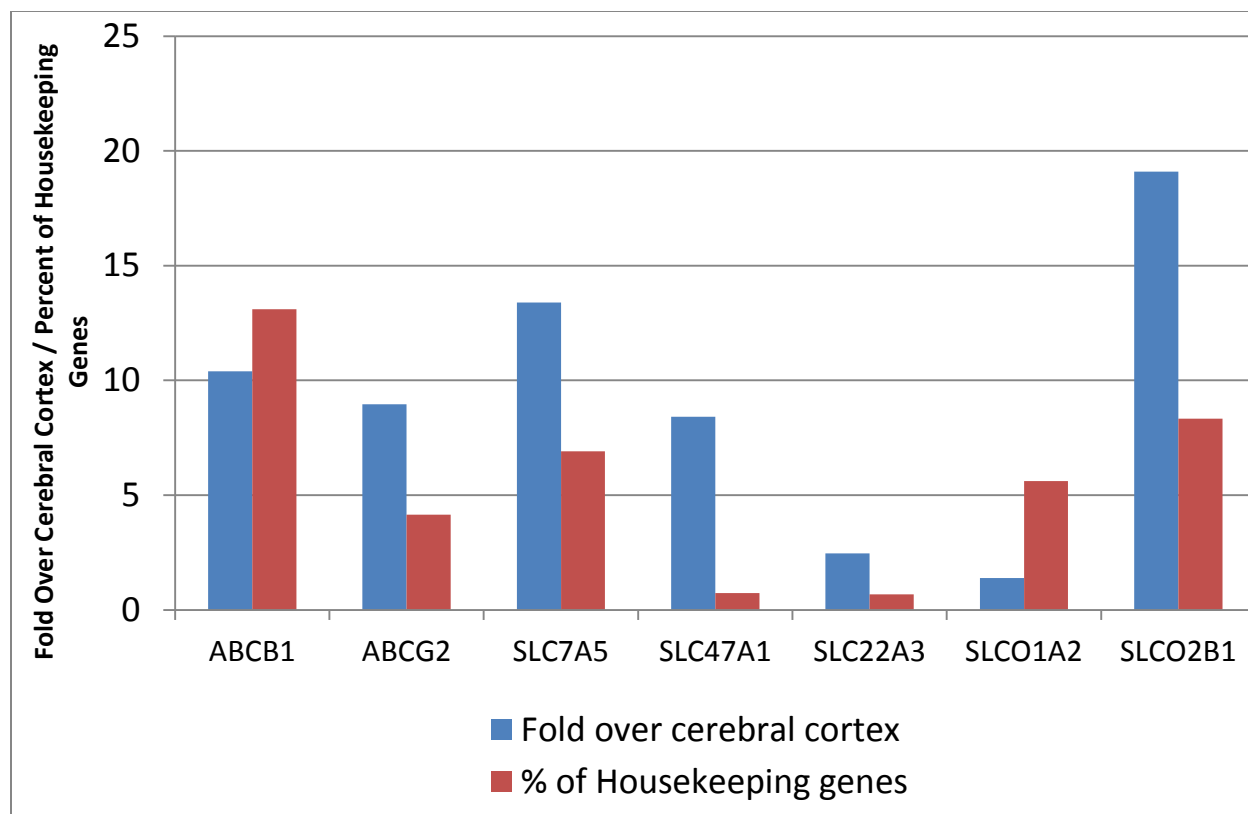
Figure 1.2. Transporter expression at the BBB. For each transporter, we show the enrichment of its expression in the BBB, *i.e.*, the fold expression in isolated human brain microvessels over whole brain, as well as the percent expression relative to housekeeping genes. Higher enrichment values suggest a greater role of the transporter in the BBB relative to the brain.

Table 1.1. Summary of substrate specificity and tissue distribution for selected transporters in

the blood-brain barrier.

	Gene	Reported Substrates	Tissue Distribution
LAT1	*SLC7A5*	gabapentin, pregabalin, melphalan, baclofen	BBB, colon, placenta, testes
MATE1	*SLC47A1*	cimetidine, metformin, topotecan, procainamide, acyclovir	BBB, kidney, adrenal gland, liver, muscle, testes
OCT3	*SLC22A3*	metformin, clonidine, desipramine, imipramine, procainamide	ubiquitous
OATP1A2	*SLCO1A2*	pitavastatin, rosuvastatin, digoxin, fexofenadine, imatinib, levofloxacin, methotrexate, saquinavir	BBB, intestine, liver, kidney
OATP2B1	*SLCO2B1*	rosuvastatin, fluvastatin, fexofenadine, estrone sulfate, bromosulphalein	BBB, liver, intestine, heart

Table 1.2. CNS-active drugs in our drug library, grouped by therapeutic class.

Sedatives	Antipsychotics	Antidepressants	Opioids	Antiepileptics	Vasoactive	Parkinson's	Alzheimer
Alprazolam	Amisulpride	Amfebutamone	Apomorphine	Carbamazepine	Eletriptan	Amantadine	Aniracetam
Bromazepam	Aripiprazole	Atomoxetine	Buprenorphine	Ethosuximide	Frovatriptan	Bromocriptine	Donepezil
Brotizolam	Chlorpromazine	Buspirone	Fentanyl	Felbamate	Lofexidine	Budipine	Galantamine
Clonazepam	Clozapine	Citalopram	Hydrocodone	Lamotrigine	Nimodipine	Cabergoline	Minaprine
Diazepam	Haloperidol	Clomipramine	Levomethadyl (LAAM)	Meprobamate	Riluzole	Carisoprodol	Moclobemide
Eszopiclone	Nemonapride	Duloxetine	Morphine	Oxcarbazepine	Sumatriptan	Cyclobenzaprine	Rasagiline
Lorazepam	Olanzapine	Escitalopram	Oxycodone	Phenytoin	Verapamil	Nicergoline	Rivastigmine
Midazolam	Paliperidone	Fluoxetine	Remifentanil	Tiagabine	Vinpocetine	Pergolide	Selegiline
Propofol	Perospirone	Fluvoxamine	Tramadol	Topiramate	Zolmitriptan	Pramipexole	Tacrine
Quazepam	Quetiapine	Indeloxazine	Dextropropoxyphene	Valproic Acid		Ropinirole	Terguride
Ramelteon	Risperidone	Milnacipran	Propoxyphene	Vigabatrin		Talipexole	
Thiopental	Sertindole	Mirtazapine		Zonisamide			
Zaleplon	Sulpiride	Nortriptyline		Levetiracetam		**Other**	**Addictive Behavior**
Zolpidem	Ziprasidone	Paroxetine				Diphenhydramine	Acamprosate
	Zotepine	Reboxetine				Gabapentin	Flumazenil
		Sertraline	**Antiemetics**	**Stimulants**	**Cannabinoids**	Pregabalin	Nalmefene
		Doxepin	Aprepitant	Amphetamine	Cannabidiol	Piracetam	Naltrexone
		Trazodone	Cevimeline	Caffeine	Dronabinol	Modafinil	Nicotrol
		Tianeptine	Metoclopramide	Dexmethyl-phenidate	Rimonabant	Ropivacaine	Varenicline
		Venlafaxine	Tropisetron	Methylphenidate			

The major questions addressed in this dissertation were:

1. Are any of the CNS active drugs shown in Table 1.2 inhibitors of BBB transporters at concentrations that are achieved clinically?

2. Do any of these CNS active drugs utilize BBB membrane transporters for brain uptake into the CNS?

3. Can *in vivo* evidence show that transporters play an important role in transporting drugs into the brain?

In this dissertation, we primarily used a human embryonic kidney cell line (HEK-293) stably transfected with different SLC transporters known to be expressed at the blood-brain barrier. We performed inhibition and substrate screening assays to understand if drugs known to have central nervous system activity were in fact inhibitors or substrates of these transporters. We further characterized the transport kinetics of these drugs for each transporter. We also developed and used rat perfusion models to demonstrate that these transporters play an important role in the brain uptake of these drugs in a whole organism.

Summary of Dissertation Chapters

Transporters mediate drug absorption, distribution, metabolism and elimination (ADME) mechanisms throughout the body by controlling the ability of a drug to cross lipid bilayers in and out of tissues and cells. Their importance in the body suggests that they might be a good target for drug development. At the BBB, transporters are present at both the luminal and abluminal membranes, where influx transporters bring nutrients into the brain and efflux transporters keep out xenobiotics and drugs. The goals of this dissertation are to further study the role of transporters recently identified at the BBB and identify new ligands of these transporters to gain a greater understanding of the role of these transporters in moving drugs in and out of various tissues. We focused on transporters from four classes of uptake transporters: the amino acid transporter LAT1, the organic cation transporter MATE1, the amine transporters OCT1, OCT3 and SERT, and the organic anion transporters OATP1A2 and OATP2B1.

Chapter 2. Solute Carrier Transporters as Therapeutic Targets

Solute carrier (SLC) transporters play important roles in the body in the handling of endogenous substrates and xenobiotics, and defects in these transporters often result in human disease. Studies of Mendelian diseases caused by defects in SLC transporters have revealed new information about the function of many of these transporters, and genome-wide association studies have implicated transporters in various diseases. While some currently marketed drugs already target SLC transporters, namely diuretics, antidepressants and antidiabetics, we present major opportunities for developing drugs that target transporters for new indications, such as gout, liver disease and cancer. We also discuss some potential strategies that may be used in the targeting of SLC transporters for therapeutic purposes, including enhancement/inhibition of transporter function, development of substrates that circumvent transporters, and gene therapy to restore transporter function.

Chapter 3. Identification of Novel Ligands of the L-Type Amino Acid Transporter 1 (LAT1)

Amino acids are essential to the function of the human body, and one of the major transport systems for large neutral amino acids is LAT1. In addition to being overexpressed in various cancers, LAT1 expression has also been found to be enhanced at the blood-brain barrier. We screened a library of CNS-active drugs using a LAT1-overexpressing cell line to identify novel ligands of this transporter. We were able to identify several new inhibitors of LAT1, namely duloxetine, paroxetine, fluphenazine and clomipramine. In addition, we performed trans-stimulation assays to find novel substrates of LAT1. While we were unable to demonstrate

that any of our compounds were indeed substrates of LAT1, identification of four new inhibitors suggests that the chemical space of this transporter is larger than previously anticipated.

Chapter 4. Identification of Novel Substrates of the Organic Cation Transporter, MATE1

Organic cation transporters are known to participate in ADME of various drugs in a variety of tissues, notably the kidney and liver. MATE1 has been shown to play an important role in the kidney in mediating elimination of various drugs, and drug-drug interactions have been demonstrated when multiple drugs that inhibit MATE1 are taken concurrently. In this study, we screened a library of CNS-active drugs and identified multiple novel substrates of MATE1, including bromocriptine, buprenorphine, caffeine, chlorpromazine, cyclobenzaprine, diphenhydramine, frovatriptan, levetiracetam, riluzole, rizatriptan, rocuronium, sulpiride, sumatriptan, talipexole, tramadol, triprolidine, valproic acid and zolmitriptan. We show that sulpiride is taken up by MATE1 with a V_{max} of 0.213 µmol/min/µg protein and K_m of 7.6 µM. These results have great implications; MATE1 may be involved in the renal clearance of many of these drugs, which was previously unknown.

Chapter 5. Prediction and Testing of Enzyme and Transporter Off-Targets for Metformin

In this chapter, we look at the role of some BBB transporters in other tissues. Metformin is an established first-line treatment for patients with type 2 diabetes. Despite its beneficial effects, metformin causes gastrointestinal (GI) side effects that limit the use of the drug. Histamine and serotonin have potent effects in the GI tract. Using chemoinformatic and experimental methods, we show that metformin interacts with amine transporters that transport histamine and serotonin (OCT1, OCT3 and SERT) and intestinal diamine oxidase (DAO), which

plays a role in histamine disposition. We found that metformin inhibited histamine and serotonin uptake by OCT1, OCT3 and SERT in a dose-dependent manner, with inhibition of OCT1-mediated histamine uptake most potent (K_i = 1.5 mM). Using Similarity Ensemble Approach, we predicted that metformin interacts with diamine oxidase (DAO) and *in vitro* experiments validated this prediction. The K_i of metformin for DAO (8.6 ± 3.1 mM) was in the expected range of intestinal concentrations of metformin after therapeutic doses. These results demonstrate that metformin inhibits intestinal amine transporters and DAO, suggesting a possible mechanism for metformin-associated side effects.

Chapter 6. Identification of Novel Substrates of the Organic Anion Transporting Polypeptide 1A2 (OATP1A2)

The blood-brain barrier (BBB) represents a significant barrier for drugs to cross from systemic circulation into the brain. However, uptake transporters including members of the organic anion transporting polypeptide family have been localized at the BBB and are known to transport drugs and other xenobiotics. Screening a library of 103 CNS-active drugs against both OATP1A2 and OATP2B1, we identified 24 novel substrates of OATP1A2, including amfebutamone, R-apomorphine, buprenorphine, donepezil, escitalopram, fluvoxamine, galantamine, haloperidol, lamotrigine, lofexidine, methylphenidate, L-threo-methylphenidate, minaprine, mirtazapine, moclobemide, olanzapine, pimozide, propranolol, rivastigmine, varenicline, ropinirole, sulpiride, tramadol and trazodone. Many of these novel substrates are basic drugs, suggesting that OATP1A2 has an important role in the disposition of organic cations. We also determined kinetic parameters (K_m and V_{max}) of several of these drugs for OATP1A2. Approximately one third of the novel substrates were found to also be substrates of

the closest rodent ortholog of OATP1A2, Oatp2.   In contrast to OATP1A2, OATP2B1 had limited interactions with the CNS active drugs, *i.e*., none of the compounds in our library were found to be substrates of OATP2B1. These results provide further insight into the substrate specificity of OATP transporters and implicate OATP1A2 in the brain uptake of many drugs.

References

1.   Hediger, M. A., Clémençon, B., Burrier, R. E. & Bruford, E. A. The ABCs of membrane transporters in health and disease (SLC series): Introduction. *Mol. Aspects Med.* **34,** 95–107 (2013).

2.   Schlessinger, A., Yee, S. W., Sali, A. & Giacomini, K. M. SLC classification: an update. *Clin. Pharmacol. Ther.* **94,** 19–23 (2013).

3.   Geier, E. G., Schlessinger, A., Fan, H., Gable, J. E., Irwin, J. J., Sali, A. & Giacomini, K. M. Structure-based ligand discovery for the Large-neutral Amino Acid Transporter 1, LAT-1. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 5480–5 (2013).

4.   Fotiadis, D., Kanai, Y. & Palacín, M. The SLC3 and SLC7 families of amino acid transporters. *Mol. Aspects Med.* **34,** 139–58 (2013).

5.   Izumi, S., Nozaki, Y., Komori, T., Maeda, K., Takenaka, O., Kusano, K., Yoshimura, T., Kusuhara, H. & Sugiyama, Y. Substrate-dependent inhibition of organic anion transporting polypeptide 1B1: Comparative analysis with prototypical probe substrates estradiol-17β-glucuronide, estrone-3-sulfate, and sulfobromophthalein. *Drug Metab. Dispos.* **41,** 1859–66 (2013).

6.   Hagenbuch, B. & Stieger, B. The SLCO (former SLC21) superfamily of transporters. *Mol. Aspects Med.* **34,** 396–412 (2013).

7.   Koepsell, H. & Endou, H. The SLC22 drug transporter family. *Pflugers Arch.* **447,** 666–76 (2004).

8.   Bröer, S. Apical transporters for neutral amino acids: physiology and pathophysiology. *Physiology (Bethesda).* **23,** 95–103 (2008).

9.   Mueckler, M. & Thorens, B. The SLC2 (GLUT) family of membrane transporters. *Mol. Aspects Med.* **34,** 121–138 (2013).

10.   Wright, E. M. Glucose transport families SLC5 and SLC50. *Mol. Aspects Med.* **34,** 183–96 (2013).

11.   Dresser, G. K., Bailey, D. G., Leake, B. F., Schwarz, U. I., Dawson, P. A., Freeman, D. J. & Kim, R. B. Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clin. Pharmacol. Ther.* **71,** 11–20 (2002).

12.   Giacomini, K. M., Huang, S. M., Tweedie, D. J., Benet, L. Z., Brouwer, K. L., Chu, X., Dahlin, A., Evers, R., Fischer, V., Hillgren, K. M., Hoffmaster, K. A., Ishikawa, T., Keppler, D., Kim, R. B., Lee, C. A., Niemi, M., Polli, J. W., Sugiyama, Y., Swaan, P. W., Ware, J. A., Wright, S. H., Yee, S. W., Zamek-Gliszczynski, M. J. & Zhang, L. Membrane transporters in drug development. *Nat. Rev. Drug Discov.* **9,** 215–236 (2010).

13.   Tweedie, D., Polli, J. W., Berglund, E. G., Huang, S. M., Zhang, L., Poirier, a, Chu, X. & Feng, B. Transporter studies in drug development: experience to date and follow-up on decision trees from the International Transporter Consortium. *Clin. Pharmacol. Ther.* **94,** 113–25 (2013).

14. Ballabh, P., Braun, A. & Nedergaard, M. The blood-brain barrier: An overview: Structure, regulation, and clinical implications. *Neurobio. of Dis.* **16,** 1–13 (2004).

15. Saunders, N. R., Daneman, R., Dziegielewska, K. M. & Liddelow, S. A. Transporters of the blood-brain and blood-CSF interfaces in development and in the adult. *Mol. Aspects Med.* **34,** 742–52 (2013).

16. Geier, E., Chen, E., Webb, A., Papp, A., Yee, S., Sadee, W. & Giacomini, K. Profiling solute carrier transporters in the human blood-brain barrier. *Clin. Pharmacol. Ther.* 1–4 (2013). doi:10.1038/clpt.2013.175

17. Amin, M. L. P-glycoprotein inhibition for optimal drug delivery. *Drug Target Insights* **7,** 27–34 (2013).

18. Kemper, E. M., Van Zandbergen, A. E., Cleypool, C., Mos, H. A., Boogerd, W., Beijnen, J. H. & Van Tellingen, O. Increased penetration of paclitaxel into the brain by inhibition of P-glycoprotein. *Clin. Cancer Res.* **9,** 2849–2855 (2003).

19. Anderson, B. D., May, M. J., Jordan, S., Song, L., Roberts, M. J. & Leggas, M. Dependence of nelfinavir brain uptake on dose and tissue concentrations of the selective P-glycoprotein inhibitor zosuquidar in rats. *Drug Metab. Dispos.* **34,** 653–659 (2006).

20. Eyal, S., Hsiao, P. & Unadkat, J. D. Drug interactions at the blood-brain barrier: fact or fantasy? *Pharmacol. Ther.* **123,** 80–104 (2009).

21. Alebouyeh, M., Takeda, M., Onozato, M. L., Tojo, A., Noshiro, R., Hasannejad, H., Inatomi, J., Narikawa, S., Huang, X.-L., Khamdang, S., Anzai, N. & Endou, H.

Expression of human organic anion transporters in the choroid plexus and their interactions with neurotransmitter metabolites. *J. Pharmacol. Sci.* **93,** 430–6 (2003).

22. Deguchi, Y., Yokoyama, Y., Sakamoto, T., Hayashi, H., Naito, T., Yamada, S. & Kimura, R. Brain distribution of 6-mercaptopurine is regulated by the efflux transport system in the blood-brain barrier. *Life Sci.* **66,** 649–662 (2000).

23. Sasongko, L., Link, J. M., Muzi, M., Mankoff, D. A., Yang, X., Collier, A. C., Shoner, S. C. & Unadkat, J. D. Imaging P-glycoprotein transport activity at the human blood-brain barrier with positron emission tomography. *Clin. Pharmacol. Ther.* **77,** 503–514 (2005).

24. Kalvass, J. C., Polli, J. W., Bourdet, D. L., Feng, B., Huang, S.-M., Liu, X., Smith, Q. R., Zhang, L. K. & Zamek-Gliszczynski, M. J. Why clinical modulation of efflux transport at the human blood-brain barrier is unlikely: the ITC evidence-based position. *Clin. Pharmacol. Ther.* **94,** 80–94 (2013).

25. Dallaire, L., Tremblay, L. & Béliveau, R. Purification and characterization of metabolically active capillaries of the blood-brain barrier. *Biochem. J.* **276 (Pt 3,** 745–752 (1991).

26. Bressler, J., Clark, K. & O'Driscoll, C. Assessing blood-brain barrier function using in vitro assays. *Methods Mol. Biol.* **1066,** 67–79 (2013).

27. Murugesan, N., MacDonald, J., Ge, S. & Pachter, J. S. Probing the CNS microvascular endothelium by immune-guided laser-capture microdissection coupled to quantitative rt-pcr. *Methods Mol. Biol.* **755,** 385–394 (2011).

28. Li, J. Y., Boado, R. J. & Pardridge, W. M. Blood-brain barrier genomics. *J. Cereb. Blood Flow Metab.* **21,** 61–68 (2001).

29. Enerson, B. E. & Drewes, L. R. The rat blood-brain barrier transcriptome. *J. Cereb. Blood Flow Metab.* **26,** 959–973 (2006).

30. Daneman, R., Zhou, L., Agalliu, D., Cahoy, J. D., Kaushal, A. & Barres, B. A. The mouse blood-brain barrier transcriptome: A new resource for understanding the development and function of brain endothelial cells. *PLoS One* **5,** (2010).

31. Uchida, Y., Ohtsuki, S., Katsukura, Y., Ikeda, C., Suzuki, T., Kamiie, J. & Terasaki, T. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *J. Neurochem.* **117,** 333–45 (2011).

32. Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **64,** 4–17 (2012).

33. Wu, C. Y. & Benet, L. Z. Predicting drug disposition via application of BCS: Transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm. Res.* **22,** 11–23 (2005).

34. Benet, L. Z., Amidon, G. L., Barends, D. M., Lennernäs, H., Polli, J. E., Shah, V. P., Stavchansky, S. A. & Yu, L. X. The use of BDDCS in classifying the permeability of marketed drugs. *Pharm. Res.* **25,** 483–8 (2008).

35. Benet, L. Z., Broccatelli, F. & Oprea, T. I. BDDCS applied to over 900 drugs. *AAPS J.* **13,** 519–47 (2011).

36. Broccatelli, F., Cruciani, G., Benet, L. Z. & Oprea, T. I. BDDCS class prediction for new molecular entities. *Mol. Pharm.* **9,** 570–80 (2012).

37. Shugarts, S. & Benet, L. Z. The role of transporters in the pharmacokinetics of orally administered drugs. *Pharm. Res.* **26,** 2039–54 (2009).

38. Broccatelli, F., Larregieu, C. A., Cruciani, G., Oprea, T. I. & Benet, L. Z. Improving the prediction of the brain disposition for orally administered drugs using BDDCS. *Adv. Drug Deliv. Rev.* **64,** 95–109 (2012).

39. Lippmann, E. S., Azarin, S. M., Kay, J. E., Nessler, R. A., Wilson, H. K., Al-Ahmad, A., Palecek, S. P. & Shusta, E. V. Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. *Nature Biotechnology* **30,** 783–791 (2012).

CHAPTER TWO

Solute Carrier Transporters as Therapeutic Targets

Introduction

Given the key physiological roles of solute carrier transporters, it is not surprising that mutations in these genes have been associated with human disease. Mutations in 20% of the genes encoding known SLC transporters in humans have been associated with Mendelian disease, and it is likely that mutations in more SLC transporters will be found to be causal for some of the remaining half of Mendelian diseases with no known cause (Figure 2.1). The defective transporters or transporter deficiencies that cause these diseases result in a wide variety of symptoms that affect almost all organ systems; although some of these diseases are considered to be benign, others cause serious illness and death (Figure 2.2).
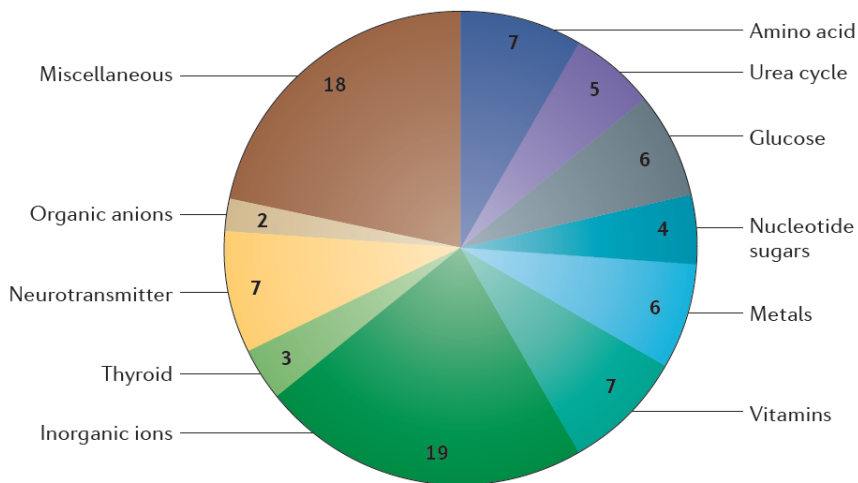
Figure 2.1. SLC transporters implicated in Mendelian disease, grouped by substrate type. A total of 84 solute carrier (SLC) transporters have been implicated in Mendelian diseases. The number in each segment indicates the number of transporters.
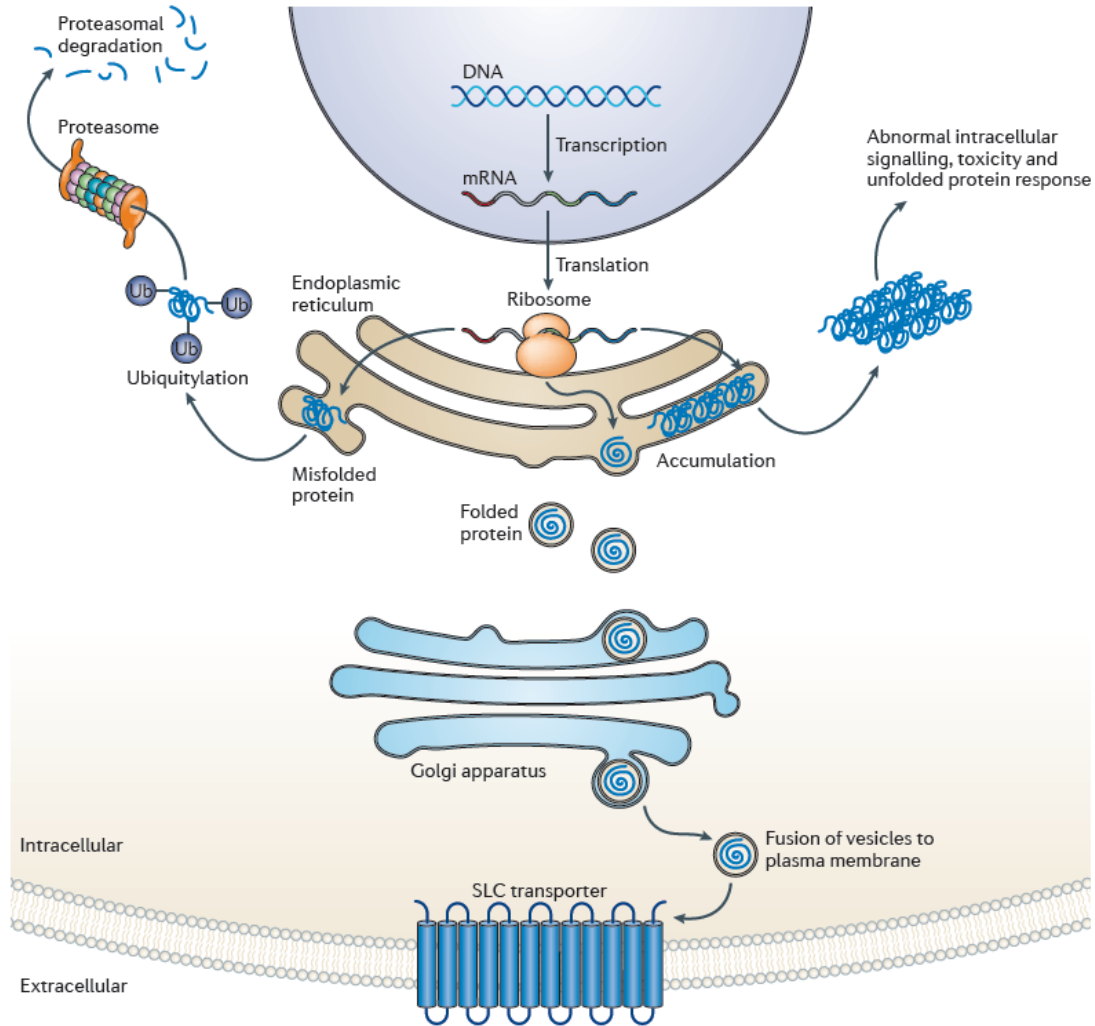
Figure 2.2. Overview of the different types of mutations in SLC transporter genes, and their effects. Transcription occurs in the nucleus and translation in the ribosomes. Normally folded proteins translocate in vesicles across the endoplasmic reticulum and to the Golgi apparatus. Once through the Golgi, proteins are trafficked to the cell surface membrane, where they fuse to the plasma membrane to form the functional solute carrier (SLC) transporter. Mutations in the gene encoding the transporter may result in poor transporter function owing to several different factors. First, improper translation could result in a misfolded protein. In this case, the misfolded protein may be ubiquitylated and degraded in the proteasome. Second, the SLC transporter

protein may not be trafficked to the cell surface membrane. Finally, mutations in the gene could cause an intracellular accumulation of the misfolded protein, resulting in abnormal intracellular signalling and initiation of the unfolded protein response to degrade the protein. Drugs can affect any of the various steps to alter transport function. For example, riluzole increases the RNA transcript levels of excitatory amino acid carrier 1 (EAAC1), ultimately resulting in an enhanced uptake of glutamate from the neuronal synapse.

SLC transporter gene polymorphisms that are associated with common disease have generally been identified through the genotyping of candidate genes or from genome-wide association studies (GWASs). Examples of transporter genes of which polymorphisms have been associated with human traits or disease include: *SLC22A4* and *SLC22A5,* which are associated with inflammatory bowel disease[1–3]; *SLC2A9, SLC22A11* and *SLC22A12,* which are associated with gout and uric acid levels[4–6]; *SLCO1B1* and *SLCO1B3,* which are associated with high bilirubin levels[7–10]; and *SLC24A5* and *SLC45A2,* which are associated with skin color[11,12]. Many of the polymorphisms implicated in human disease are non-synonymous —that is, they cause amino-acid substitutions in the encoded transporter that reduce transporter function or expression levels. For example, non-synonymous variants of the uric acid transporters encoded by *SLC2A9*, *SLC22A11* and *SLC22A12* show reduced uric acid transport[13]. A reduced-function variant will reabsorb less uric acid from the urine. As a result, hypouricemia can occur, thus reducing the risk of gout. Polymorphisms in the genes encoding the hepatic transporters OATP1B1 (*SLCO1B1)* and OATP1B3 (*SLCO1B3*) provide a second example. These transporters are responsible for the uptake of conjugated bilirubin — the metabolic breakdown product of heme catabolism — into hepatocytes[14]. From there, it is excreted into the bile. Common polymorphisms in the genes of

transporters that are involved in the hepatic bilirubin elimination pathway may lead to hyperbilirubinemia and jaundice. Moreover, the complete loss of the genes encoding OATP1B1 and OATP1B3 results in a rare disease known as Rotor syndrome[15]. This example, wherein a common reduced-function gene polymorphism is associated with a less serious phenotype, whereas a rare, more severe defect in the same gene causes a Mendelian disease, is not uncommon in human genetic studies.

There have been several transporters discovered through GWA studies that were not previously known to be important in human disease, including: SLC30A8, a pancreatic beta-cell-specific zinc transporter, a genetic polymorphism of which has been associated with diabetes[16,17]; SLC14A1, a urea transporter, variants of which have been associated with bladder cancer[18]; SLC4A7, a bicarbonate transporter for which certain polymorphisms have been associated with abnormal blood pressure[19,20] or with breast cancer[21]; and SLC35F3, a nucleoside-sugar transporter that also transports thiamine, some polymorphisms of which have been associated with increased blood pressure and predicted to cause disturbances in cardiac and autonomic function[22]. Variants in *SLC30A8* have been associated with type 2 diabetes in several GWA studies and across different ethnic groups. A non-synonymous variant in *SLC30A8*, with a high allele frequency (>25% in Caucasians and Asians), Arg325Trp (rs13266634), was identified through a GWAS[16]. This risk allele, p.Arg325Trp, is associated with reduced susceptibility to type 2 diabetes and higher zinc transport activity. It was later demonstrated in *Slc30a8*-knockout mice that the transporter plays a role in transporting zinc ions from the cytoplasm into insulin granules[23], and further studies suggested that zinc may have an important role as an endogenous regulator of insulin homeostasis[24,25]. Furthermore, although *Slc30a8*-deficient mice exhibit phenotypic variability, they generally show lower blood-insulin levels and increased insulin

clearance[26]. Interestingly, a recent paper by Flannick *et al.*[27] demonstrated that carriers of loss-of-function and rare missense variants in *SLC30A8* were at reduced risk for type 2 diabetes. The group performed sequencing or genotyping on over 150,000 individuals and demonstrated that loss-of-function missense *SLC30A8* variants that led to truncation of the transporter protein actually protected individuals from type 2 diabetes. Overall, these human genetic studies suggest that *SLC30A8* could be a potential target for the treatment and/or prevention of type 2 diabetes.

Several classes of marketed drugs target well-known SLC transporters, such as neurotransmitter transporters, and human genetic studies have provided powerful insights into the roles of more recently characterized SLC transporters in both rare and common diseases, indicating a wealth of new therapeutic opportunities. Many SLC transporters have been identified as 'druggable'— that is, they contribute to a disease phenotype and could be modulated by drugs[28]. In Table 2.1, we list transporters implicated in Mendelian disease that may potentially be targets of new drugs. Databases[29–33] such as the Sophic Integrated Druggable Genome Database, the DrugBank database and recent review articles[30,34] provide useful and detailed information about already approved drugs that target SLC transporters for various diseases. In this chapter, we provide several examples of approved drug classes for which the primary mechanisms of action involve inhibition of SLC transporters and highlight several other examples of transporters that are being targeted by drugs in development. We conclude with a discussion of potential strategies through which transporters may be a drug target.

Table 2.1. SLC transporters implicated in Mendelian diseases that may potentially be targets of new drugs.

Substrate Class	Protein (gene)	Disease (disease prevalence)	Current or Potential Drug Target
Neurotransmitter	EAAT1 (*SLC1A3*)	Episodic Ataxia Type 6 (N/A)	• Riluzole, which facilitates SLC1A3, is used for ALS[35]. • Loss of EAAT1 has been found in patients with amyotrophic lateral sclerosis[36]. • EAAT1 is upregulated in chronic brain ischemia[37]. • EAAT1 inhibitors have been developed as pharmacological tools[38–40].
Glucose	GLUT1 (*SLC2A1*)	GLUT1 Deficiency Syndrome / Dystonia 9 / Dystonia 18 (1:90,000 in Australia)	• Triheptanoin is in Phase II trials to treat GLUT1 deficiency syndrome (NCT02036853, NCT02018315). • GLUT1 expression levels are correlated to [^{18}F] 2-fluoro-2-deoxy-D-glucose uptake values[41,42]. • A small molecule inhibitor of GLUT1 was found to inhibit cancer cell growth *in vitro* and *in vivo*[43].
Glucose	SGLT1 (*SLC5A1*)	Glucose-Galactose Malabsorption (300 cases to date)	• Several inhibitors of SGLT1 and/or SGLT2 have been approved to treat type 2 Diabetes, including canagliflozin, dapagliflozin and empagliflozin[44,45]. Tofogliflozin and ipragliflozin are approved for use in Japan. • Clinical trials are ongoing for other SLC5A1 and SLC5A2 inhibitors including: remogliflozin in Phase I trials (NCT01571661, NCT00519480, NCT00376038), GSK1614235 in Phase I trials (NCT01607385), sotagliflozin in Phase II trials (NCT01742208, NCT00962065, NCT01376557) and ertugliflozin in Phase III trials (NCT01986881, NCT01999218, NCT01958671, NCT01986855, NCT02036515, NCT02226003)
Glucose	SGLT2 (*SLC5A2*)	Familial Renal Glucosuria (0.16-6.3% in US[46])	See above.
Thyroid	NIS (*SLC5A5*)	Thyroid Dyshormonogenesis (1:100,000 newborns)	A PET imaging probe, [99mTc]-pertechnetate is being studied for imaging and radioiodine therapy by NIS[47,48].
Neurotransmitter	NET (*SLC6A2*)	Orthostatic Intolerance (N/A)	Many antidepressants, including fluoxetine, citalopram, sertraline, venlafaxine, and duloxetine target neurotransmitter reuptake transporters including NET, DAT and SERT.

Neurotransmitter	DAT (*SLC6A3*)	Infantile Parkinsonism-Dystonia (8 cases to date[49])	• See above. • A radioligand for DAT, (1R)-2beta-Carbomethoxy-3beta-(4-[123I]iodophenyl)tropane ([123I]beta-CIT), could be used clinically as a nuclear imaging marker for Parkinson's disease[50,51].
Neurotransmitter	GLYT2 (*SLC6A5*)	Hyperekplexia (<1:1,000,000)	Inhibitors of GlyT2 may be used for the glycinergic control of pain[52] or overactive bladder[53].
Miscellaneous	CRTR (*SLC6A8*)	Cerebral Creatine Deficiency Syndrome (0.3-3.5% in males)	Creatine analogues may be used to enhance creatine levels in the brain[54–56].
Inorganic Ions	NHE6 (*SLC9A6*)	Christianson Syndrome (<30 cases to date[57])	This transporter is upregulated in tumor cells under hypoxic conditions[58,59].
Miscellaneous	ASBT (*SLC10A2*)	Primary Bile Acid Malabsorption (N/A)	• ASBT is a potential drug target in diabetes and in individuals with hyper-triglyceride levels[60,61]. • Drugs can be conjugated to bile acids to target ASBT to enhance drug uptake into intestine[62,63]. • Several inhibitors of ASBT are in development. LUM001 is in Phase II trials for cholestatic liver diseases (NCT02057718, NCT01904058, NCT02057692, NCT02117713, NCT02061540). SHP626 will begin a Phase I trial in 2015 for the treatment of NASH (NCT02287779). Elobixibat is in Phase II trials for chronic idiopathic constipation (NCT01895543). A4250 is in Phase II trials for PBC (NCT02360852).
Inorganic Ions	NKCC2 (*SLC12A1*)	Bartter Syndrome Type I (1:1,000,000)	Diuretic drugs such as bumetanide and furosemide inhibit SLC12A1.
Miscellaneous	MCT1 (*SLC16A1*)	Erythrocyte Lactate Transporter Defect (N/A)	• MCT1 is upregulated in cancer[64,65]. Inhibitors of MCT1 may be used as a target for immunosuppression or cancer[64,66,67]. • A SLC16A1 inhibitor, AZD3965, is in Phase I trials for patients with cancer (NCT01791595).
Neurotransmitter	VGLUT3 (*SLC17A8*)	Autosomal Dominant Deafness Type 25 (N/A)	VGLUT inhibitors block the uptake of glutamate into vesicles and reduce excitotoxic events to prevent acute CNS injury and chronic neurodegenerative disease[68–70].

Vitamin	THTR2 (*SLC19A3*)	Thiamine Metabolism Dysfunction Syndrome 2 (N/A)	A Janus Kinase 2 inhibitor, fedratinib, led to thiamine deficiency and Wernicke's encephalopathy, resulting in the withdrawal of the drug during Phase III trials[71].
Urea Cycle	URAT1 (*SLC22A12*)	Renal Hypouricemia 1 (2.37% in Japan[72])	• Lesinurad, a URAT1 inhibitor, is FDA-approved for the treatment of gout. • Clinical trials are ongoing for other URAT1 inhibitors, including RDEA3170 in Phase II trials (NCT01927198) and KUX-1151 in Phase II trials (NCT01927198)[73].
Miscellaneous	CACT (*SLC25A20*)	Carnitine-Acylcarnitine Translocase Deficiency (N/A)	Statins, fibrates and retinoic acid upregulate CACT gene expression[74].
Thyroid	PDS (*SLC26A4*)	Pendred Syndrome (7.5% of congenital deafness[75])	PDS plays an important role in the distal tubule salt reabsorption and nay represent a novel target for a new diuretic[76].
Glucose	G6PT1, SPX4 (*SLC37A4*)	Glycogen Storage Disease Type Ib / Ic (1:100,000[77])	Mice with glycogen storage disease type-Ia show increased G6TP1 mRNA levels after a 24-hour fast. Gene therapy to restore G6PT function has been shown in these mice[78,79].
Miscellaneous	FLVCR1 (*SLC49A1*)	Posterior Column Ataxia With Retinitis Pigmentosa (20 cases to date[80])	Inhibition of FLVCR results in decreased heme export in an erythroid cell line, yet FLVCR knockout mice develop iron overload at microphages[81].

SLC12 Transporters as Targets of Diuretic Drugs

Diuretics are important drugs used to treat hypertension and heart failure. The discovery of the first diuretic, chlorothiazide (which belongs to the thiazide class), in 1957 was followed by the discoveries of the loop diuretics bumetanide and furosemide. Later, Wiley and Cooper observed that furosemide inhibits the influx and efflux of sodium and potassium in human red blood cells, and concluded that the drug inhibits the co-transport of sodium and potassium[82]. In addition, using isolated perfused rabbit kidney tubules, Burg and co-workers described the inhibitory effect of furosemide on active chloride transport in the thick ascending limbs of Henle's loop, which results in decreased net absorption of sodium chloride and a decrease in

electrical potential (more positive electrical potential) in tubule cells[83]. Sodium–chloride co-transport was recognized in 1970s and has since been studied in a wide variety of animal cells and tissues.

The role of a sodium–chloride co-transporter is to maintain and regulate cell volumes and ion gradients; thus, diuretics that inhibit such co-transporters in the renal tubules decrease extracellular fluid volume and regulate electrolyte levels in the body[84]. The functional expression of the Na-K-Cl cotransporter in HEK293 kidney cells was first revealed by Xu *et al.*[85], who used radiolabelled rubidium as a tracer for potassium movement and showed that cells transfected with the Na–K–Cl co-transporter had greater influx of ^{86}Rb, which was inhibited by bumetanide, a loop diuretic. Consequently, it was established that diuretics inhibit the sodium–potassium cotransporter NKCC2 (SLC12A1) to reduce sodium reabsorption in the thick ascending limb of the Loop of Henle. The reduction in sodium reabsorption results in increased sodium levels in the tubule fluid and a corresponding loss of body water, thus increasing urine production and decreasing blood volume.

Interestingly, mutations in the gene coding for NKCC2 (*SLC12A1*), which is expressed only in the kidney were found to cause Bartter's syndrome, an inherited hypokalemic alkalosis featuring hypercalciuria and severe volume depletion[86]. Moreover, mutations in the gene encoding the sodium-chloride cotransporter TSC (*SLC12A3*; normally expressed in the renal distal tubule) were found to cause Gitelman syndrome, in which patients present with hypokalemic alkalosis and abnormalities in electrolyte homeostasis[87]. Unlike NKCC2, which is sensitive to loop diuretics such as bumetanide, but insensitive to thiazides, TSC is sensitive to the thiazides chlorothiazide and hydrochlorothiazide, but is insensitive to loop diuretics. At high doses, thiazides are used to relieve systemic and pulmonary edema due to chronic heart failure

and, at lower doses, to reduce blood pressure. One of the major adverse effects of thiazides and loop diuretics is hypokalemia, which is especially dangerous in patients with severe cardiovascular disease. Furthermore, high doses of thiazides can also cause hyperuricemia. One possible explanation for this effect is that diuretics may inhibit renal transporters such as OAT1 (SLC22A6), OAT3 (SLC22A8), NPT1 (SLC17A1), and NPT4 (SLC17A3) that are involved in uric acid secretion, and thus increase serum uric acid levels[88,89].

Urea transporters (UTs) of the SLC14 family also play an important role in the recycling of urea and in concentrating urine. As a result, several research groups are screening for small-molecule inhibitors to target UTs, particularly UT-B (SLC14A1) and UT-A (SLC14A2). Such inhibitors cause an increase in urine output due to decreased urea concentrations in the urine[46,112,113]. Perhaps in the near future, a new class of diuretics that inhibit UT-B and UT-A transporters will be developed.

SLC6 Transporters as Targets of Neuropsychiatric Drugs

Drugs for treating depression can be classified according to their presumed targets, which may be transporters. For example, the monoamine-reuptake inhibitors — which include the serotonin-selective reuptake inhibitors (SSRIs) and noradrenaline-reuptake inhibitors (NRIs) — inhibit neurotransmitter transporters. Over the past several decades, the development of novel antidepressants has moved from 'discovery by chance' to single-target strategies, and then to multiple-target strategies. Several transporters in the SLC6 family have important roles in the uptake of monoamines in the synapses of the central nervous system (CNS). These include NET (SLC6A2), DAT (SLC6A3) and SERT (SLC6A4), which are transporters for noradrenaline (NE), dopamine (DA) and serotonin (5-HT), respectively, although they all exhibit overlapping

substrate specificity. Inhibition of these transporters by drugs reduces clearance of the neurotransmitter monoamines from the synapse, thus increasing their dwell time in the synaptic cleft. The resulting increased concentrations of the monoamines in the synaptic cleft enhance receptor occupancy, leading to increased activation of ligand-gated ion channels and modulation of G protein-coupled receptor signaling[90]. However, the downstream mechanisms by which such drugs ultimately exert antidepressant effects (which can take weeks to become apparent) are not yet clear[91].

The first-generation tricyclic antidepressants (TCAs) inhibit 5-HT and NE reuptake, but also interact with other targets in the CNS. In the 1960s, several investigators showed that TCAs such as imipramine competitively inhibit 5-HT and NE uptake in several tissue types, including platelet-rich plasma, brain slices and synaptosomes. TCAs were used as the primary treatment for depression until the FDA's approval of the first SSRI, fluoxetine, in 1987. Fluoxetine was found to inhibit serotonin uptake into rat brain synaptosomes, without inhibiting uptake of noradrenaline into rat hearts[92,93].

Since the discovery of fluoxetine and other SSRIs, other classes of compounds that affect neurotransmitter reuptake have been developed. For example, serotonin–noradrenaline reuptake inhibitors (SNRIs), such as venlafaxine, are a newer class of antidepressants that selectively target two transporters, NET and SERT. The use of SSRIs and SNRIs has also expanded to other neuropsychiatric disorders such as anorexia nervosa, bulimia nervosa, obsessive-compulsive disorder, relief of menopausal symptoms, panic disorders and anxiety disorders.

Another therapeutic target for the treatment of neuropsychiatric disorders is the vesicular monoamine transporter 2, which transports monoamines from the cellular cytosol into synaptic vesicles. An inhibitor of VMAT2 (SLC18A2), tetrabenazine, is currently FDA-approved for the

treatment of hyperkinetic disorders associated with Huntington disorder[94], and others are in clinical development, including NBI-98854 (Neurocrine) in Phase III trials for dyskinesia (NCT02274558) and a natural product, lobeline, in Phase II trials for the treatment of attention deficit hyperactivity disorder in adults (NCT00664703). VMAT2 inhibitors may also be developed for the treatment of psychostimulant abuse and addiction[95].

Glucose Transporter Inhibitors

The SGLT2 (SLC5A2) transporter, which has low affinity and high capacity for glucose, plays a major part in renal glucose reabsorption, whereas the high-affinity, low capacity SGLT1 (SLC5A1) transporter has a major role in glucose absorption in the small intestine[96]. Mimicking the effects of a loss-of-function mutation in *SGLT2*, SGLT2 inhibitors enhance renal glucose excretion and consequently lower plasma glucose levels. Many companies have been developing drugs that target SGLT1 and SGLT2 for type 2 diabetes (T2D), in which patients exhibit hyperglycemia owing to insulin resistance. In such programs, compound libraries were screened for lead compounds inhibiting glucose uptake in cell lines that stably expressed human SGLT1 or SGLT2[97,98]. Once the lead compounds were identified, their *in vivo* effects were determined in murine and other animal models to confirm that the compound could increase urinary glucose excretion by inhibiting renal glucose reabsorption. Importantly, oral glucose tolerance tests in diabetic rats revealed that these animals were responsive to treatment with SGLT2 inhibitors[99]. Moreover, T2D patients treated with the SGLT2 inhibitor empagliflozin showed improved beta cell function and insulin sensitivity of tissue glucose uptake. Chronic dosing with empagliflozin for four weeks in these patients led to a significant decline in both HbA$_{1c}$ and fasting glucose levels[100].

Canagliflozin (developed by Johnson and Johnson) was the first SGLT2 inhibitor to be approved by the FDA in 2013, and was followed by dapagliflozin (Bristol-Myers Squibb/AstraZeneca) and empagliflozin (Boehringer Ingelheim/Eli Lilly) in 2014. All three drugs are being prescribed as second-line therapy for patients whose diabetes is inadequately controlled by a single antidiabetic agent[101]. Tofogliflozin (Chugai/Sanofi/Kowa) and ipragliflozin (Astellas/Kotobuki) have both been approved for the treatment of T2D in Japan, and both have active Phase IV trials ongoing (Tofogliflozin: NCT02201004, NCT0228469; Ipragliflozin: NCT02291874, NCT02175784). Other SGLT1 and/or SGLT2 inhibitors are being studied in early- and late-phase clinical studies either alone or in combination with metformin (which suppresses glucose production by the liver)[102–104]. These SGLT inhibitors include remogliflozin (GlaxoSmithKline) in Phase I trials (NCT01571661, NCT00519480, NCT00376038), GSK1614235 (GlaxoSmithKline) in Phase I trials (NCT01607385), sotagliflozin (Lexicon) in Phase II trials (NCT01742208, NCT00962065, NCT01376557), and ertugliflozin (Merck/Pfizer) in Phase III trials (NCT01986881, NCT01999218, NCT01958671, NCT01986855, NCT02036515, NCT02226003). In addition to having notable effects on reducing plasma glucose levels and increasing urinary glucose excretion, SGLT2 inhibitors have also been associated with weight loss and lowering of blood pressure, owing to their osmotic diuretic effects[105].

Uric Acid Transporter Inhibitors

Gout is the most common inflammatory arthritic disease, and has been increasing in prevalence in the United States and in other countries, in part as a consequence of the obesity epidemic[106]. Gout is caused by the accumulation of monosodium urate monohydrate crystals in

the joints and soft tissue, as a result of hyperuricemia. Most current treatments for gout generally target the inflammation that occurs during an acute gout attack, or reduce uric acid production by inhibiting the enzymes involved (for example, allopurinol, which inhibits xanthine oxidase); however, many patients fail to respond to these treatments or suffer from serious side effects (for instance, allopurinol can cause drug-hypersensitivity reactions)[107].

Recent advances in the understanding of uric acid transporters, through GWA studies and other studies, have opened the door for the development of novel compounds that target these transporters in the kidney as a way to block reuptake of uric acid and thereby promote elimination of uric acid in the urine. The main uric acid reabsorption transporters include URAT1 (SLC22A12) and GLUT9 (SLC2A9), which are expressed in the proximal tubule, although other transporters have also been identified that transport urate or that have been associated with gout[107,108]. Although probenecid, which has been used for decades, blocks uric acid reabsorption through organic anion transporters, several novel compounds are currently in development for this indication that are more selective, and most target URAT1. Lesinurad, a URAT1 inhibitor developed by Ardea Bioscience/AstraZeneca was recently approved by the European Medicines Agency in 2015 for the treatment of hyperuricemia in combination with xanthine oxidase inhibitors in gout patients (NCT01808144, NCT01510158, NCT01493531). Another URAT1 inhibitor, RDEA3170, is also being developed by AstraZeneca and completed Phase II trials (NCT01927198)[73]. Meanwhile, Pfizer recently licensed KUX-1151 from Kissei Pharmaceutical; this dual xanthine oxidase and URAT1 inhibitor is currently recruiting for a Phase II trial in Japan (NCT02190786). Since URAT1 inhibitors selectively inhibit URAT1 and reduce uric acid reabsorption in the kidney, their effects mimic those observed in patients with URAT1 mutations that lead to renal hypouricemia type 1.

Glycine Transporter Inhibitors

In the CNS, glycine has important roles in neuronal inhibition and excitation. It functions as an inhibitory neurotransmitter by activating ionotropic glycine receptors, enabling an influx of chloride ions and hyperpolarization of the post-synaptic membrane. Glycine also binds to excitatory N-methyl-D-aspartate (NMDA) receptors to enable receptor activation by glutamate[109]. The glycine transporters GlyT1 (SLC6A9) and GlyT2 (SLC6A5) on neurons, astrocytes and glial cells regulate levels of extracellular glycine in the brain, and thereby regulate NMDA-receptor activity. According to the glutamate hypothesis of schizophrenia, symptoms of the disease are caused by deficient glutamatergic (NMDA) signaling, and so GlyT1 inhibitors have been developed to inhibit glycine reuptake and increase levels of glycine at NMDA receptors to enhance NMDA signaling in this disorder.

Bitopertin, a highly selective and potent GlyT1 inhibitor developed by Roche, is furthest along in development[110]. However, several Phase III studies of bitopertin in schizophrenia (NCT01235559, NCT01235585, NCT01192906, NCT011928880) failed to reach their primary end point, leaving bitopertin with an uncertain future. Nevertheless, there is a Phase II trial currently ongoing using bitopertin with SSRIs in patients with obsessive-compulsive disorder (NCT01674361). The GlyT1 inhibitor PF-04958242 is still listed in Pfizer's Phase I pipeline and has an active Phase I study ongoing (NCT02341482), but the development of several other investigational GlyT1 inhibitors for the treatment of schizophrenia, including compounds from Pfizer, GlaxoSmithKline, Amgen, Merck, Johnson & Johnson and Sanofi appears to have stopped. However, there is evidence from animal models that GlyT2 inhibitors reduce pain in animal models of chronic pain, and thus there is probably work ongoing now in the development of partial GlyT2 inhibitors that can be used for pain relief without serious side effects[111]. One

such compound, VVZ-149, a dual inhibitor of GlyT2 and 5HT2A, is under development by Vivozon and completed a Phase I study in 2014 (NCT01905410).

Bile Acid Transporter Inhibitors

Bile acids are the main components of bile and their primary function is to solubilize fat and other dietary nutrients, although they can also function as signaling molecules[112]. New bile acids are synthesized in the liver from cholesterol through the action of the rate-limiting enzyme CYP7A1. Bile acid uptake and efflux transporters in the intestine and liver play key roles in the highly efficient enterohepatic recycling pathway, through which over 90% of the total pool of bile acids are reabsorbed from intestine and transported back to the liver via portal circulation[113]. The apical sodium-dependent bile acid cotransporter, ASBT (SLC10A2) mediates the reabsorption of bile acids from the lumen of the intestine[114]. Inhibition of bile acid reabsorption in the intestine would result in a loss of bile acids and increased conversion of cholesterol to bile acid in the liver, thereby lowering circulating cholesterol levels and potentially ameliorating or preventing the progression of cardiovascular disease. Thus, not surprisingly, inhibiting ASBT has received a substantial amount of attention as a potential therapeutic strategy for lowering cholesterol[61] and several pharmaceutical companies are developing potent and specific inhibitors of ASBT as therapeutic agents with this aim[114]. Interestingly, the rare genetic disorder primary bile acid malabsorption (PBAM) syndrome is caused by loss-of-function mutations in *ASBT*[115]. Those who have this disorder suffer from chronic diarrhea and fat-soluble vitamin deficiency and, importantly, exhibit lower plasma cholesterol levels[115].

Given the potency and efficacy of the statins, as well as newer agents such as ezetimibe that target cholesterol absorption in the intestine, the extent of clinical benefit from ASBT

inhibition (relative to the potential side effects) remains to be clarified. However, there now appears to be more-compelling evidence for targeting ASBT for cholestatic liver diseases such as primary biliary cirrhosis (PBC), for which treatment options have been limited. LUM001, an ASBT inhibitor developed by Lumena Pharmaceuticals (now Shire), is currently in Phase II clinical trials for the treatment of cholestatic liver diseases, including PBC[116] (NCT02057718, NCT01904058, NCT02057692, NCT02117713, NCT02061540). Another ASBT inhibitor developed by Shire, SHP626, is scheduled to start Phase I trials in 2015 for the treatment of nonalcoholic steatohepatitis in overweight adults (NCT02287779). Albiero Pharma is also currently developing ASBT inhibitors of its own — elobixibat is in Phase III trials for chronic idiopathic constipation (NCT01895543). A4250 (Albireo) received orphan drug designation from the FDA in 2012 for the treatment of progressive familial intrahepatic cholestasis (PFIC) and PBC and is currently in Phase II trials in PBC patients (NCT02360852). It is likely that there are other pharmaceutical companies pursuing ASBT as a therapeutic target for cholestatic liver disease, as many have done so for reducing cholesterol.

Because bile acids promote the secretion of intestinal incretin peptides (such as glucagon-like peptide 1, GLP-1), which in turn stimulate pancreatic insulin secretion, inhibition of ASBT may also be a viable strategy for the treatment of type 2 diabetes[117]. GlaxoSmithKline recently completed a Phase II study using GSK2330672 with metformin to treat diabetic patients in 2015 (NCT02202161). Furthermore, targeting ASBT has also been proposed as a prodrug strategy for compounds that have poor oral bioavailability, as an active drug molecule could be coupled with a bile acid moiety that would increase the absorption of the drug in the intestine[63].

Nutrient Transporter Inhibitors

Tumor cells have an increased demand for nutrients, owing to their rapid proliferation and growth. However, many amino acids and carbohydrates are too polar to diffuse across cell membranes, and thus require transporter proteins for cellular uptake. In theory, tumor growth can be controlled by starving the cells of the metabolic precursors needed for sustained growth. Such an approach can be used to selectively kill tumor cells, which are generally locked into a state of rapid growth by oncogenes and are thus more sensitive to starvation than normal cells. Moreover, nutrient transporters have been found to be overexpressed in a wide variety of cancers[118]. To date, no anticancer drug has been developed to specifically inhibit a nutrient transporter with the explicit goal of controlling cell growth. However, inhibition of key nutrient transporters such as the glucose transporters of the SLC2 and SLC5 families, amino acid transporters of the SLC7 family, and lactate transporters of the SLC16 family may be a reasonable approach for discovering new anticancer drugs[119].

As glucose is the primary energy source in most cells, it is no surprise that glucose transporters such as GLUT1 (SLC2A1) are often overexpressed in tumor cells in a variety of tissues[120,121]. Indeed, [18]FDG-PET imaging, which is used to detect and stage tumors, is based on the rate of cellular glucose uptake. However, with increased glycolysis comes an increase in the generation of lactate, which cells need to remove to maintain cellular pH levels. There is often also an upregulation of lactate transporters (such as MCT1 (SLC16A1))[122,123], or transporters important in pH regulation (such as NHE1 (SLC9A1))[59,124] in cancer cells.

Various research groups have identified MCT1 inhibitors. For example, Cancer Research UK's MCT1 inhibitor AZD3965 is currently in Phase I clinical trials for patients with advanced solid tumors or lymphoma (NCT01791595). Similarly, amino acid transporters (such as LAT1

(SLC7A5) [125,126], ASCT2 (SLC1A5)[127] and xCT (SLC7A11)[128]) are also overexpressed in a variety of tumors and may be potential anticancer targets. Sulfasalazine, which was initially developed as an anti-inflammatory drug to treat rheumatoid arthritis, was later found to inhibit xCT[129] and has anticancer effects in various cancer xenograft models[128]. As our understanding of the importance of transporters in tumor cell metabolism increases, it is very likely that the development of anticancer drugs that target different nutrient transporters will continue.

SLC-targeting Imaging Agents

Positron emission tomography (PET) imaging probes, some of which exploit SLC transporters for uptake into cells, are widely used in the clinic, particularly in diagnosing disease[130]. [^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG), a widely used PET probe, is transported into cells via GLUT1, which is upregulated in many cancers. GLUT1 upregulation, which enables higher ^{18}F-FDG uptake in tumor cells, is frequently associated with poor cancer prognosis[131,132].

Besides cancer diagnosis, several PET probes that inhibit SLC transporters are in clinical development for guiding drug treatment in other disorders. For example, PET radioligands that inhibit SERT, DAT or NET can provide information on occupancy of these neurotransmitter transporters by various drugs in patients with depression to understand mechanisms of nonresponse to drug therapies[133–135]. Another interesting use of PET imaging is the detection of monoaminergic degeneration in Parkinson's disease using ^{18}F-9-fluoroprolyl-(+)-dihydrotetrabenazine (^{18}F-DTBZ), a radioligand targeting VMAT2, which transports monoamine neurotransmitters from the neuronal cytosol into synaptic vesicles. As VMAT2 density is correlated with the integrity of dopaminergic neurons in the brain, measuring VMAT2

occupancy using ^{18}F-DTBZ enables early diagnosis and monitoring of Parkinson disease[136]. This imaging agent is currently in Phase II trials as an *in vivo* biomarker for Parkinson disease (NCT0128347). As VMAT2 can be a marker of islet beta cells, observational studies using the same agent are ongoing to measure beta cell mass in healthy individuals and patients with diabetes (NCT02236754, NCT00771576). Similarly, an imaging probe for the vesicular acetylcholine transporter VAChT is currently under investigation to assist in the detection of early Alzheimer's disease[137], which typically leads to deficits in acetylcholine transmission.

Drug–drug interactions can also be studied using PET imaging by administering a radiolabelled drug known to be a transporter substrate in conjunction with an unlabeled drug[138]. In this way, an interaction between a substrate and an inhibitor of a transporter may be observed in multiple organs simultaneously. For example, [^{11}C]-metformin may be used as a PET probe to study drug-drug interactions mediated by MATE1 in the liver and kidney[139].

Strategies for Targeting SLC Transporters

Inhibition of Transporter Function

Most current drugs — as well as novel drugs in clinical trials — that modulate SLC transporters do so by inhibiting transporter activity. For diseases in which decreased transporter activity leads to a potentially beneficial effect, high-throughput screening (HTS) of large compound libraries using cell lines that overexpress the transporter of interest can be used to discover lead molecules[140,141].

Many SLC transporters may function in both influx and efflux of their substrates, and the net direction of flux across a cellular membrane is dependent on the substrate gradient (or in the

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case of secondary or tertiary transporters, the gradient of the co- or counter-transported ions). Because of their simplicity, influx assays are frequently used in screening studies. In particular, cells that are grown on solid support are typically used to screen for inhibitors that prevent the uptake or influx of a fluorescent substrate probe[142–144]. Even for transporters that require a co- or counter-transported ion, the assays may be set up in the influx mode for convenience. For example, a high concentration of extracellular sodium may be used to drive the influx of a fluorescent probe, or the cells may be pre-loaded with a counterion to drive fluorescent probe influx. For transporters that are primarily efflux transporters, such as the ATP-binding cassette transporters ABCB11, ABCC2 and ABCG2, a vesicular-transport assay is frequently used. Instead of adherent cells, membrane fractions that contain inside-out vesicles are prepared from cells that overexpress the efflux transporter[145–148].

A fluorescent or radiolabelled substrate probe that can be quantitatively measured is added to each well in the presence and absence of each test compound to determine the transporter-inhibiting activity of the compounds. The concentration of the substrate probe and uptake time that give the best Z' assay sensitivity factor will be selected; a Z' > 0.8 will be ideal for high-throughput screening, although Z' > 0.5 is also acceptable[149]. Such methods have been used to screen thousands of compounds in HTS campaigns. However, HTS also suffers from limitations. One commonly observed issue relates to assay interference, whereby aggregation of a test compound that causes protein sequestration or non-specific inhibition results in a false-positive 'hit'; this problem affects 1.7–1.9% of total compounds in compound libraries[150–152]. Another problem arises from the interference of fluorescent test compounds or contaminating particulate matter with the measurement of the fluorescent substrate probe. A 'pre-read' control measurement after the addition of the test compound but prior to substrate addition can prevent

this problem. Use of an orange or red fluorophore as the substrate also helps to avoid this interference, as fluorescence and spurious light emissions from test compounds have been found to be more likely to occur at shorter wavelengths[150].

The structure of identified potential inhibitors can be modified to produce more potent or selective molecules. Pharmacophore modelling and quantitative structure–activity relationship (QSAR) modelling may be used to engineer better compounds with more desirable properties[153,154]. Novel inhibitors for many transporters have been identified through these methods, including inhibitors of ASBT (SLC10A2)[155,156], EAAT3 (SLC1A1)[157], DAT (SLC6A3), SERT (SLC6A4), NET (SLC6A2)[158–160] and VGLUT2 (SLC17A6)[69]. For example, the *O*-spiroketal C-arylglucoside scaffold — which forms the basis of tofogliflozin, a selective SGLT2 inhibitor recently approved in Japan for the treatment of diabetes — was found by pharmacophore modelling of SGLT2 inhibitors and a search of the Cambridge Structural Database[161].

Homology modelling and docking have been used widely to identify and optimize lead compounds. As the crystal structures of SLC transporters from various species become increasingly available[162,163], homology models of human orthologues are being constructed that can be used in molecular-docking studies to design more-selective transporter inhibitors. These methods have been successfully used to identify binding sites, accurately predict inhibitory actions of already prescribed drugs on SLC transporters and identify potent transporter inhibitors for treating diseases[164–166]. For example, high-throughput docking of the glutamate-binding site on the *Pyrococcus horikoshii* glutamate transporter homologue has been used to identify a potent inhibitor of EAAT2 (SLC1A2)[167], and the crystal structure of the same concentrative nucleoside transporter from *Vibrio cholerae* was used to identify a selective anticancer drug[168,169]. The

crystal structures of bacterial homologs of neurotransmitter transporters bound to antidepressant drugs have been recently elucidated, allowing researchers to identify the amino-acid residues that comprise the binding sites of these transporters[170–172]. Such information may be implemented to design and synthesize more potent serotonin- and/or norepinephrine-reuptake inhibitors[173].

Enhancement of Transporter Function

For the vast majority of Mendelian diseases for which causal variants in the genes coding for SLC transporters result in loss of transporter function, compounds that enhance the function of the affected transporter are needed; these compounds are also of interest in the treatment of common diseases. To our knowledge, no current drugs were originally developed specifically to activate SLC transporters. Riluzole, a drug used to treat amyotrophic lateral sclerosis (ALS), inhibits glutamate release, thereby preventing glutamate-induced activation of sodium channels on postsynaptic neurons and reducing excitotoxicity[174]. However, riluzole was later found to enhance the transport activity of EAAT1 (SLC1A3), which takes up extracellular glutamate from the synapse and thus reduces glutamate levels in the synaptic cleft[35,175]. Another study showed that riluzole stimulates glutamate uptake by increasing EAAC1 (SLC1A1) transcript levels in astroglial cells[176], although the exact mechanism for this effect is not fully understood.

There is interest in identifying additional targets to increase glutamate clearance. Similarly to EAAT1, the major glutamate transporter in astrocytes, EAAT2, reduces glutamate levels in the synaptic cleft, thereby attenuating excitotoxicity. Some examples of neurodegenerative diseases that could benefit from upregulated EAAT2 function include Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease — all of which have been associated with decreased EAAT2 protein expression levels[177,178] — as well as epilepsy,

stroke and neurotrauma[179]. Therefore, developing a drug that enhances EAAT2 activity represents a promising approach in CNS drug development[180]. Two approaches are currently being used to identify enhancers of EAAT2. First, a HTS assay that uses a reporter gene that contains the *EAAT2* promoter upstream of the luciferase gene has found that several currently prescribed drugs, such as ceftriaxone, increased luciferase transcript levels[181]. Second, an astrocyte-based enzyme-linked immunosorbent assay can screen large compound libraries for molecules that can induce translation of silenced *EAAT2* transcripts, as the expression of EAAT2 protein is highly regulated at the translational level by extracellular factors[182].

These and other screening technologies — for example, high-throughput fluorescence assays — could be used to identify compounds that enhance SLC transporter activity. The development of drugs that enhance the activity of CFTR (ABCC7), the ATP-binding cassette transporter that is defective in cystic fibrosis, could provide a model for the identification of small molecules that may rescue mutated SLC transporters. Small-molecule therapies for modulating CFTR fall into two categories: potentiators and correctors. Various assays have been developed to identify potentiators and correctors. Ivacaftor, the first U.S. Food and Drug Administration-approved drug to target a specific CFTR mutant, CFTR-G551D, acts as a potentiator; it increases the activity of CFTR-G551D, which is already present on the plasma membrane, by increasing the probability of the open state of the CFTR channel[183]. By contrast, correctors enhance the trafficking of mutant proteins to the plasma membrane, such as the most common disease-causing mutant protein, CFTR-F508del. Early clinical results suggest that the combination of a corrector and a potentiator results in enhanced efficacy in the treatment of individuals with cystic fibrosis who harbor the *CFTR*-F508del mutation[184].

Circumventing Transporters

Another potential therapeutic strategy for treatment of rare disease caused by mutant SLC transporters is to develop substrates that circumvent transporters. SLC transporter gene mutations that lead to Mendelian disease often also lead to low levels of an essential nutrient, such as thiamine or carnitine, in specific tissues. Therapies that circumvent the use of the mutant transporter may be developed. For example, creation of a hydrolyzable hydrophobic derivative of an essential nutrient that is normally hydrophilic may result in enhanced permeability of the nutrient. Thus, the hydrophobic derivative would enter the cell (without the aid of a transporter) where it would undergo hydrolysis and release the nutrient, thus enhancing the availability of the nutrient in the cell to treat, for instance, thiamine deficiency[185]. However, a potential problem in the development of diffusible analogs that circumvent transporters may be the low cellular availability of the analogue. For example, many transporters are secondary-active and therefore concentrate their substrates intracellularly, whereas diffusible analogues may not accumulate to the same extent. Furthermore, hydrophobic diffusible analogs may be substrates for efflux pumps of the ABC transporter families, such as P-glycoprotein, that preferentially handle hydrophobic substrates and may therefore exhibit poor cellular availability. Finally, diffusible analogues may distribute widely into body tissues, rather than selectively targeting the tissues of greatest need (for example, in the case of carnitine deficiency, the liver).

Gene Therapy

Gene therapy, in principle, may be a strategy for the treatment of diseases caused by mutant transporter proteins. Several animal studies have provided proof of concept. For example, Yiu *et al*. infused an adenoviral vector containing the human glucose-6-phosphate transporter

gene (*G6PT*) into *G6pt*-deficient mice (which recapitulate the human glycogen-storage disease type Ib[186] that is attributable to mutations in *G6PT* (*SLC37A4*)). The gene therapy restored *G6PT* expression in various tissues, including the liver; normalized serum-glucose and -lipid profiles; and reduced glycogen deposition in the liver. Though historically fraught with issues, gene therapy may become a viable strategy for the treatment of Mendelian disorders involving defective membrane transporters as more advanced methods are applied.

Future Directions

SLC transporters represent a plethora of new therapeutic targets for rare diseases, and may be particularly amenable to small-molecule targeting. Of importance, many SLC transporters are expressed on the cell surface and hence are targetable by both small molecules and therapeutic antibodies. Further, there are many examples of SLC transporters that are targets of already approved drugs, as well as of drugs in development. Several challenges remain, however, for targeting SLC transporters. Although 92% of known drug-target structures have been deposited in the Protein Data Base (PDB), most SLC transporters have not been crystallized, thus limiting computer-aided drug discovery efforts[162]. Moreover, many SLC transporters remain orphans, with unknown function and unknown substrates. Finally, SLC transporters located in intracellular compartments may not be as amenable to drug targeting. For example, to our knowledge, the SLC25 family of mitochondrial transporters, some of which are associated with Mendelian disease, have not yet been targeted.

Despite these obstacles, the potential for discovering inhibitors of transporters or modulating transporter activity is enormous. The development of small molecules that can target specific amino-acid substitutions in the ABC transporter CFTR suggests that efforts to target

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mutant SLC transporters may be possible. In some rare diseases, hydrophobic nutrient-derivatives that can circumvent dysfunctional membrane transporters could be used to deliver necessary nutrients to cells. A comprehensive understanding of the mechanisms responsible for rare and common diseases — including the function of the transporters and the pathways in which they act — will be crucial for recognizing other proteins that may be therapeutically targeted.

References

1.   Jostins, L., Ripke, S., Weersma, R. K., Duerr, R. H., McGovern, D. P., Hui, K. Y., Lee, J. C., Schumm, L. P., Sharma, Y., Anderson, C. A., Essers, J., Mitrovic, M., Ning, K., Cleynen, I., Theatre, E., Spain, S. L., Raychaudhuri, S., Goyette, P., Wei, Z., Abraham, C., Achkar, J. P., Ahmad, T., Amininejad, L., Ananthakrishnan, A. N., Andersen, V., Andrews, J. M., Baidoo, L., Balschun, T., Bampton, P. A., Bitton, A., Boucher, G., Brand, S., Buning, C., Cohain, A., Cichon, S., D'Amato, M., De Jong, D., Devaney, K. L., Dubinsky, M., Edwards, C., Ellinghaus, D., Ferguson, L. R., Franchimont, D., Fransen, K., Gearry, R., Georges, M., Gieger, C., Glas, J., Haritunians, T., Hart, A., Hawkey, C., Hedl, M., Hu, X., Karlsen, T. H., Kupcinskas, L., Kugathasan, S., Latiano, A., Laukens, D., Lawrance, I. C., Lees, C. W., Louis, E., Mahy, G., Mansfield, J., Morgan, A. R., Mowat, C., Newman, W., Palmieri, O., Ponsioen, C. Y., Potocnik, U., Prescott, N. J., Regueiro, M., Rotter, J. I., Russell, R. K., Sanderson, J. D., Sans, M., Satsangi, J., Schreiber, S., Simms, L. A., Sventoraityte, J., Targan, S. R., Taylor, K. D., Tremelling, M., Verspaget, H. W., De Vos, M., Wijmenga, C., Wilson, D. C., Winkelmann, J., Xavier, R. J., Zeissig, S., Zhang, B., Zhang, C. K., Zhao, H., Silverberg, M. S., Annese, V., Hakonarson, H., Brant, S. R., Radford-Smith, G., Mathew, C. G., Rioux, J. D., Schadt, E. E., Daly, M. J., Franke, A., Parkes, M., Vermeire, S., Barrett, J. C. & Cho, J. H. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491,** 119–124 (2012).

2.   Kenny, E. E., Pe'er, I., Karban, A., Ozelius, L., Mitchell, A. A., Ng, S. M., Erazo, M., Ostrer, H., Abraham, C., Abreu, M. T., Atzmon, G., Barzilai, N., Brant, S. R., Bressman, S., Burns, E. R., Chowers, Y., Clark, L. N., Darvasi, A., Doheny, D., Duerr, R. H.,

Eliakim, R., Giladi, N., Gregersen, P. K., Hakonarson, H., Jones, M. R., Marder, K., McGovern, D. P., Mulle, J., Orr-Urtreger, A., Proctor, D. D., Pulver, A., Rotter, J. I., Silverberg, M. S., Ullman, T., Warren, S. T., Waterman, M., Zhang, W., Bergman, A., Mayer, L., Katz, S., Desnick, R. J., Cho, J. H. & Peter, I. A genome-wide scan of Ashkenazi Jewish Crohn's disease suggests novel susceptibility loci. *PLoS Genet.* **8,** e1002559 (2012).

3.   Franke, A., McGovern, D. P., Barrett, J. C., Wang, K., Radford-Smith, G. L., Ahmad, T., Lees, C. W., Balschun, T., Lee, J., Roberts, R., Anderson, C. A., Bis, J. C., Bumpstead, S., Ellinghaus, D., Festen, E. M., Georges, M., Green, T., Haritunians, T., Jostins, L., Latiano, A., Mathew, C. G., Montgomery, G. W., Prescott, N. J., Raychaudhuri, S., Rotter, J. I., Schumm, P., Sharma, Y., Simms, L. A., Taylor, K. D., Whiteman, D., Wijmenga, C., Baldassano, R. N., Barclay, M., Bayless, T. M., Brand, S., Buning, C., Cohen, A., Colombel, J. F., Cottone, M., Stronati, L., Denson, T., De Vos, M., D'Inca, R., Dubinsky, M., Edwards, C., Florin, T., Franchimont, D., Gearry, R., Glas, J., Van Gossum, A., Guthery, S. L., Halfvarson, J., Verspaget, H. W., Hugot, J. P., Karban, A., Laukens, D., Lawrance, I., Lemann, M., Levine, A., Libioulle, C., Louis, E., Mowat, C., Newman, W., Panes, J., Phillips, A., Proctor, D. D., Regueiro, M., Russell, R., Rutgeerts, P., Sanderson, J., Sans, M., Seibold, F., Steinhart, A. H., Stokkers, P. C., Torkvist, L., Kullak-Ublick, G., Wilson, D., Walters, T., Targan, S. R., Brant, S. R., Rioux, J. D., D'Amato, M., Weersma, R. K., Kugathasan, S., Griffiths, A. M., Mansfield, J. C., Vermeire, S., Duerr, R. H., Silverberg, M. S., Satsangi, J., Schreiber, S., Cho, J. H., Annese, V., Hakonarson, H., Daly, M. J. & Parkes, M. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* **42,** 1118–1125 (2010).

4. Kottgen, A., Albrecht, E., Teumer, A., Vitart, V., Krumsiek, J., Hundertmark, C., Pistis, G., Ruggiero, D., O'Seaghdha, C. M., Haller, T., Yang, Q., Tanaka, T., Johnson, A. D., Kutalik, Z., Smith, A. V, Shi, J., Struchalin, M., Middelberg, R. P., Brown, M. J., Gaffo, A. L., Pirastu, N., Li, G., Hayward, C., Zemunik, T., Huffman, J., Yengo, L., Zhao, J. H., Demirkan, A., Feitosa, M. F., Liu, X., Malerba, G., Lopez, L. M., van der Harst, P., Li, X., Kleber, M. E., Hicks, A. A., Nolte, I. M., Johansson, A., Murgia, F., Wild, S. H., Bakker, S. J., Peden, J. F., Dehghan, A., Steri, M., Tenesa, A., Lagou, V., Salo, P., Mangino, M., Rose, L. M., Lehtimaki, T., Woodward, O. M., Okada, Y., Tin, A., Muller, C., Oldmeadow, C., Putku, M., Czamara, D., Kraft, P., Frogheri, L., Thun, G. A., Grotevendt, A., Gislason, G. K., Harris, T. B., Launer, L. J., McArdle, P., Shuldiner, A. R., Boerwinkle, E., Coresh, J., Schmidt, H., Schallert, M., Martin, N. G., Montgomery, G. W., Kubo, M., Nakamura, Y., Munroe, P. B., Samani, N. J., Jacobs Jr., D. R., Liu, K., D'Adamo, P., Ulivi, S., Rotter, J. I., Psaty, B. M., Vollenweider, P., Waeber, G., Campbell, S., Devuyst, O., Navarro, P., Kolcic, I., Hastie, N., Balkau, B., Froguel, P., Esko, T., Salumets, A., Khaw, K. T., Langenberg, C., Wareham, N. J., Isaacs, A., Kraja, A., Zhang, Q., Wild, P. S., Scott, R. J., Holliday, E. G., Org, E., Viigimaa, M., Bandinelli, S., Metter, J. E., Lupo, A., Trabetti, E., Sorice, R., Doring, A., Lattka, E., Strauch, K., Theis, F., Waldenberger, M., Wichmann, H. E., Davies, G., Gow, A. J., Bruinenberg, M., Stolk, R. P., Kooner, J. S., Zhang, W., Winkelmann, B. R., Boehm, B. O., Lucae, S., Penninx, B. W., Smit, J. H., Curhan, G., Mudgal, P., Plenge, R. M., Portas, L., Persico, I., Kirin, M., Wilson, J. F., Mateo Leach, I., van Gilst, W. H., Goel, A., Ongen, H., Hofman, A., Rivadeneira, F., Uitterlinden, A. G., Imboden, M., von Eckardstein, A., Cucca, F., Nagaraja, R., Piras, M. G., Nauck, M., Schurmann, C., Budde, K., Ernst, F., Farrington, S.

M., Theodoratou, E., Prokopenko, I., Stumvoll, M., Jula, A., Perola, M., Salomaa, V., Shin, S. Y., Spector, T. D., Sala, C., Ridker, P. M., Kahonen, M., Viikari, J., Hengstenberg, C., Nelson, C. P., Meschia, J. F., Nalls, M. A., Sharma, P., Singleton, A. B., Kamatani, N., Zeller, T., Burnier, M., Attia, J., Laan, M., Klopp, N., Hillege, H. L., Kloiber, S., Choi, H., Pirastu, M., Tore, S., Probst-Hensch, N. M., Volzke, H., Gudnason, V., Parsa, A., Schmidt, R., Whitfield, J. B., Fornage, M., Gasparini, P., Siscovick, D. S., Polasek, O., Campbell, H., Rudan, I., Bouatia-Naji, N., Metspalu, A., Loos, R. J., van Duijn, C. M., Borecki, I. B., Ferrucci, L., Gambaro, G., Deary, I. J., Wolffenbuttel, B. H., Chambers, J. C., Marz, W., Pramstaller, P. P., Snieder, H., Gyllensten, U., Wright, A. F., Navis, G., Watkins, H., Witteman, J. C., Sanna, S., Schipf, S., Dunlop, M. G., Tonjes, A., Ripatti, S., Soranzo, N., Toniolo, D., Chasman, D. I., Raitakari, O., Kao, W. H., Ciullo, M., Fox, C. S., Caulfield, M., Bochud, M. & Gieger, C. Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. *Nat. Genet.* **45,** 145–154 (2013).

5.   Tin, A., Woodward, O. M., Kao, W. H., Liu, C. T., Lu, X., Nalls, M. A., Shriner, D., Semmo, M., Akylbekova, E. L., Wyatt, S. B., Hwang, S. J., Yang, Q., Zonderman, A. B., Adeyemo, A. A., Palmer, C., Meng, Y., Reilly, M., Shlipak, M. G., Siscovick, D., Evans, M. K., Rotimi, C. N., Flessner, M. F., Kottgen, M., Cupples, L. A., Fox, C. S. & Kottgen, A. Genome-wide association study for serum urate concentrations and gout among African Americans identifies genomic risk loci and a novel URAT1 loss-of-function allele. *Hum. Mol. Genet.* **20,** 4056–4068 (2011).

6.   Li, S., Sanna, S., Maschio, A., Busonero, F., Usala, G., Mulas, A., Lai, S., Dei, M., Orru, M., Albai, G., Bandinelli, S., Schlessinger, D., Lakatta, E., Scuteri, A., Najjar, S. S.,

Guralnik, J., Naitza, S., Crisponi, L., Cao, A., Abecasis, G., Ferrucci, L., Uda, M., Chen, W. M. & Nagaraja, R. The GLUT9 gene is associated with serum uric acid levels in Sardinia and Chianti cohorts. *PLoS Genet.* **3,** e194 (2007).

7. Dai, X., Wu, C., He, Y., Gui, L., Zhou, L., Guo, H., Yuan, J., Yang, B., Li, J., Deng, Q., Huang, S., Guan, L., Hu, D., Zhu, J., Min, X., Lang, M., Li, D., Yang, H., Hu, F. B., Lin, D., Wu, T. & He, M. A genome-wide association study for serum bilirubin levels and gene-environment interaction in a Chinese population. *Genet. Epidemiol.* **37,** 293–300 (2013).

8. Bielinski, S. J., Chai, H. S., Pathak, J., Talwalkar, J. A., Limburg, P. J., Gullerud, R. E., Sicotte, H., Klee, E. W., Ross, J. L., Kocher, J. P., Kullo, I. J., Heit, J. A., Petersen, G. M., de Andrade, M. & Chute, C. G. Mayo Genome Consortia: a genotype-phenotype resource for genome-wide association studies with an application to the analysis of circulating bilirubin levels. *Mayo Clin. Proc.* **86,** 606–614 (2011).

9. Sanna, S., Busonero, F., Maschio, A., McArdle, P. F., Usala, G., Dei, M., Lai, S., Mulas, A., Piras, M. G., Perseu, L., Masala, M., Marongiu, M., Crisponi, L., Naitza, S., Galanello, R., Abecasis, G. R., Shuldiner, A. R., Schlessinger, D., Cao, A. & Uda, M. Common variants in the SLCO1B3 locus are associated with bilirubin levels and unconjugated hyperbilirubinemia. *Hum. Mol. Genet.* **18,** 2711–2718 (2009).

10. Johnson, A. D., Kavousi, M., Smith, A. V, Chen, M. H., Dehghan, A., Aspelund, T., Lin, J. P., van Duijn, C. M., Harris, T. B., Cupples, L. A., Uitterlinden, A. G., Launer, L., Hofman, A., Rivadeneira, F., Stricker, B., Yang, Q., O'Donnell, C. J., Gudnason, V. & Witteman, J. C. Genome-wide association meta-analysis for total serum bilirubin levels. *Hum. Mol. Genet.* **18,** 2700–2710 (2009).

11. Nan, H., Kraft, P., Qureshi, A. A., Guo, Q., Chen, C., Hankinson, S. E., Hu, F. B., Thomas, G., Hoover, R. N., Chanock, S., Hunter, D. J. & Han, J. Genome-wide association study of tanning phenotype in a population of European ancestry. *J. Invest. Dermatol.* **129,** 2250–2257 (2009).

12. Stokowski, R. P., Pant, P. V, Dadd, T., Fereday, A., Hinds, D. A., Jarman, C., Filsell, W., Ginger, R. S., Green, M. R., van der Ouderaa, F. J. & Cox, D. R. A genomewide association study of skin pigmentation in a South Asian population. *Am. J. Hum. Genet.* **81,** 1119–1132 (2007).

13. Kolz, M., Johnson, T., Sanna, S., Teumer, A., Vitart, V., Perola, M., Mangino, M., Albrecht, E., Wallace, C., Farrall, M., Johansson, A., Nyholt, D. R., Aulchenko, Y., Beckmann, J. S., Bergmann, S., Bochud, M., Brown, M., Campbell, H., Connell, J., Dominiczak, A., Homuth, G., Lamina, C., McCarthy, M. I., Meitinger, T., Mooser, V., Munroe, P., Nauck, M., Peden, J., Prokisch, H., Salo, P., Salomaa, V., Samani, N. J., Schlessinger, D., Uda, M., Volker, U., Waeber, G., Waterworth, D., Wang-Sattler, R., Wright, A. F., Adamski, J., Whitfield, J. B., Gyllensten, U., Wilson, J. F., Rudan, I., Pramstaller, P., Watkins, H., Doering, A., Wichmann, H. E., Spector, T. D., Peltonen, L., Volzke, H., Nagaraja, R., Vollenweider, P., Caulfield, M., Illig, T. & Gieger, C. Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations. *PLoS Genet.* **5,** e1000504 (2009).

14. Van De Steeg, E., Stránecký, V., Hartmannová, H., Nosková, L., Hřebíček, M., Wagenaar, E., Van Esch, A., De Waart, D. R., Oude Elferink, R. P. J., Kenworthy, K. E., Sticová, E., Al-Edreesi, M., Knisely, A. S., Kmoch, S., Jirsa, M. & Schinkel, A. H. Complete

OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver. *J. Clin. Invest.* **122,** 519–528 (2012).

15.   Dhumeaux, D. & Erlinger, S. Hereditary conjugated hyperbilirubinaemia: 37 years later. *Journal of Hepatology* **58,** 388–390 (2013).

16.   Sladek, R., Rocheleau, G., Rung, J., Dina, C., Shen, L., Serre, D., Boutin, P., Vincent, D., Belisle, A., Hadjadj, S., Balkau, B., Heude, B., Charpentier, G., Hudson, T. J., Montpetit, A., Pshezhetsky, A. V, Prentki, M., Posner, B. I., Balding, D. J., Meyre, D., Polychronakos, C. & Froguel, P. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* **445,** 881–885 (2007).

17.   Scott, L. J., Mohlke, K. L., Bonnycastle, L. L., Willer, C. J., Li, Y., Duren, W. L., Erdos, M. R., Stringham, H. M., Chines, P. S., Jackson, A. U., Prokunina-Olsson, L., Ding, C.-J., Swift, A. J., Narisu, N., Hu, T., Pruim, R., Xiao, R., Li, X.-Y., Conneely, K. N., Riebow, N. L., Sprau, A. G., Tong, M., White, P. P., Hetrick, K. N., Barnhart, M. W., Bark, C. W., Goldstein, J. L., Watkins, L., Xiang, F., Saramies, J., Buchanan, T. A., Watanabe, R. M., Valle, T. T., Kinnunen, L., Abecasis, G. R., Pugh, E. W., Doheny, K. F., Bergman, R. N., Tuomilehto, J., Collins, F. S. & Boehnke, M. A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. *Science* **316,** 1341–1345 (2007).

18.   Rafnar, T., Vermeulen, S. H., Sulem, P., Thorleifsson, G., Aben, K. K., Witjes, J. A., Grotenhuis, A. J., Verhaegh, G. W., van de Kaa, C. A. H., Besenbacher, S., Gudbjartsson, D., Stacey, S. N., Gudmundsson, J., Johannsdottir, H., Bjarnason, H., Zanon, C., Helgadottir, H., Jonasson, J. G., Tryggvadottir, L., Jonsson, E., Geirsson, G., Nikulasson, S., Petursdottir, V., Bishop, D. T., Chung-Sak, S., Choudhury, A., Elliott, F., Barrett, J. H., Knowles, M. A., de Verdier, P. J., Ryk, C., Lindblom, A., Rudnai, P., Gurzau, E.,

Koppova, K., Vineis, P., Polidoro, S., Guarrera, S., Sacerdote, C., Panadero, A., Sanz-Velez, J. I., Sanchez, M., Valdivia, G., Garcia-Prats, M. D., Hengstler, J. G., Selinski, S., Gerullis, H., Ovsiannikov, D., Khezri, A., Aminsharifi, A., Malekzadeh, M., van den Berg, L. H., Ophoff, R. A., Veldink, J. H., Zeegers, M. P., Kellen, E., Fostinelli, J., Andreoli, D., Arici, C., Porru, S., Buntinx, F., Ghaderi, A., Golka, K., Mayordomo, J. I., Matullo, G., Kumar, R., Steineck, G., Kiltie, A. E., Kong, A., Thorsteinsdottir, U., Stefansson, K. & Kiemeney, L. A. European genome-wide association study identifies SLC14A1 as a new urinary bladder cancer susceptibility gene. *Hum. Mol. Genet.* **20,** 4268–4281 (2011).

19.   Ehret, G. B., Munroe, P. B., Rice, K. M., Bochud, M., Johnson, A. D., Chasman, D. I., Smith, A. V, Tobin, M. D., Verwoert, G. C., Hwang, S. J., Pihur, V., Vollenweider, P., O'Reilly, P. F., Amin, N., Bragg-Gresham, J. L., Teumer, A., Glazer, N. L., Launer, L., Zhao, J. H., Aulchenko, Y., Heath, S., Sober, S., Parsa, A., Luan, J., Arora, P., Dehghan, A., Zhang, F., Lucas, G., Hicks, A. A., Jackson, A. U., Peden, J. F., Tanaka, T., Wild, S. H., Rudan, I., Igl, W., Milaneschi, Y., Parker, A. N., Fava, C., Chambers, J. C., Fox, E. R., Kumari, M., Go, M. J., van der Harst, P., Kao, W. H., Sjogren, M., Vinay, D. G., Alexander, M., Tabara, Y., Shaw-Hawkins, S., Whincup, P. H., Liu, Y., Shi, G., Kuusisto, J., Tayo, B., Seielstad, M., Sim, X., Nguyen, K. D., Lehtimaki, T., Matullo, G., Wu, Y., Gaunt, T. R., Onland-Moret, N. C., Cooper, M. N., Platou, C. G., Org, E., Hardy, R., Dahgam, S., Palmen, J., Vitart, V., Braund, P. S., Kuznetsova, T., Uiterwaal, C. S., Adeyemo, A., Palmas, W., Campbell, H., Ludwig, B., Tomaszewski, M., Tzoulaki, I., Palmer, N. D., Aspelund, T., Garcia, M., Chang, Y. P., O'Connell, J. R., Steinle, N. I., Grobbee, D. E., Arking, D. E., Kardia, S. L., Morrison, A. C., Hernandez, D., Najjar, S.,

McArdle, W. L., Hadley, D., Brown, M. J., Connell, J. M., Hingorani, A. D., Day, I. N., Lawlor, D. A., Beilby, J. P., Lawrence, R. W., Clarke, R., Hopewell, J. C., Ongen, H., Dreisbach, A. W., Li, Y., Young, J. H., Bis, J. C., Kahonen, M., Viikari, J., Adair, L. S., Lee, N. R., Chen, M. H., Olden, M., Pattaro, C., Bolton, J. A., Kottgen, A., Bergmann, S., Mooser, V., Chaturvedi, N., Frayling, T. M., Islam, M., Jafar, T. H., Erdmann, J., Kulkarni, S. R., Bornstein, S. R., Grassler, J., Groop, L., Voight, B. F., Kettunen, J., Howard, P., Taylor, A., Guarrera, S., Ricceri, F., Emilsson, V., Plump, A., Barroso, I., Khaw, K. T., Weder, A. B., Hunt, S. C., Sun, Y. V, Bergman, R. N., Collins, F. S., Bonnycastle, L. L., Scott, L. J., Stringham, H. M., Peltonen, L., Perola, M., Vartiainen, E., Brand, S. M., Staessen, J. A., Wang, T. J., Burton, P. R., Soler Artigas, M., Dong, Y., Snieder, H., Wang, X., Zhu, H., Lohman, K. K., Rudock, M. E., Heckbert, S. R., Smith, N. L., Wiggins, K. L., Doumatey, A., Shriner, D., Veldre, G., Viigimaa, M., Kinra, S., Prabhakaran, D., Tripathy, V., Langefeld, C. D., Rosengren, A., Thelle, D. S., Corsi, A. M., Singleton, A., Forrester, T., Hilton, G., McKenzie, C. A., Salako, T., Iwai, N., Kita, Y., Ogihara, T., Ohkubo, T., Okamura, T., Ueshima, H., Umemura, S., Eyheramendy, S., Meitinger, T., Wichmann, H. E., Cho, Y. S., Kim, H. L., Lee, J. Y., Scott, J., Sehmi, J. S., Zhang, W., Hedblad, B., Nilsson, P., Smith, G. D., Wong, A., Narisu, N., Stancakova, A., Raffel, L. J., Yao, J., Kathiresan, S., O'Donnell, C. J., Schwartz, S. M., Ikram, M. A., Longstreth Jr., W. T., Mosley, T. H., Seshadri, S., Shrine, N. R., Wain, L. V, Morken, M. A., Swift, A. J., Laitinen, J., Prokopenko, I., Zitting, P., Cooper, J. A., Humphries, S. E., Danesh, J., Rasheed, A., Goel, A., Hamsten, A., Watkins, H., Bakker, S. J., van Gilst, W. H., Janipalli, C. S., Mani, K. R., Yajnik, C. S., Hofman, A., Mattace-Raso, F. U., Oostra, B. A., Demirkan, A., Isaacs, A., Rivadeneira, F., Lakatta, E. G., Orru, M., Scuteri, A.,

Ala-Korpela, M., Kangas, A. J., Lyytikainen, L. P., Soininen, P., Tukiainen, T., Wurtz, P., Ong, R. T., Dorr, M., Kroemer, H. K., Volker, U., Volzke, H., Galan, P., Hercberg, S., Lathrop, M., Zelenika, D., Deloukas, P., Mangino, M., Spector, T. D., Zhai, G., Meschia, J. F., Nalls, M. A., Sharma, P., Terzic, J., Kumar, M. V, Denniff, M., Zukowska-Szczechowska, E., Wagenknecht, L. E., Fowkes, F. G., Charchar, F. J., Schwarz, P. E., Hayward, C., Guo, X., Rotimi, C., Bots, M. L., Brand, E., Samani, N. J., Polasek, O., Talmud, P. J., Nyberg, F., Kuh, D., Laan, M., Hveem, K., Palmer, L. J., van der Schouw, Y. T., Casas, J. P., Mohlke, K. L., Vineis, P., Raitakari, O., Ganesh, S. K., Wong, T. Y., Tai, E. S., Cooper, R. S., Laakso, M., Rao, D. C., Harris, T. B., Morris, R. W., Dominiczak, A. F., Kivimaki, M., Marmot, M. G., Miki, T., Saleheen, D., Chandak, G. R., Coresh, J., Navis, G., Salomaa, V., Han, B. G., Zhu, X., Kooner, J. S., Melander, O., Ridker, P. M., Bandinelli, S., Gyllensten, U. B., Wright, A. F., Wilson, J. F., Ferrucci, L., Farrall, M., Tuomilehto, J., Pramstaller, P. P., Elosua, R., Soranzo, N., Sijbrands, E. J., Altshuler, D., Loos, R. J., Shuldiner, A. R., Gieger, C., Meneton, P., Uitterlinden, A. G., Wareham, N. J., Gudnason, V., Rotter, J. I., Rettig, R., Uda, M., Strachan, D. P., Witteman, J. C., Hartikainen, A. L., Beckmann, J. S., Boerwinkle, E., Vasan, R. S., Boehnke, M., Larson, M. G., Jarvelin, M. R., Psaty, B. M., Abecasis, G. R., Chakravarti, A., Elliott, P., van Duijn, C. M., Newton-Cheh, C., Levy, D., Caulfield, M. J. & Johnson, T. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. *Nature* **478,** 103–109 (2011).

20.   Wain, L. V, Verwoert, G. C., O'Reilly, P. F., Shi, G., Johnson, T., Johnson, A. D., Bochud, M., Rice, K. M., Henneman, P., Smith, A. V, Ehret, G. B., Amin, N., Larson, M. G., Mooser, V., Hadley, D., Dorr, M., Bis, J. C., Aspelund, T., Esko, T., Janssens, A. C.,

Zhao, J. H., Heath, S., Laan, M., Fu, J., Pistis, G., Luan, J., Arora, P., Lucas, G., Pirastu, N., Pichler, I., Jackson, A. U., Webster, R. J., Zhang, F., Peden, J. F., Schmidt, H., Tanaka, T., Campbell, H., Igl, W., Milaneschi, Y., Hottenga, J. J., Vitart, V., Chasman, D. I., Trompet, S., Bragg-Gresham, J. L., Alizadeh, B. Z., Chambers, J. C., Guo, X., Lehtimaki, T., Kuhnel, B., Lopez, L. M., Polasek, O., Boban, M., Nelson, C. P., Morrison, A. C., Pihur, V., Ganesh, S. K., Hofman, A., Kundu, S., Mattace-Raso, F. U., Rivadeneira, F., Sijbrands, E. J., Uitterlinden, A. G., Hwang, S. J., Vasan, R. S., Wang, T. J., Bergmann, S., Vollenweider, P., Waeber, G., Laitinen, J., Pouta, A., Zitting, P., McArdle, W. L., Kroemer, H. K., Volker, U., Volzke, H., Glazer, N. L., Taylor, K. D., Harris, T. B., Alavere, H., Haller, T., Keis, A., Tammesoo, M. L., Aulchenko, Y., Barroso, I., Khaw, K. T., Galan, P., Hercberg, S., Lathrop, M., Eyheramendy, S., Org, E., Sober, S., Lu, X., Nolte, I. M., Penninx, B. W., Corre, T., Masciullo, C., Sala, C., Groop, L., Voight, B. F., Melander, O., O'Donnell, C. J., Salomaa, V., d'Adamo, A. P., Fabretto, A., Faletra, F., Ulivi, S., Del Greco, F., Facheris, M., Collins, F. S., Bergman, R. N., Beilby, J. P., Hung, J., Musk, A. W., Mangino, M., Shin, S. Y., Soranzo, N., Watkins, H., Goel, A., Hamsten, A., Gider, P., Loitfelder, M., Zeginigg, M., Hernandez, D., Najjar, S. S., Navarro, P., Wild, S. H., Corsi, A. M., Singleton, A., de Geus, E. J., Willemsen, G., Parker, A. N., Rose, L. M., Buckley, B., Stott, D., Orru, M., Uda, M., van der Klauw, M. M., Zhang, W., Li, X., Scott, J., Chen, Y. D., Burke, G. L., Kahonen, M., Viikari, J., Doring, A., Meitinger, T., Davies, G., Starr, J. M., Emilsson, V., Plump, A., Lindeman, J. H., Hoen, P. A., Konig, I. R., Felix, J. F., Clarke, R., Hopewell, J. C., Ongen, H., Breteler, M., Debette, S., Destefano, A. L., Fornage, M., Mitchell, G. F., Smith, N. L., Holm, H., Stefansson, K., Thorleifsson, G., Thorsteinsdottir, U., Samani, N. J., Preuss, M., Rudan, I., Hayward, C.,

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Deary, I. J., Wichmann, H. E., Raitakari, O. T., Palmas, W., Kooner, J. S., Stolk, R. P., Jukema, J. W., Wright, A. F., Boomsma, D. I., Bandinelli, S., Gyllensten, U. B., Wilson, J. F., Ferrucci, L., Schmidt, R., Farrall, M., Spector, T. D., Palmer, L. J., Tuomilehto, J., Pfeufer, A., Gasparini, P., Siscovick, D., Altshuler, D., Loos, R. J., Toniolo, D., Snieder, H., Gieger, C., Meneton, P., Wareham, N. J., Oostra, B. A., Metspalu, A., Launer, L., Rettig, R., Strachan, D. P., Beckmann, J. S., Witteman, J. C., Erdmann, J., van Dijk, K. W., Boerwinkle, E., Boehnke, M., Ridker, P. M., Jarvelin, M. R., Chakravarti, A., Abecasis, G. R., Gudnason, V., Newton-Cheh, C., Levy, D., Munroe, P. B., Psaty, B. M., Caulfield, M. J., Rao, D. C., Tobin, M. D., Elliott, P. & van Duijn, C. M. Genome-wide association study identifies six new loci influencing pulse pressure and mean arterial pressure. *Nat. Genet.* **43,** 1005–1011 (2011).

21.   Fletcher, O., Johnson, N., Orr, N., Hosking, F. J., Gibson, L. J., Walker, K., Zelenika, D., Gut, I., Heath, S., Palles, C., Coupland, B., Broderick, P., Schoemaker, M., Jones, M., Williamson, J., Chilcott-Burns, S., Tomczyk, K., Simpson, G., Jacobs, K. B., Chanock, S. J., Hunter, D. J., Tomlinson, I. P., Swerdlow, A., Ashworth, A., Ross, G., dos Santos Silva, I., Lathrop, M., Houlston, R. S. & Peto, J. Novel breast cancer susceptibility locus at 9q31.2: results of a genome-wide association study. *J. Natl. Cancer Inst.* **103,** 425–435 (2011).

22.   Zhang, K., Huentelman, M. J., Rao, F., Sun, E. I., Corneveaux, J. J., Schork, A. J., Wei, Z., Waalen, J., Miramontes-Gonzalez, J. P., Hightower, C. M., Maihofer, A. X., Mahata, M., Pastinen, T., Ehret, G. B., Schork, N. J., Eskin, E., Nievergelt, C. M., Saier, M. H. & O'Connor, D. T. Genetic implication of a novel thiamine transporter in human hypertension. *J. Am. Coll. Cardiol.* **63,** 1542–1555 (2014).

23. Nicolson, T. J., Bellomo, E. A., Wijesekara, N., Loder, M. K., Baldwin, J. M., Gyulkhandanyan, A. V., Koshkin, V., Tarasov, A. I., Carzaniga, R., Kronenberger, K., Taneja, T. K., Da Silva Xavier, G., Libert, S., Froguel, P., Scharfmann, R., Stetsyuk, V., Ravassard, P., Parker, H., Gribble, F. M., Reimann, F., Sladek, R., Hughes, S. J., Johnson, P. R. V, Masseboeuf, M., Burcelin, R., Baldwin, S. A., Liu, M., Lara-Lemus, R., Arvan, P., Schuit, F. C., Wheeler, M. B., Chimienti, F. & Rutter, G. A. Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. *Diabetes* **58,** 2070–2083 (2009).

24. Ishihara, H., Maechler, P., Gjinovci, A., Herrera, P.-L. & Wollheim, C. B. Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. *Nat. Cell Biol.* **5,** 330–335 (2003).

25. Kim, B. J., Kim, Y. H., Kim, S., Kim, J. W., Koh, J. Y., Oh, S. H., Lee, M. S. K. & Kim, K. W. Zinc as a paracrine effector in pancreatic islet cell death. *Diabetes* **49,** 367–372 (2000).

26. Tamaki, M., Fujitani, Y., Hara, A., Uchida, T., Tamura, Y., Takeno, K., Kawaguchi, M., Watanabe, T., Ogihara, T., Fukunaka, A., Shimizu, T., Mita, T., Kanazawa, A., Imaizumi, M. O., Abe, T., Kiyonari, H., Hojyo, S., Fukada, T., Kawauchi, T., Nagamatsu, S., Hirano, T., Kawamori, R. & Watada, H. The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. *J. Clin. Invest.* **123,** 4513–4524 (2013).

27. Flannick, J., Thorleifsson, G., Beer, N. L., Jacobs, S. B. R., Grarup, N., Burtt, N. P., Mahajan, A., Fuchsberger, C., Atzmon, G., Benediktsson, R., Blangero, J., Bowden, D. W., Brandslund, I., Brosnan, J., Burslem, F., Chambers, J., Cho, Y. S., Christensen, C., Douglas, D. a, Duggirala, R., Dymek, Z., Farjoun, Y., Fennell, T., Fontanillas, P., Forsén,

T., Gabriel, S., Glaser, B., Gudbjartsson, D. F., Hanis, C., Hansen, T., Hreidarsson, A. B., Hveem, K., Ingelsson, E., Isomaa, B., Johansson, S., Jørgensen, T., Jørgensen, M. E., Kathiresan, S., Kong, A., Kooner, J., Kravic, J., Laakso, M., Lee, J.-Y., Lind, L., Lindgren, C. M., Linneberg, A., Masson, G., Meitinger, T., Mohlke, K. L., Molven, A., Morris, A. P., Potluri, S., Rauramaa, R., Ribel-Madsen, R., Richard, A.-M., Rolph, T., Salomaa, V., Segrè, A. V, Skärstrand, H., Steinthorsdottir, V., Stringham, H. M., Sulem, P., Tai, E. S., Teo, Y. Y., Teslovich, T., Thorsteinsdottir, U., Trimmer, J. K., Tuomi, T., Tuomilehto, J., Vaziri-Sani, F., Voight, B. F., Wilson, J. G., Boehnke, M., McCarthy, M. I., Njølstad, P. R., Pedersen, O., Groop, L., Cox, D. R., Stefansson, K. & Altshuler, D. Loss-of-function mutations in SLC30A8 protect against type 2 diabetes. *Nat. Genet.* **46,** 357–363 (2014).

28.   Hopkins, A. L. & Groom, C. R. The druggable genome. *Nat. Rev. Drug Discov.* **1,** 727–730 (2002).

29.   Rask-Andersen, M., Masuram, S., Fredriksson, R., Schiöth, H. B. & Schioth, H. B. Solute carriers as drug targets: Current use, clinical trials and prospective. *Mol. Aspects Med.* **34,** 702–710 (2013).

30.   El-Gebali, S., Bentz, S., Hediger, M. A. & Anderle, P. Solute carriers (SLCs) in cancer. *Mol. Aspects Med.* **34,** 719–734 (2013).

31.   Sophic   Alliance.   The   Integrated   Druggable   Genome   Database.   at <http://www.sophicalliance.com/documents/The Druggable Genome012511.pdf>

32.   Zhu, F., Shi, Z., Qin, C., Tao, L., Liu, X., Xu, F., Zhang, L., Song, Y., Zhang, J., Han, B., Zhang, P. & Chen, Y. Therapeutic target database update 2012: a resource for facilitating target-oriented drug discovery. *Nucleic Acids Res.* **40,** D1128–36 (2012).

33. Knox, C., Law, V., Jewison, T., Liu, P., Ly, S., Frolkis, A., Pon, A., Banco, K., Mak, C., Neveu, V., Djoumbou, Y., Eisner, R., Guo, A. C. & Wishart, D. S. DrugBank 3.0: A comprehensive resource for 'Omics' research on drugs. *Nucleic Acids Res.* **39,** D1035–D1041 (2011).

34. Rask-Andersen, M., Masuram, S. & Schiöth, H. B. The druggable genome: Evaluation of drug targets in clinical trials suggests major shifts in molecular class and indication. *Annu. Rev. Pharmacol. Toxicol.* **54,** 9–26 (2014).

35. Fumagalli, E., Funicello, M., Rauen, T., Gobbi, M. & Mennini, T. Riluzole enhances the activity of glutamate transporters GLAST, GLT1 and EAAC1. *Eur J Pharmacol* **578,** 171–176 (2008).

36. Lin, C. L. G., Bristol, L. A., Jin, L., Dykes-Hoberg, M., Crawford, T., Clawson, L. & Rothstein, J. D. Aberrant RNA processing in a neurodegenerative disease: The cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron* **20,** 589–602 (1998).

37. Yatomi, Y., Tanaka, R., Shimura, H., Miyamoto, N., Yamashiro, K., Takanashi, M., Urabe, T. & Hattori, N. Chronic brain ischemia induces the expression of glial glutamate transporter EAAT2 in subcortical white matter. *Neuroscience* **244,** 113–121 (2013).

38. Abrahamsen, B., Schneider, N., Erichsen, M. N., Huynh, T. H., Fahlke, C., Bunch, L. & Jensen, A. A. Allosteric modulation of an excitatory amino acid transporter: the subtype-selective inhibitor UCPH-101 exerts sustained inhibition of EAAT1 through an intramonomeric site in the trimerization domain. *J. Neurosci.* **33,** 1068–1087 (2013).

39. Huynh, T. H. V, Shim, I., Bohr, H., Abrahamsen, B., Nielsen, B., Jensen, A. A. & Bunch, L. Structure-activity relationship study of selective excitatory amino acid transporter

subtype 1 (EAAT1) inhibitor 2-amino-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile (UCPH-101) and absolute configurational as. *J. Med. Chem.* **55,** 5403–5412 (2012).

40. Kanai, Y., Clémençon, B., Simonin, A., Leuenberger, M., Lochner, M., Weisstanner, M. & Hediger, M. A. The SLC1 high-affinity glutamate and neutral amino acid transporter family. *Mol. Aspects Med.* **34,** 108–120 (2013).

41. Takebayashi, R., Izuishi, K., Yamamoto, Y., Kameyama, R., Mori, H., Masaki, T. & Suzuki, Y. [18F]Fluorodeoxyglucose accumulation as a biological marker of hypoxic status but not glucose transport ability in gastric cancer. *J. Exp. Clin. Cancer Res.* **32,** 34 (2013).

42. Mertens, K., Mees, G., Lambert, B., Van de Wiele, C. & Goethals, I. In vitro 2-deoxy-2-[18F]fluoro-D-glucose uptake: practical considerations. *Cancer Biother. Radiopharm.* **27,** 183–188 (2012).

43. Liu, Y., Cao, Y., Zhang, W., Bergmeier, S., Qian, Y., Akbar, H., Colvin, R., Ding, J., Tong, L., Wu, S., Hines, J. & Chen, X. A small-molecule inhibitor of glucose transporter 1 downregulates glycolysis, induces cell-cycle arrest, and inhibits cancer cell growth in vitro and in vivo. *Mol. Cancer Ther.* **11,** 1672–1682 (2012).

44. Zambrowicz, B., Freiman, J., Brown, P. M., Frazier, K. S., Turnage, A., Bronner, J., Ruff, D., Shadoan, M., Banks, P., Mseeh, F., Rawlins, D. B., Goodwin, N. C., Mabon, R., Harrison, B. A., Wilson, A., Sands, A. & Powell, D. R. LX4211, a dual SGLT1/SGLT2 inhibitor, improved glycemic control in patients with type 2 diabetes in a randomized, placebo-controlled trial. *Clin. Pharmacol. Ther.* **92,** 158–169 (2012).

45. Gorboulev, V., Schurmann, A., Vallon, V., Kipp, H., Jaschke, A., Klessen, D., Friedrich, A., Scherneck, S., Rieg, T., Cunard, R., Veyhl-Wichmann, M., Srinivasan, A., Balen, D., Breljak, D., Rexhepaj, R., Parker, H. E., Gribble, F. M., Reimann, F., Lang, F., Wiese, S., Sabolic, I., Sendtner, M. & Koepsell, H. Na(+)-D-glucose cotransporter SGLT1 is pivotal for intestinal glucose absorption and glucose-dependent incretin secretion. *Diabetes* **61,** 187–196 (2012).

46. Feld, L. G. Renal Glycosuria. *Pediatric Nephrology Resource Center* (2004). at <http://www.childrensdayton.org/cms/resource_library/nephrology_files/29a7a3e7e2ad2c 63/glycosuria_renal_benign.pdf>

47. Weeks, A. J., Jauregui-Osoro, M., Cleij, M., Blower, J. E., Ballinger, J. R. & Blower, P. J. Evaluation of [18F]-tetrafluoroborate as a potential PET imaging agent for the human sodium/iodide symporter in a new colon carcinoma cell line, HCT116, expressing hNIS. *Nucl. Med. Commun.* **32,** 98–105 (2011).

48. Jauregui-Osoro, M., Sunassee, K., Weeks, A. J., Berry, D. J., Paul, R. L., Cleij, M., Banga, J. P., O'Doherty, M. J., Marsden, P. K., Clarke, S. E., Ballinger, J. R., Szanda, I., Cheng, S. Y. & Blower, P. J. Synthesis and biological evaluation of [(18)F]tetrafluoroborate: a PET imaging agent for thyroid disease and reporter gene imaging of the sodium/iodide symporter. *Eur. J. Nucl. Med. Mol. Imaging* **37,** 2108–2116 (2010).

49. Kamm, C. Infantile dystonia-parkinsonism. *Orphanet Encyclopedia* (2013). at <http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=238455>

50. Jeon, B., Kim, J. M., Jeong, J. M., Kim, K. M., Chang, Y. S., Lee, D. S. & Lee, M. C. Dopamine transporter imaging with [123I]-beta-CIT demonstrates presynaptic

nigrostriatal dopaminergic damage in Wilson's disease. *J. Neurol. Neurosurg. Psychiatry* **65,** 60–64 (1998).

51.   Jeon, B. S., Jeong, J. M., Park, S. S., Kim, J. M., Chang, Y. S., Song, H. C., Kim, K. M., Yoon, K. Y., Lee, M. C. & Lee, S. B. Dopamine transporter density measured by [123I]beta-CIT single-photon emission computed tomography is normal in dopa-responsive dystonia. *Ann. Neurol.* **43,** 792–800 (1998).

52.   Morita, K., Motoyama, N., Kitayama, T., Morioka, N., Kifune, K. & Dohi, T. Spinal antiallodynia action of glycine transporter inhibitors in neuropathic pain models in mice. *J. Pharmacol. Exp. Ther.* **326,** 633–645 (2008).

53.   Yoshikawa, S., Oguchi, T., Funahashi, Y., de Groat, W. C. & Yoshimura, N. Glycine transporter type 2 (GlyT2) inhibitor ameliorates bladder overactivity and nociceptive behavior in rats. *Eur. Urol.* **62,** 704–712 (2012).

54.   Kurosawa, Y., Degrauw, T. J., Lindquist, D. M., Blanco, V. M., Pyne-Geithman, G. J., Daikoku, T., Chambers, J. B., Benoit, S. C. & Clark, J. F. Cyclocreatine treatment improves cognition in mice with creatine transporter deficiency. *J. Clin. Invest.* **122,** 2837–2846 (2012).

55.   Trotier-Faurion, A., Dezard, S., Taran, F., Valayannopoulos, V., de Lonlay, P. & Mabondzo, A. Synthesis and biological evaluation of new creatine Fatty esters revealed dodecyl creatine ester as a promising drug candidate for the treatment of the creatine transporter deficiency. *J. Med. Chem.* **56,** 5173–5181 (2013).

56.   Mercimek-Mahmutoglu, S., Connolly, M. B., Poskitt, K. J., Horvath, G. A., Lowry, N., Salomons, G. S., Casey, B., Sinclair, G., Davis, C., Jakobs, C. & Stockler-Ipsiroglu, S.

Treatment of intractable epilepsy in a female with SLC6A8 deficiency. *Mol. Genet. Metab.* **101,** 409–412 (2010).

57. Zanni, G. Christianson syndrome. *Orphanet Encyclopedia* (2013). at <http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=85278>

58. Reshkin, S. J., Cardone, R. A. & Harguindey, S. Na+-H+ exchanger, pH regulation and cancer. *Recent Pat. Anticancer Drug Discov.* **8,** 85–99 (2013).

59. Loo, S. Y., Chang, M. K., Chua, C. S., Kumar, A. P., Pervaiz, S. & Clement, M. V. NHE-1: a promising target for novel anti-cancer therapeutics. *Curr. Pharm. Des.* **18,** 1372–1382 (2012).

60. Sakamoto, S., Kusuhara, H., Horie, K., Takahashi, K., Baba, T., Ishizaki, J. & Sugiyama, Y. Identification of the transporters involved in the hepatobiliary transport and intestinal efflux of methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) glucuronide, a pharmacologically active metabolite of S-. *Drug Metab. Dispos.* **36,** 1553–1561 (2008).

61. Sakamoto, S., Kusuhara, H., Miyata, K., Shimaoka, H., Kanazu, T., Matsuo, Y., Nomura, K., Okamura, N., Hara, S., Horie, K., Baba, T. & Sugiyama, Y. Glucuronidation converting methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) to a potent apical sodium-dependent bile acid transporter inhibitor, resulting in a hypocholesterolemic action. *J. Pharmacol. Exp. Ther.* **322,** 610–618 (2007).

62. Rais, R., Fletcher, S. & Polli, J. E. Synthesis and in vitro evaluation of gabapentin prodrugs that target the human apical sodium-dependent bile acid transporter (hASBT). *J. Pharm. Sci.* **100,** 1184–1195 (2011).

63. Balakrishnan, A. & Polli, J. E. Apical sodium dependent bile acid transporter (ASBT, SLC10A2): a potential prodrug target. *Mol. Pharm.* **3,** 223–230 (2006).

64. Pinheiro, C., Albergaria, A., Paredes, J., Sousa, B., Dufloth, R., Vieira, D., Schmitt, F. & Baltazar, F. Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma. *Histopathology* **56,** 860–867 (2010).

65. Kennedy, K. M. & Dewhirst, M. W. Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation. *Futur. Oncol.* **6,** 127–148 (2010).

66. Murray, C. M., Hutchinson, R., Bantick, J. R., Belfield, G. P., Benjamin, A. D., Brazma, D., Bundick, R. V, Cook, I. D., Craggs, R. I., Edwards, S., Evans, L. R., Harrison, R., Holness, E., Jackson, A. P., Jackson, C. G., Kingston, L. P., Perry, M. W., Ross, A. R., Rugman, P. A., Sidhu, S. S., Sullivan, M., Taylor-Fishwick, D. A., Walker, P. C., Whitehead, Y. M., Wilkinson, D. J., Wright, A. & Donald, D. K. Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nat. Chem. Biol.* **1,** 371–376 (2005).

67. Polański, R., Hodgkinson, C. L., Fusi, A., Nonaka, D., Priest, L., Kelly, P., Trapani, F., Bishop, P. W., White, A., Critchlow, S. E., Smith, P. D., Blackhall, F., Dive, C., Morrow, C. J. & Polanski, R. Activity of the monocarboxylate transporter 1 inhibitor AZD3965 in small cell lung cancer. *Clin. Cancer Res.* **20,** 926–937 (2014).

68. Patel, S. A., Nagy, J. O., Bolstad, E. D., Gerdes, J. M. & Thompson, C. M. Tetrapeptide inhibitors of the glutamate vesicular transporter (VGLUT). *Bioorg. Med. Chem. Lett.* **17,** 5125–5128 (2007).

69. Thompson, C. M., Davis, E., Carrigan, C. N., Cox, H. D., Bridges, R. J. & Gerdes, J. M. Inhibitor of the glutamate vesicular transporter (VGLUT). *Curr. Med. Chem.* **12,** 2041–2056 (2005).

70. Carrigan, C. N., Bartlett, R. D., Esslinger, C. S., Cybulski, K. A., Tongcharoensirikul, P., Bridges, R. J. & Thompson, C. M. Synthesis and in vitro pharmacology of substituted quinoline-2,4-dicarboxylic acids as inhibitors of vesicular glutamate transport. *J. Med. Chem.* **45,** 2260–2276 (2002).

71. Zhang, Q., Zhang, Y., Diamond, S., Boer, J., Harris, J. J., Li, Y., Rupar, M., Behshad, E., Gardiner, C., Collier, P., Liu, P., Burn, T., Wynn, R., Hollis, G. & Yeleswaram, S. The Janus Kinase 2 Inhibitor Fedratinib Inhibits Thiamine Uptake: A Putative Mechanism for the Onset of Wernicke's Encephalopathy. *Drug Metab. Dispos.* **42,** 1656–1662 (2014).

72. Iwai, N., Mino, Y., Hosoyamada, M., Tago, N., Kokubo, Y. & Endou, H. A high prevalence of renal hypouricemia caused by inactive SLC22A12 in Japanese. *Kidney Int.* **66,** 935–944 (2004).

73. Crittenden, D. B. & Pillinger, M. H. New therapies for gout. *Annu. Rev. Med.* **64,** 325–337 (2013).

74. Iacobazzi, V., Convertini, P., Infantino, V., Scarcia, P., Todisco, S. & Palmieri, F. Statins, fibrates and retinoic acid upregulate mitochondrial acylcarnitine carrier gene expression. *Biochem Biophys Res Commun* **388,** 643–647 (2009).

75. Alasti, F., Van Camp, G. & Smith, R. J. Pendred Syndrome/DFNB4. *GeneReviews* (2014). at <http://www.ncbi.nlm.nih.gov/books/NBK1467/>

76. Soleimani, M. A novel target for diuretic therapy. *Iran J. Kidney Dis.* **6,** 419–425 (2012).

77. Bali, D. S., Chen, Y.-T. & Goldstein, J. L. Glycogen Storage Disease Type I. *GeneReviews* (2013). at <http://www.ncbi.nlm.nih.gov/books/NBK1312/>

78. Yiu, W. H., Pan, C. J., Mead, P. A., Starost, M. F., Mansfield, B. C. & Chou, J. Y. Normoglycemia alone is insufficient to prevent long-term complications of hepatocellular adenoma in glycogen storage disease type Ib mice. *J. Hepatol.* **51,** 909–917 (2009).

79. Lee, Y. M., Jun, H. S., Pan, C. J., Lin, S. R., Wilson, L. H., Mansfield, B. C. & Chou, J. Y. Prevention of hepatocellular adenoma and correction of metabolic abnormalities in murine glycogen storage disease type Ia by gene therapy. *Hepatology* **56,** 1719–1729 (2012).

80. Posterior column ataxia - retinitis pigmentosa. *Orphanet Encyclopedia* (2011). at <http://www.orpha.net/consor/cgi-bin/OC_Exp.php?Expert=88628&lng=EN>

81. Yang, Z., Philips, J. D., Doty, R. T., Giraudi, P., Ostrow, J. D., Tiribelli, C., Smith, A. & Abkowitz, J. L. Kinetics and specificity of Feline Leukemia Virus Subgroup C Receptor (FLVCR) export function and its dependence on hemopexin. *J. Biol. Chem.* **285,** 28874–28882 (2010).

82. Wiley, J. S. & Cooper, R. A. A furosemide-sensitive cotransport of sodium plus potassium in the human red cell. *J. Clin. Invest.* **53,** 745–755 (1974).

83. Burg, M., Stoner, L., Cardinal, J. & Green, N. Furosemide effect on isolated perfused tubules. *Am. J. Physiol.* **225,** 119–124 (1973).

84. Markadieu, N. & Delpire, E. Physiology and pathophysiology of SLC12A1/2 transporters. *Pflugers Arch.* **466,** 91–105 (2014).

85. Xu, J. C., Lytle, C., Zhu, T. T., Payne, J. A., Benz, E. & Forbush, B. Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2201–2205 (1994).

86.   Simon, D. B., Karet, F. E., Hamdan, J. M., DiPietro, A., Sanjad, S. A. & Lifton, R. P. Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter NKCC2. *Nat. Genet.* **13,** 183–188 (1996).

87.   Simon, D. B., Nelson-Williams, C., Bia, M. J., Ellison, D., Karet, F. E., Molina, A. M., Vaara, I., Iwata, F., Cushner, H. M., Koolen, M., Gainza, F. J., Gitleman, H. J. & Lifton, R. P. Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat. Genet.* **12,** 24–30 (1996).

88.   Cha, S. H., Sekine, T., Fukushima, J. I., Kanai, Y., Kobayashi, Y., Goya, T. & Endou, H. Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol. Pharmacol.* **59,** 1277–1286 (2001).

89.   Jutabha, P., Anzai, N., Kitamura, K., Taniguchi, A., Kaneko, S., Yan, K., Yamada, H., Shimada, H., Kimura, T., Katada, T., Fukutomi, T., Tomita, K., Urano, W., Yamanaka, H., Seki, G., Fujita, T., Moriyama, Y., Yamada, A., Uchida, S., Wempe, M. F., Endou, H. & Sakurai, H. Human sodium phosphate transporter 4 (hNPT4/SLC17A3) as a common renal secretory pathway for drugs and urate. *J. Biol. Chem.* **285,** 35123–35132 (2010).

90.   Kristensen, A. S., Andersen, J., Jørgensen, T. N., Sørensen, L., Eriksen, J., Loland, C. J., Strømgaard, K. & Gether, U. SLC6 neurotransmitter transporters: structure, function, and regulation. *Pharmacol. Rev.* **63,** 585–640 (2011).

91.   Haase, J. & Brown, E. Integrating the monoamine, neurotrophin and cytokine hypotheses of depression — A central role for the serotonin transporter? *Pharmacol. Ther.* **147,** 1–11 (2015).

92. Wong, D. T., Horng, J. S., Bymaster, F. P., Hauser, K. L. & Molloy, B. B. A selective inhibitor of serotonin uptake: Lilly 110140, 3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine. *Life Sci.* **15,** 471–479 (1974).

93. Wong, D. T., Bymaster, F. P., Horng, J. S. & Molloy, B. B. A new selective inhibitor for uptake of serotonin into synaptosomes of rat brain: 3-(p-trifluoromethylphenoxy). N-methyl-3-phenylpropylamine. *J. Pharmacol. Exp. Ther.* **193,** 804–811 (1975).

94. Jankovic, J. & Clarence-Smith, K. Tetrabenazine for the treatment of chorea and other hyperkinetic movement disorders. *Expert Rev. Neurother.* **11,** 1509–1523 (2011).

95. Nickell, J. R., Siripurapu, K. B., Vartak, A., Crooks, P. A. & Dwoskin, L. P. The vesicular monoamine transporter-2: An important pharmacological target for the discovery of novel therapeutics to treat methamphetamine abuse. *Adv. Pharmacol.* **69,** 71–106 (2014).

96. Shibazaki, T., Tomae, M., Ishikawa-Takemura, Y., Fushimi, N., Itoh, F., Yamada, M. & Isaji, M. KGA-2727, a novel selective inhibitor of a high-affinity sodium glucose cotransporter (SGLT1), exhibits antidiabetic efficacy in rodent models. *J. Pharmacol. Exp. Ther.* **342,** 288–296 (2012).

97. Katsuno, K., Fujimori, Y., Takemura, Y., Hiratochi, M., Itoh, F., Komatsu, Y., Fujikura, H. & Isaji, M. Sergliflozin, a novel selective inhibitor of low-affinity sodium glucose cotransporter (SGLT2), validates the critical role of SGLT2 in renal glucose reabsorption and modulates plasma glucose level. *J. Pharmacol. Exp. Ther.* **320,** 323–330 (2007).

98. Fujimori, Y., Katsuno, K., Nakashima, I., Ishikawa-Takemura, Y., Fujikura, H. & Isaji, M. Remogliflozin etabonate, in a novel category of selective low-affinity sodium glucose cotransporter (SGLT2) inhibitors, exhibits antidiabetic efficacy in rodent models. *J. Pharmacol. Exp. Ther.* **327,** 268–76 (2008).

99. Fujimori, Y., Katsuno, K., Ojima, K., Nakashima, I., Nakano, S., Ishikawa-Takemura, Y., Kusama, H. & Isaji, M. Sergliflozin etabonate, a selective SGLT2 inhibitor, improves glycemic control in streptozotocin-induced diabetic rats and Zucker fatty rats. *Eur. J. Pharmacol.* **609,** 148–154 (2009).

100. Ferrannini, E., Muscelli, E., Frascerra, S., Baldi, S., Mari, A., Heise, T., Broedl, U. C. & Woerle, H.-J. Metabolic response to sodium-glucose cotransporter 2 inhibition in type 2 diabetic patients. *J. Clin. Invest.* **124,** 499–508 (2014).

101. Plosker, G. L. Canagliflozin: a review of its use in patients with type 2 diabetes mellitus. *Drugs* **74,** 807–824 (2014).

102. Forst, T., Guthrie, R., Goldenberg, R., Yee, J., Vijapurkar, U., Meininger, G. & Stein, P. Efficacy and safety of canagliflozin over 52 weeks in patients with type 2 diabetes on background metformin and pioglitazone. *Diabetes. Obes. Metab.* **16,** 467–477 (2014).

103. Cefalu, W. T., Leiter, L. A., Yoon, K.-H., Arias, P., Niskanen, L., Xie, J., Balis, D. A., Canovatchel, W. & Meininger, G. Efficacy and safety of canagliflozin versus glimepiride in patients with type 2 diabetes inadequately controlled with metformin (CANTATA-SU): 52 week results from a randomised, double-blind, phase 3 non-inferiority trial. *Lancet* **382,** 941–950 (2013).

104. Lapuerta, P., Rosenstock, J., Zambrowicz, B., Powell, D. R., Ogbaa, I., Freiman, J., Cefalu, W. T., Banks, P., Frazier, K., Kelly, M. & Sands, A. Study design and rationale of a dose-ranging trial of LX4211, a dual inhibitor of SGLT1 and SGLT2, in type 2 diabetes inadequately controlled on metformin monotherapy. *Clin. Cardiol.* **36,** 367–71 (2013).

105. Oliva, R. V & Bakris, G. L. Blood pressure effects of sodium-glucose co-transport 2 (SGLT2) inhibitors. *J. Am. Soc. Hypertens.* **8,** 330–339 (2014).

106. George, R. L. & Keenan, R. T. Genetics of hyperuricemia and gout: implications for the present and future. *Curr. Rheumatol. Rep.* **15,** 309 (2013).

107. Terkeltaub, R. Update on gout: new therapeutic strategies and options. *Nat. Rev. Rheumatol.* **6,** 30–38 (2010).

108. Anzai, N. & Endou, H. Urate Transporters: An Evolving Field. *Semin. Nephrol.* **31,** 400–409 (2011).

109. Harvey, R. J. & Yee, B. K. Glycine transporters as novel therapeutic targets in schizophrenia, alcohol dependence and pain. *Nat. Rev. Drug Discov.* **12,** 866–885 (2013).

110. Goff, D. C. Bitopertin: The good news and bad news. *JAMA psychiatry* **71,** 621–622 (2014).

111. Vandenberg, R. J., Ryan, R. M., Carland, J. E., Imlach, W. L. & Christie, M. J. Glycine transport inhibitors for the treatment of pain. *Trends in Pharmacological Sciences* **35,** 423–430 (2014).

112. Claro da Silva, T., Polli, J. E. & Swaan, P. W. The solute carrier family 10 (SLC10): Beyond bile acid transport. *Mol. Aspects Med.* **34,** 252–269 (2013).

113. Staels, B. & Fonseca, V. A. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. *Diabetes care* **32 Suppl 2,** S237–S245 (2009).

114. Dawson, P. A. Role of the intestinal bile acid transporters in bile acid and drug disposition. *Handb. Exp. Pharmacol.* **201,** 169–203 (2011).

115. Oelkers, P., Kirby, L. C., Heubi, J. E. & Dawson, P. A. *Primary bile acid malabsorption caused by mutations in the ileal sodium-dependent bile acid transporter gene (SLC10A2). The Journal of clinical investigation* **99,** 1880–1887 (1997).

116. Lundasen, T., Andersson, E. M., Snaith, M., Lindmark, H., Lundberg, J., Ostlund-Lindqvist, A. M., Angelin, B. & Rudling, M. Inhibition of intestinal bile acid transporter Slc10a2 improves triglyceride metabolism and normalizes elevated plasma glucose levels in mice. *PLoS One* **7,** e37787 (2012).

117. Wu, Y., Aquino, C. J., Cowan, D. J., Anderson, D. L., Ambroso, J. L., Bishop, M. J., Boros, E. E., Chen, L., Cunningham, A., Dobbins, R. L., Feldman, P. L., Harston, L. T., Kaldor, I. W., Klein, R., Liang, X., McIntyre, M. S., Merrill, C. L., Patterson, K. M., Prescott, J. S., Ray, J. S., Roller, S. G., Yao, X., Young, A., Yuen, J. & Collins, J. L. Discovery of a highly potent, nonabsorbable apical sodium-dependent bile acid transporter inhibitor (GSK2330672) for treatment of type 2 diabetes. *J. Med. Chem.* **56,** 5094–5114 (2013).

118. McCracken, A. N. & Edinger, A. L. Nutrient transporters: The Achilles' heel of anabolism. *Trends in Endocrinology and Metabolism* **24,** 200–208 (2013).

119. Ganapathy, V., Thangaraju, M. & Prasad, P. D. Nutrient transporters in cancer: relevance to Warburg hypothesis and beyond. *Pharmacol. Ther.* **121,** 29–40 (2009).

120. Chan, D. A., Sutphin, P. D., Nguyen, P., Turcotte, S., Lai, E. W., Banh, A., Reynolds, G. E., Chi, J.-T., Wu, J., Solow-Cordero, D. E., Bonnet, M., Flanagan, J. U., Bouley, D. M., Graves, E. E., Denny, W. A., Hay, M. P. & Giaccia, A. J. Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. *Sci. Transl. Med.* **3,** 94ra70 (2011).

121. Airley, R. E. & Mobasheri, A. Hypoxic regulation of glucose transport, anaerobic metabolism and angiogenesis in cancer: Novel pathways and targets for anticancer therapeutics. *Chemotherapy* **53,** 233–256 (2007).

122. Miranda-Goncalves, V., Honavar, M., Pinheiro, C., Martinho, O., Pires, M. M., Cordeiro, M., Bebiano, G., Costa, P., Palmeirim, I., Reis, R. M. & Baltazar, F. Monocarboxylate transporters (MCTs) in gliomas: expression and exploitation as therapeutic targets. *Neuro. Oncol.* **15,** 172–188 (2013).

123. Le Floch, R., Chiche, J., Marchiq, I., Naiken, T., Ilc, K., Murray, C. M., Critchlow, S. E., Roux, D., Simon, M. P. & Pouyssegur, J. CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 16663–16668 (2011).

124. Provost, J. J. & Wallert, M. A. Inside out: targeting NHE1 as an intracellular and extracellular regulator of cancer progression. *Chem. Biol. Drug. Des.* **81,** 85–101 (2013).

125. Imai, H., Kaira, K., Oriuchi, N., Shimizu, K., Tominaga, H., Yanagitani, N., Sunaga, N., Ishizuka, T., Nagamori, S., Promchan, K., Nakajima, T., Yamamoto, N., Mori, M. & Kanai, Y. Inhibition of L-type amino acid transporter 1 has antitumor activity in non-small cell lung cancer. *Anticancer Res.* **30,** 4819–4828 (2010).

126. Nawashiro, H., Otani, N., Shinomiya, N., Fukui, S., Ooigawa, H., Shima, K., Matsuo, H., Kanai, Y. & Endou, H. L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors. *Int. J. Cancer* **119,** 484–492 (2006).

127. Hassanein, M., Hoeksema, M. D., Shiota, M., Qian, J., Harris, B. K., Chen, H., Clark, J. E., Alborn, W. E., Eisenberg, R. & Massion, P. P. SLC1A5 mediates glutamine transport required for lung cancer cell growth and survival. *Clin. Cancer Res.* **19,** 560–570 (2013).

128. Timmerman, L., Holton, T., Yuneva, M., Louie, R., Padró, M., Daemen, A., Hu, M., Chan, D., Ethier, S., Van'tVeer, L., Polyak, K., McCormick, F. & Gray, J. Glutamine

Sensitivity Analysis Identifies the xCT Antiporter as a Common Triple-Negative Breast Tumor Therapeutic Target. *Cancer Cell* **24,** 450–465 (2013).

129. Gout, P. W., Buckley, A. R., Simms, C. R. & Bruchovsky, N. Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the x(c)- cystine transporter: a new action for an old drug. *Leukemia* **15,** 1633–1640 (2001).

130. Kong, F.-L. & J. Yang, D. Amino Acid Transporter-Targeted Radiotracers for Molecular Imaging in Oncology. *Current Medicinal Chemistry* **19,** 3271–3281 (2012).

131. Guan, Z., Xu, B., Wang, R., Sun, L. & Tian, J. Hyperaccumulation of (18)F-FDG in order to differentiate solid pseudopapillary tumors from adenocarcinomas and from neuroendocrine pancreatic tumors and review of the literature. *Hell. J. Nucl. Med.* **16,** 97– 102

132. Kaira, K., Sunaga, N., Ishizuka, T., Shimizu, K. & Yamamoto, N. The role of [^{18}F]fluorodeoxyglucose positron emission tomography in thymic epithelial tumors. *Cancer Imaging* **11,** 195–201 (2011).

133. Nogami, T., Takano, H., Arakawa, R., Ichimiya, T., Fujiwara, H., Kimura, Y., Kodaka, F., Sasaki, T., Takahata, K., Suzuki, M., Nagashima, T., Mori, T., Shimada, H., Fukuda, H., Sekine, M., Tateno, A., Takahashi, H., Ito, H., Okubo, Y. & Suhara, T. Occupancy of serotonin and norepinephrine transporter by milnacipran in patients with major depressive disorder: a positron emission tomography study with [11C]DASB and (S,S)-[18F]FMeNER-D2. *The International Journal of Neuropsychopharmacology* **16,** 1–7 (2012).

134. Takano, A., Halldin, C. & Farde, L. SERT and NET occupancy by venlafaxine and milnacipran in nonhuman primates: A PET study. *Psychopharmacology (Berl).* **226,** 147–153 (2013).

135. Comley, R. A., Salinas, C. A., Slifstein, M., Petrone, M., Marzano, C., Bennacef, I., Shotbolt, P., Van der Aart, J., Neve, M., Iavarone, L., Gomeni, R., Laruelle, M., Gray, F. A., Gunn, R. N. & Rabiner, E. A. Monoamine transporter occupancy of a novel triple reuptake inhibitor in baboons and humans using positron emission tomography. *J. Pharmacol. Exp. Ther.* **346,** 311–317 (2013).

136. Lin, S.-C., Lin, K.-J., Hsiao, I.-T., Hsieh, C.-J., Lin, W.-Y., Lu, C.-S., Wey, S.-P., Yen, T.-C., Kung, M.-P. & Weng, Y.-H. In Vivo Detection of Monoaminergic Degeneration in Early Parkinson Disease by 18F-9-Fluoropropyl-(+)-Dihydrotetrabenzazine PET. *J. Nucl. Med.* **55,** 73–79 (2014).

137. Giboureau, N., Som, I. M., Boucher-Arnold, A., Guilloteau, D. & Kassiou, M. PET radioligands for the vesicular acetylcholine transporter (VAChT). *Curr. Top. Med. Chem.* **10,** 1569–1583 (2010).

138. Wulkersdorfer, B., Wanek, T., Bauer, M., Zeitlinger, M., Müller, M. & Langer, O. Using positron emission tomography to study transporter-mediated drug-drug interactions in tissues. *Clin. Pharmacol. Ther.* **96,** 206–13 (2014).

139. Hume, W. E., Shingaki, T., Takashima, T., Hashizume, Y., Okauchi, T., Katayama, Y., Hayashinaka, E., Wada, Y., Kusuhara, H., Sugiyama, Y. & Watanabe, Y. The synthesis and biodistribution of [11C]metformin as a PET probe to study hepatobiliary transport mediated by the multi-drug and toxin extrusion transporter 1 (MATE1) in vivo. *Bioorganic Med. Chem.* **21,** 7584–7590 (2013).

140. Hu, G., Henke, A., Karpowicz, R. J., Sonders, M. S., Farrimond, F., Edwards, R., Sulzer, D. & Sames, D. New fluorescent substrate enables quantitative and high-throughput examination of vesicular monoamine transporter 2 (VMAT2). *ACS Chem. Biol.* **8,** 1947–1954 (2013).

141. Ulanovskaya, O. A., Cui, J., Kron, S. J. & Kozmin, S. A. A pairwise chemical genetic screen identifies new inhibitors of glucose transport. *Chem. Biol.* **18,** 222–230 (2011).

142. Wittwer, M. B., Zur, A. a., Khuri, N., Kido, Y., Kosaka, A., Zhang, X., Morrissey, K. M., Sali, A., Huang, Y. & Giacomini, K. M. Discovery of potent, selective multidrug and toxin extrusion transporter 1 (MATE1, SLC47A1) inhibitors through prescription drug profiling and computational modeling. *J. Med. Chem.* **56,** 781–795 (2013).

143. Kido, Y., Matsson, P. & Giacomini, K. M. Profiling of a prescription drug library for potential renal drug-drug interactions mediated by the organic cation transporter 2. *J. Med. Chem.* **54,** 4548–4558 (2011).

144. Gui, C., Obaidat, A., Chaguturu, R. & Hagenbuch, B. Development of a cell-based high-throughput assay to screen for inhibitors of organic anion transporting polypeptides 1B1 and 1B3. *Curr. Chem. Genomics* **4,** 1–8 (2010).

145. Jani, M. & Krajcsi, P. In vitro methods in drug transporter interaction assessment. *Drug Discov. Today. Technol.* **12,** e105–12 (2014).

146. Pedersen, J. M., Matsson, P., Bergström, C. A., Hoogstraate, J., Norén, A., LeCluyse, E. L. & Artursson, P. Early identification of clinically relevant drug interactions with the human bile salt export pump (BSEP/ABCB11). *Toxicol. Sci.* **136,** 328–343 (2013).

147. Sodani, K., Patel, A., Anreddy, N., Singh, S., Yang, D. H., Kathawala, R. J., Kumar, P., Talele, T. T. & Chen, Z. S. Telatinib reverses chemotherapeutic multidrug resistance

mediated by ABCG2 efflux transporter in vitro and in vivo. *Biochem. Pharmacol.* **89,** 52–61 (2014).

148. Pedersen, J. M., Matsson, P., Bergström, C. A., Norinder, U., Hoogstraate, J. & Artursson, P. Prediction and identification of drug interactions with the human ATP-binding cassette transporter multidrug-resistance associated protein 2 (MRP2; ABCC2). *J. Med. Chem.* **51,** 3275–3287 (2008).

149. Zhang, J.-H. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4,** 67–73 (1999).

150. Thorne, N., Auld, D. S. & Inglese, J. Apparent activity in high-throughput screening: origins of compound-dependent assay interference. *Current Opinion in Chemical Biology* **14,** 315–324 (2010).

151. Feng, B. Y., Simeonov, A., Jadhav, A., Babaoglu, K., Inglese, J., Shoichet, B. K. & Austin, C. P. A high-throughput screen for aggregation-based inhibition in a large compound library. *J. Med. Chem.* **50,** 2385–2390 (2007).

152. Jadhav, A., Ferreira, R. S., Klumpp, C., Mott, B. T., Austin, C. P., Inglese, J., Thomas, C. J., Maloney, D. J., Shoichet, B. K. & Simeonov, A. Quantitative analyses of aggregation, autofluorescence, and reactivity artifacts in a screen for inhibitors of a thiol protease. *J. Med. Chem.* **53,** 37–51 (2010).

153. Caporuscio, F. & Tafi, A. Pharmacophore modelling: a forty year old approach and its modern synergies. *Curr. Med. Chem.* **18,** 2543–2553 (2011).

154. Cumming, J. G., Davis, A. M., Muresan, S., Haeberlein, M. & Chen, H. Chemical predictive modelling to improve compound quality. *Nat. Rev. Drug Discov.* **12,** 948–962 (2013).

155. Zheng, X., Ekins, S., Raufman, J. & Polli, J. E. Computational models for drug inhibition of the human apical sodium-dependent bile acid transporter. *Mol. Pharm.* **6,** 1591–1603 (2009).

156. Zheng, X., Pan, Y., Acharya, C., Swaan, P. W. & Polli, J. E. Structural requirements of the ASBT by 3D-QSAR analysis using aminopyridine conjugates of chenodeoxycholic acid. *Bioconjug. Chem.* **21,** 2038–2048 (2010).

157. Esslinger, C. S., Agarwal, S., Gerdes, J., Wilson, P. A., Davis, E. S., Awes, A. N., O'Brien, E., Mavencamp, T., Koch, H. P., Poulsen, D. J., Rhoderick, J. F., Chamberlin, A. R., Kavanaugh, M. P. & Bridges, R. J. The substituted aspartate analogue L-??-threo-benzyl-aspartate preferentially inhibits the neuronal excitatory amino acid transporter EAAT3. *Neuropharmacology* **49,** 850–861 (2005).

158. MacDougall, I. J. A. & Griffith, R. Pharmacophore design and database searching for selective monoamine neurotransmitter transporter ligands. *J. Mol. Graph. Model.* **26,** 1113–1124 (2008).

159. Sharma, H., Santra, S., Debnath, J., Antonio, T., Reith, M. & Dutta, A. Flexible and biomimetic analogs of triple uptake inhibitor 4-((((3S,6S)-6-benzhydryltetrahydro-2H-pyran-3-yl)amino)methyl)phenol: Synthesis, biological characterization, and development of a pharmacophore model. *Bioorganic Med. Chem.* **22,** 311–324 (2014).

160. Santra, S., Gogoi, S., Gopishetty, B., Antonio, T., Zhen, J., Reith, M. E. & Dutta, A. K. Structural Exploration of (3S,6S)-6-Benzhydryl-N-benzyltetrahydro-2H-pyran-3-amine Analogues: Identification of Potent Triple Monoamine Reuptake Inhibitors as Potential Antidepressants. *Chem. Med. Chem.* **7,** 2093–2100 (2012).

161. Ohtake, Y., Sato, T., Kobayashi, T., Nishimoto, M., Taka, N., Takano, K., Yamamoto, K., Ohmori, M., Yamaguchi, M., Takami, K., Yeu, S. Y., Ahn, K. H., Matsuoka, H., Morikawa, K., Suzuki, M., Hagita, H., Ozawa, K., Yamaguchi, K., Kato, M. & Ikeda, S. Discovery of tofogliflozin, a novel C-arylglucoside with an O-spiroketal ring system, as a highly selective sodium glucose cotransporter 2 (SGLT2) inhibitor for the treatment of type 2 diabetes. *J. Med. Chem.* **55,** 7828–7840 (2012).

162. Schlessinger, A., Yee, S. W., Sali, A. & Giacomini, K. M. SLC classification: an update. *Clin. Pharmacol. Ther.* **94,** 19–23 (2013).

163. Schlessinger, A., Khuri, N., Giacomini, K. M. & Sali, A. Molecular modeling and ligand docking for solute carrier (SLC) transporters. *Curr. Top. Med. Chem.* **13,** 843–856 (2013).

164. Schlessinger, A., Wittwer, M. B., Dahlin, A., Khuri, N., Bonomi, M., Fan, H., Giacomini, K. M. & Sali, A. High selectivity of the ??-aminobutyric acid transporter 2 (GAT-2, SLC6A13) revealed by structure-based approach. *J. Biol. Chem.* **287,** 37745–37756 (2012).

165. Geier, E. G., Schlessinger, A., Fan, H., Gable, J. E., Irwin, J. J., Sali, A. & Giacomini, K. M. Structure-based ligand discovery for the Large-neutral Amino Acid Transporter 1, LAT-1. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 5480–5485 (2013).

166. Schlessinger, A., Geier, E., Fan, H., Irwin, J. J., Shoichet, B. K., Giacomini, K. M. & Sali, A. Structure-based discovery of prescription drugs that interact with the norepinephrine transporter, NET. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 15810–15815 (2011).

167. Luethi, E., Nguyen, K. T., Bürzle, M., Blum, L. C., Suzuki, Y., Hediger, M. & Reymond, J. L. Identification of selective norbornane-type aspartate analogue inhibitors of the

glutamate transporter 1 (GLT-1) from the chemical universe generated database (GDB). *J. Med. Chem.* **53,** 7236–7250 (2010).

168. Johnson, Z. L., Lee, J.-H., Lee, K., Lee, M., Kwon, D.-Y., Hong, J. & Lee, S.-Y. Structural basis of nucleoside and nucleoside drug selectivity by concentrative nucleoside transporters. *Elife* **3,** e03604 (2014).

169. Johnson, Z. L., Cheong, C.-G. & Lee, S.-Y. Crystal structure of a concentrative nucleoside transporter from Vibrio cholerae at 2.4 Å. *Nature* **483,** 489–493 (2012).

170. Zhou, Z., Zhen, J., Karpowich, N. K., Goetz, R. M., Law, C. J., Reith, M. E. a & Wang, D.-N. LeuT-desipramine structure reveals how antidepressants block neurotransmitter reuptake. *Science* **317,** 1390–1393 (2007).

171. Singh, S. K., Yamashita, A. & Gouaux, E. Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. *Nature* **448,** 952–956 (2007).

172. Penmatsa, A., Wang, K. H. & Gouaux, E. X-ray structure of dopamine transporter elucidates antidepressant mechanism. *Nature* **503,** 85–90 (2013).

173. Cuboni, S. & Hausch, F. Snapshot of antidepressants at work: The structure of neurotransmitter transporter proteins. *Angew. Chem. Int. Ed. Engl.* **53,** 5008–5009 (2014).

174. Cheah, B. C., Vucic, S., Krishnan, A. V & Kiernan, M. C. Riluzole, neuroprotection and amyotrophic lateral sclerosis. *Curr. Med. Chem.* **17,** 1942–1959 (2010).

175. Carbone, M., Duty, S. & Rattray, M. Riluzole elevates GLT-1 activity and levels in striatal astrocytes. *Neurochem. Int.* **60,** 31–38 (2012).

176. Dall'Igna, O. P., Bobermin, L. D., Souza, D. O. & Quincozes-Santos, A. Riluzole increases glutamate uptake by cultured C6 astroglial cells. *Int. J. Dev. Neurosci.* **31,** 482–486 (2013).

177. Woltjer, R. L., Duerson, K., Fullmer, J. M., Mookherjee, P., Ryan, A. M., Montine, T. J., Kaye, J. A., Quinn, J. F., Silbert, L., Erten-Lyons, D., Leverenz, J. B., Bird, T. D., Pow, D. V, Tanaka, K., Watson, G. S. & Cook, D. G. Aberrant detergent-insoluble excitatory amino acid transporter 2 accumulates in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **69,** 667–676 (2010).

178. Guo, H., Lai, L., Butchbach E.R., M. E. R., Stockinger, M. P., Shan, X., Bishop, G. A. & Lin, C. L. G. Increased expression of the glial glutamate transporter EAAT2 modulates excitotoxicity and delays the onset but not the outcome of ALS in mice. *Hum. Mol. Genet.* **12,** 2519–2532 (2003).

179. Kong, Q., Chang, L. C., Takahashi, K., Liu, Q., Schulte, D. A., Lai, L., Ibabao, B., Lin, Y., Stouffer, N., Mukhopadhyay, C. Das, Xing, X., Seyb, K. I., Cuny, G. D., Glicksman, M. A. & Lin, C. L. Small-molecule activator of glutamate transporter EAAT2 translation provides neuroprotection. *J. Clin. Invest.* **124,** 1255–1267 (2014).

180. Lin, C.-L. G., Kong, Q., Cuny, G. D. & Glicksman, M. A. Glutamate transporter EAAT2: a new target for the treatment of neurodegenerative diseases. *Future Medicinal Chemistry* **4,** 1689–1700 (2012).

181. Su, Z., Leszczyniecka, M., Kang, D., Sarkar, D., Chao, W., Volsky, D. J. & Fisher, P. B. Insights into glutamate transport regulation in human astrocytes: cloning of the promoter for excitatory amino acid transporter 2 (EAAT2). *Proc. Natl. Acad. Sci. U. S. A.* **100,** 1955–1960 (2003).

182. Colton, C. K., Kong, Q., Lai, L., Zhu, M. X., Seyb, K. I., Cuny, G. D., Xian, J., Glicksman, M. A. & Lin, C.-L. G. Identification of translational activators of glial

glutamate transporter EAAT2 through cell-based high-throughput screening: an approach to prevent excitotoxicity. *J. Biomol. Screen.* **15,** 653–662 (2010).

183. Van Goor, F., Hadida, S., Grootenhuis, P. D., Burton, B., Cao, D., Neuberger, T., Turnbull, A., Singh, A., Joubran, J., Hazlewood, A., Zhou, J., McCartney, J., Arumugam, V., Decker, C., Yang, J., Young, C., Olson, E. R., Wine, J. J., Frizzell, R. A., Ashlock, M. & Negulescu, P. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 18825–18830 (2009).

184. Hoffman, L. R. & Ramsey, B. W. Cystic fibrosis therapeutics: The road ahead. *Chest* **143,** 207–213 (2013).

185. Manzardo, A. M., He, J., Poje, A., Penick, E. C., Campbell, J. & Butler, M. G. Double-blind, randomized placebo-controlled clinical trial of benfotiamine for severe alcohol dependence. *Drug Alcohol Depend.* **133,** 562–570 (2013).

186. Yiu, W. H., Pan, C. J., Allamarvdasht, M., Kim, S. Y. & Chou, J. Y. Glucose-6-phosphate transporter gene therapy corrects metabolic and myeloid abnormalities in glycogen storage disease type Ib mice. *Gene. Ther.* **14,** 219–226 (2007).

CHAPTER THREE

Identification of Novel Ligands of the L-Type Amino Acid Transporter 1 (LAT1)

Introduction

Amino acids are essential building blocks of proteins but require transporters in order to cross the plasma membrane. One of the major transporters for large neutral amino acids is the L-type amino acid transporter 1 (LAT1, SLC7A5). Found in a heterodimer with 4F2hc (SLC3A2), LAT1 is a sodium-independent obligatory exchanger that primarily transports large neutral amino acids such as leucine, isoleucine, tryptophan, tyrosine and phenylalanine. Expressed in various tissues across the body, LAT1 has been detected particularly in tissues with barrier functions, including the colon[1], placenta[2], the blood-retinal barrier[3] and the blood-brain barrier (BBB)[4]. Interestingly, LAT1 has been found to be highly expressed in various cancer cell lines[5], and enhanced expression has been correlated with poor prognosis in patients with different cancer types[6–9].

In addition to protein synthesis, amino acids are used in biosynthetic pathways for neurotransmitters such as dopamine, norepinephrine, epinephrine, histamine and serotonin. For example, phenylalanine and tyrosine can be used in the synthesis of dopamine and norepinephrine, whereas serotonin is derived from tryptophan. It is not surprising, then, that LAT1 was found to be one of the most highly expressed transporters in the blood-brain barrier[4]. At the blood-brain barrier, LAT1 has been localized to both the luminal and abluminal membranes of the bovine[10] and rodent[11] blood-brain barrier, though its exact localization in the human BBB has not yet been determined. LAT1 is believed to primarily serve as an influx

transporter and is the most important transporter of neutral amino acids from the blood to the brain[12].

In addition to its role in amino acid transport, LAT1 is known to transport several drugs, including melphalan[13], levodopa[14], gabapentin[15] and baclofen[16]. Its high expression level and presence on both membranes of the blood-brain barrier make it an attractive target for drug delivery, through which otherwise brain-impenetrable drugs could gain access to the central nervous system. One potential strategy of targeting a drug to the brain through LAT1 may involve creating a drug conjugated to an amino acid; this technique has been successfully demonstrated to enhance brain penetration of various drugs in rats[17,18].

Recent studies using molecular models suggest that LAT1 may be capable of transporting other compounds beyond amino acid derivatives[16,19]. Identification of novel LAT1 substrates may provide information on the mechanisms through which drugs enter the brain, and help guide the development of novel therapeutics that utilize LAT1 as a drug delivery platform into the brain. Furthermore, identification of novel LAT1 inhibitors may help to expose potential drug-drug interactions and optimize drug dosing strategies. Notably, for many approved CNS-active drugs, the mechanism of transport across the BBB is not known.

In this study, novel ligands of LAT1 were identified through multiple approaches in order to expand the known chemical space of this transporter that is highly-expressed in the brain. First, a library of 115 CNS-active small molecule drugs was screened to identify chemical inhibitors of LAT1. Compounds that showed inhibition activity were further tested as substrates using cellular uptake studies with LC/MS/MS quantification. In addition, a trans-stimulation assay was developed and a subset of non-LAT1 inhibitors was tested as potential substrates of

LAT1. Finally, an *in situ* rat perfusion model was developed which can be used to test the *in vivo* relevance of new LAT1 substrates at the blood-brain barrier.

Materials and Methods

Chemicals. Of the 115 CNS-active compounds that were tested, 47 compounds were obtained from Pfizer (Groton, CT). The remaining 68 compounds were purchased from various sources, including Sigma Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ), Santa Cruz Biotechnology (Santa Cruz, CA), Enzo Life Sciences (Farmingdale, NY) and TCI America (Portland, OR). 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) was purchased from Sigma Aldrich (St. Louis, MO). Cell culture media (DMEM H-21), blasticidin and the Pierce BCA Protein Assay Kit were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum, penicillin/streptomycin, non-essential amino acids, L-glutamine, and Fungizone were obtained from the University of California San Francisco Cell Culture Facility. [^{3}H]-gabapentin was purchased from PerkinElmer (Waltham, MA). Krebs-Henseleit powder was purchased from Sigma Aldrich (St. Louis, MO).

Cell Culture. Stably-transfected and inducible HEK-hLAT1 cells were generously provided by Pfizer. These cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) H-21 media supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, 1 µg/mL Fungizone, 2 mM L-glutamine and 3 µg/mL blasticidin. Cells were grown at 37 °C in a humidified incubator with 5% CO_2.

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Inhibition Screen and IC$_{50}$ Determinations. HEK-hLAT1 cells were grown on poly-D-lysine coated 96-well plates in DMEM medium described above to at least 90% confluence at 48 hours post-seeding. Twenty-four hours before the experiment, cells were treated with 1 µg/mL doxycycline and 2 mM sodium butyrate to induce expression of hLAT1. On the day of the experiment, cells were preincubated in pre-warmed, sodium-free choline buffer (140 mM choline chloride, 2 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 1 M Tris) for 10 minutes. The buffer was removed and replaced with uptake buffer (sodium-free choline buffer containing 6 nM of [^{3}H]-gabapentin) and each inhibitor at 200 µM. For IC$_{50}$ determinations, varying concentrations of each inhibitor were added, from 15 µM to 2 mM. Uptake was performed at 37 °C for 3 minutes, and then terminated by washing the cells twice with ice-cold choline buffer. Cells were lysed by the addition of 100 µL of lysis buffer (0.1 N NaOH, 0.1% SDS) and allowed to sit at room temperature for 3 hours. Intracellular radioactivity was determined by scintillation counting on a Topcount NXT Scintillation Counter (Packard). Results were analyzed using Graphpad Prism 5.0.

Trans-stimulation Studies. HEK-hLAT1 cells were grown on poly-D-lysine coated 24-well plates in DMEM medium described above to at least 90% confluence at 48 hours post-seeding. Twenty-four hours before the experiment, cells were treated with 1 µg/mL doxycycline and 2 mM sodium butyrate to induce expression of hLAT1. On the day of the experiment, cells were washed with pre-warmed, sodium-free choline buffer (140 mM choline chloride, 2 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 1 M Tris), then incubated in the same pre-incubation solution containing 6 nM of [^{3}H]-gabapentin. After 30 minutes, the pre-incubation solution was quickly removed and cells were washed twice with

sodium-free choline buffer. Fresh sodium-free choline buffer containing each of the tested compounds at 2 mM were added to the cells, and an aliquot of the buffer was obtained at 5 seconds, 1 minute and 3 minutes after addition of the test compound. The extracellular radioactivity in each aliquot was determined by scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter). The efflux rate was determined by graphing each time point on a line and averaging the slope of four technical replicates per time point. Compounds dissolved in a DMSO stock were tested separately from compounds dissolved in water.

Uptake Studies Using LC/MS/MS Quantitation. HEK-hLAT1 cells were grown on poly-D-lysine coated 24-well plates in DMEM medium described above to at least 90% confluence at least 48 hours post-seeding. Twenty-four hours before the experiment, cells were treated with 1 µg/mL doxycycline and 2 mM sodium butyrate to induce expression of hLAT1. Empty vector control cells were treated with sodium butyrate only. On the day of the experiment, cells were preincubated in pre-warmed, sodium-free choline buffer (140 mM choline chloride, 2 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 1 M Tris) for 10 minutes. The buffer was removed and replaced with uptake buffer (sodium-free choline buffer containing 5 µM of the test compound). Samples with inhibitor included the addition of 2 mM BCH. Uptake was performed at 37 °C for 3 minutes and then terminated by washing the cells twice with ice-cold HBSS. Plates containing cells were dried overnight at 4 °C. Samples were extracted by adding 200 µL of ice-cold methanol for 10 minutes, followed by two 10-minute incubations with 200 µL of 70:30 methanol:water. The methanol:water solution was pooled in a 96-well plate and evaporated in a Savant centrifugal evaporator overnight. Dried plates were sent to York Bioanalytical Solutions (York, UK) for quantitation by liquid chromatography-

tandem mass spectrometry (LC/MS/MS). Results were normalized by protein content as determined with a Pierce BCA Protein Assay Kit (Life Technologies) and analyzed using Graphpad Prism 5.0.

Rat Perfusion Studies. Adult Sprague-Dawley rats approximately 12-16 weeks of age were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. A catheter was inserted into the left interior carotid artery and the external carotid artery was ligated shut. Animals were then infused with the infusion solution, which consisted of Krebs-Henseleit buffer at pH 7.4, warmed to 37 °C and bubbled with a mixture of 5% CO_2 and 95% O_2. The infusion solution included 0.5 µCi/mL of [^{3}H]-gabapentin and 0.2 µCi/mL of [^{14}C]-inulin as a vascular marker, with or without the addition of 2 mM BCH as an inhibitor. The infusion pump was set to infuse 8 mL of the infusion solution in one minute. Animals were decapitated immediately following infusion and the brain was removed from the skull, bisected with a razor blade into the left and right hemispheres, and incubated in 2.5 mL Solvable tissue solubilizer (PerkinElmer) overnight at 50 °C. The radioactivity in each sample was determined by scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter).

Results

Validation of HEK-hLAT1 Cell Line. Gabapentin is a known substrate of LAT1. Cells treated with doxycycline and sodium butyrate to induce expression of LAT1 showed significantly higher uptake of [^{3}H]-gabapentin, compared with cells treated with sodium butyrate only (marked as EV). Linear uptake kinetics of [^{3}H]-gabapentin was observed through 3 minutes (Figure 3.1A).

The K_m of LAT1 for gabapentin was determined to be 49 µM using non-linear regression (Figure 3.1B and Figure 3.1C). BCH, a known LAT1 inhibitor, was found to significantly reduce gabapentin uptake at 2 mM (data not shown). These results validate the HEK-hLAT1 cell line as an *in vitro* model for studying LAT1-mediated uptake.
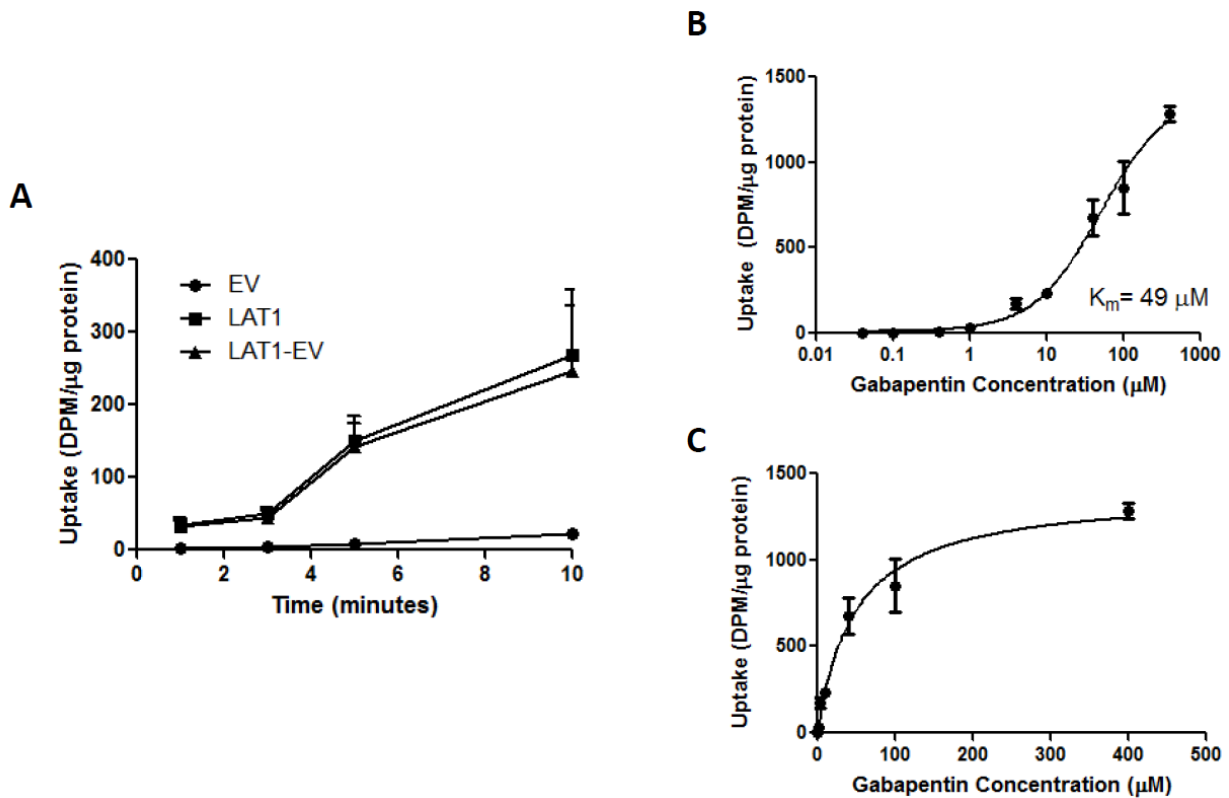
Figure 3.1. Validation of HEK-hLAT1 cell line. (A) LAT1-mediated uptake of [^{3}H]-gabapentin shows linear uptake kinetics up to 3 minutes. (B) Uptake of [^{3}H]-gabapentin in HEK-hLAT1 cells ($K_m = 49$ µM). (C) Same data as (B), but shown on a linear graph.

Inhibition screen for LAT1 Inhibitors Identified Novel Inhibitors. A total of 115 compounds were screened at 200 µM for the ability to inhibit LAT1-mediated [³H]-gabapentin uptake (Figure 3.2A and Figure 3.2B). Data were normalized such that the uptake of [³H]-gabapentin without the addition of an inhibitor was determined to be 100%. The positive control contained 2 mM BCH, a known LAT1 inhibitor. Eleven compounds were found to inhibit LAT1 greater than 50% at 200 µM, including fluphenazine, clonazepam, chlorpromazine, gabapentin, LAAM, fluoxetine, clomipramine, duloxetine, sertraline, paroxetine, and cyclobenzaprine. Gabapentin is a known substrate of LAT1 and thus served as an additional positive control. In addition, some compounds (e.g., buspirone) enhanced [³H]-gabapentin uptake via LAT1. Several studies in the literature have demonstrated that buspirone improves the pharmacologic action of gabapentin[20]. Though various mechanisms have been proposed for this effect, it is possible that buspirone enhances BBB uptake of gabapentin *in vivo*, contributing to the improved efficacy of the drug.
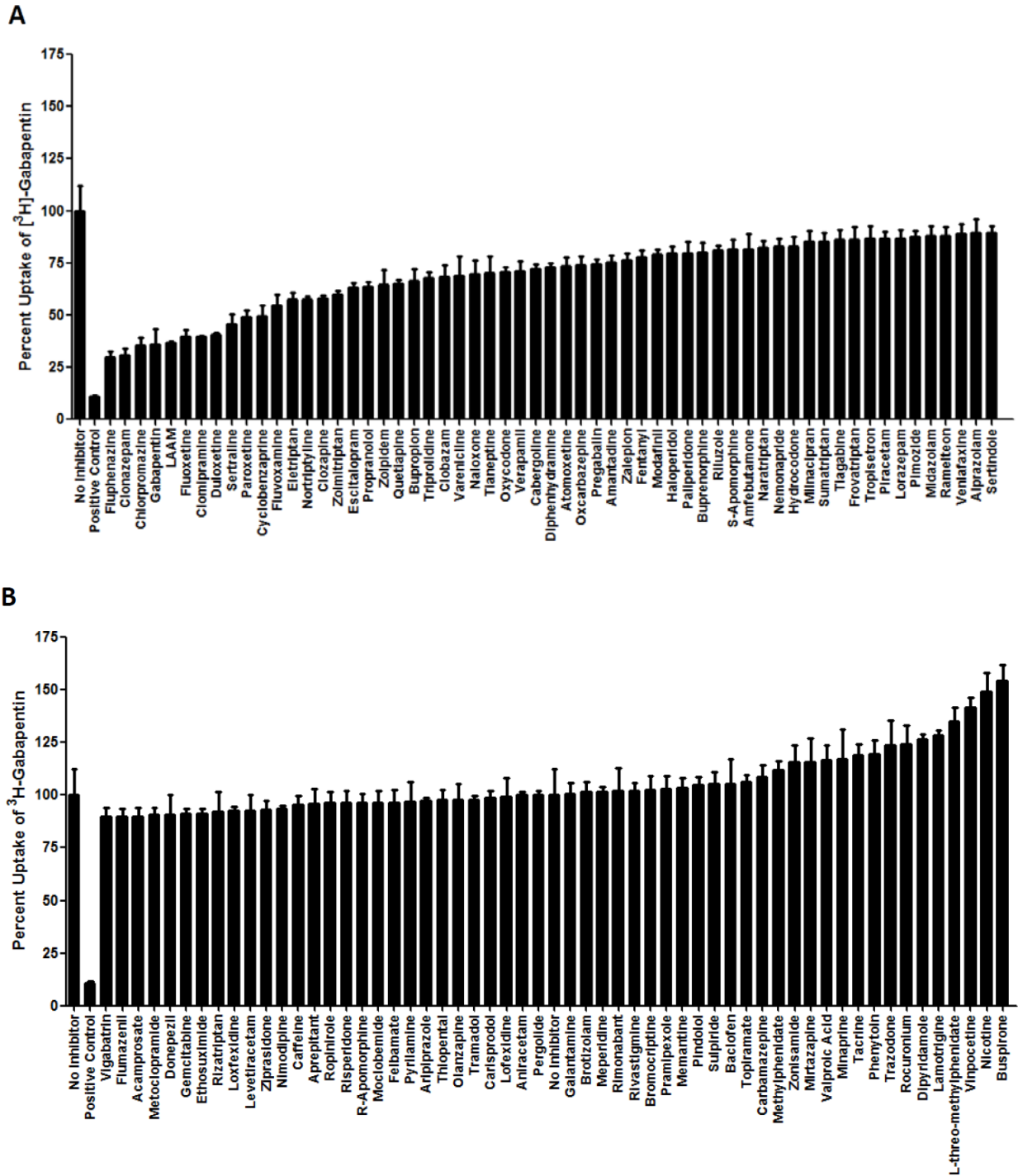
Figure 3.2. Inhibition screen for LAT1 inhibitors. Compounds are sorted by percent of inhibition

of [³H]-gabapentin uptake from highest to lowest. The results were split into two graphs, 3.2A

and 3.2B, for ease of viewing.

Kinetic Determination of LAT1 Inhibitors. For several of the compounds that showed greater than 50% inhibition of LAT1-mediated [^{3}H]-gabapentin uptake, kinetic studies were performed to determine the IC_{50} values. Duloxetine (IC_{50} = 578 µM, 95% CI: 329-1013 µM) (Figure 3.3A), paroxetine (IC_{50} = 481 µM, 95% CI: 387-599 µM) (Figure 3.3B), fluphenazine (IC_{50} = 725 µM, 95% CI: 71-7434 µM) (Figure 3.3C) and clomipramine (IC_{50} = 383 µM, 95% CI: 285-516 µM) (Figure 3.3D) were all found to be novel inhibitors of LAT1, with an IC_{50} value similar to that of a known LAT1 substrate, pregabalin (IC_{50} = 405 µM, 95% CI: 231-711 µM) (Figure 3.3E).
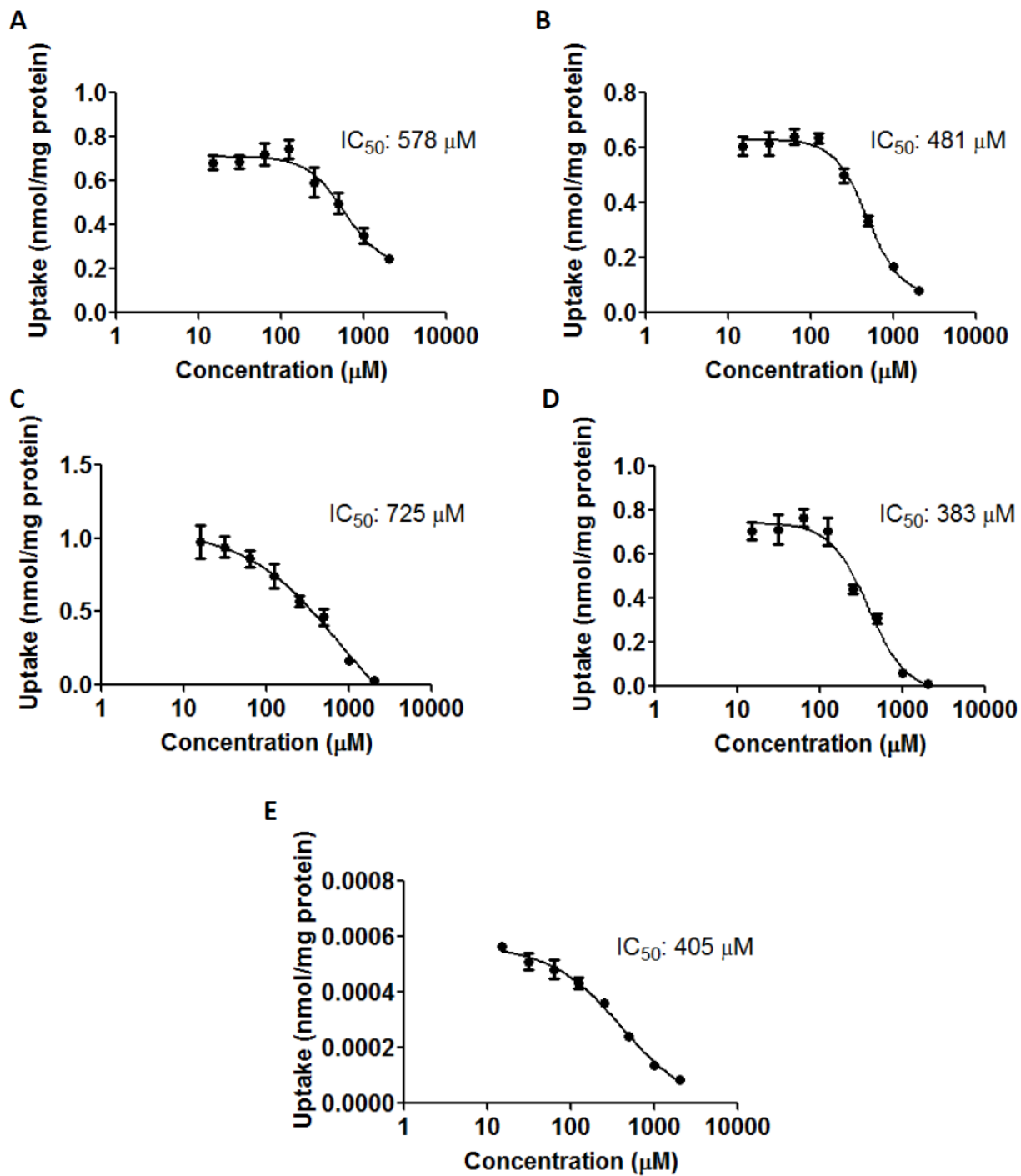
Figure 3.3. Kinetic characterization of LAT1 inhibitors. IC_{50} curves are shown for (A) duloxetine, (B) paroxetine, (C) fluphenazine and (D) clomipramine. (E) The IC_{50} values are similar to the IC_{50} of a known LAT1 substrate, pregabalin.

Novel LAT1 Inhibitors Were Not Substrates of LAT1. Following up on the inhibition screen results, duloxetine, paroxetine, fluphenazine and clomipramine were tested as substrates of LAT1 in an uptake assay using LC/MS/MS quantitation (Figure 3.4). None of the compounds showed increased uptake in LAT1-overexpressing cells compared to empty vector, nor uptake that was inhibited by the presence of 2 mM BCH, a known LAT1 substrate.
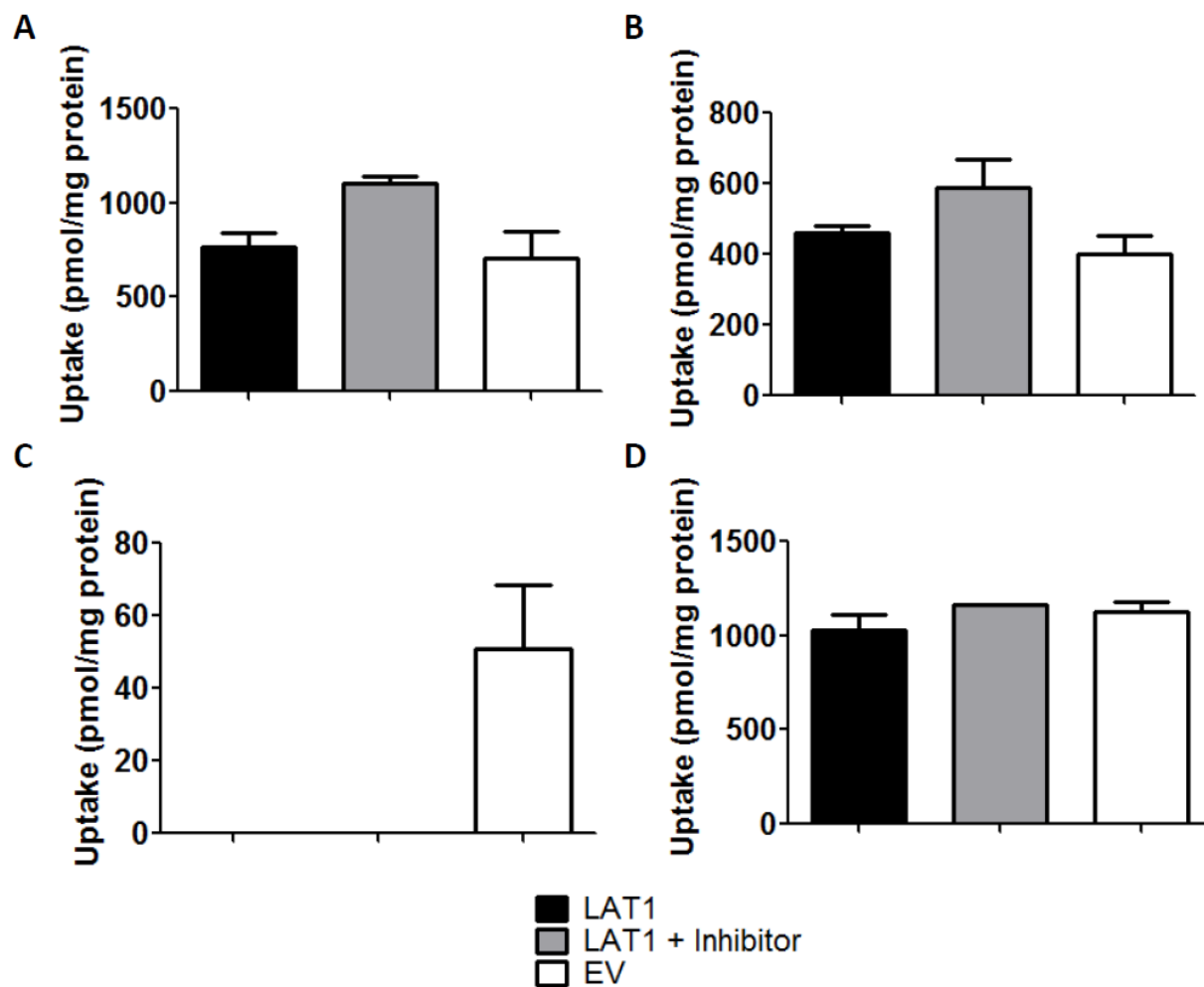
Figure 3.4. Testing of identified inhibitors as LAT1 substrates. Cells were exposed to 5 μM of (A) duloxetine, (B) paroxetine, (C) fluphenazine and (D) clomipramine for 3 minutes, then samples were extracted and quantified by LC/MS/MS. The presence of 2 mM BCH did not inhibit uptake of any of these compounds.

Trans-stimulation Assays Identified Some Compounds Capable of Enhancing Efflux of a Known LAT1 Substrate. Since the trans-stimulation assay is sensitive to DMSO concentrations, water-soluble compounds were tested separately from compounds in a DMSO stock solution. Twenty-one water-soluble compounds and 13 compounds in a DMSO stock solution were tested separately in the trans-stimulation assay. For the trans-stimulation assay with water-soluble compounds, gabapentin and leucine were used as positive controls and glycine and no drug were used as the negative controls. The positive controls show an average efflux rate of 0.74 fmol/min, whereas the negative controls had an average efflux rate of 0.26 fmol/min. Several water-soluble compounds were identified that were able to trans-stimulate the efflux of [^{3}H]-gabapentin (efflux rate > 0.35 fmol/min), including ethosuximide, vigabatrin, clonazepam, ropinirole, and methylphenidate (Table 3.1A). For the trans-stimulation assay with compounds dissolved in DMSO, gabapentin was the positive control and no drug was the negative control. Gabapentin showed an efflux rate of 0.19 fmol/min whereas the no drug negative control showed an efflux rate of 0.11 fmol/min. Lamotrigine, varenicline, mirtazapine, vinpocetine, zolpidem and fluvoxamine were able to trans-stimulate the efflux of [^{3}H]-gabapentin when the compound was exposed to the cells extracellularly (efflux rate > 0.15 fmol/min). These results suggest that some of the compounds might be substrates of LAT1 since they are able to stimulate efflux of gabapentin in LAT1-overexpressing cells.

Table 3.1A. Trans-stimulation assay of compounds dissolved in water. Positive controls (gabapentin and leucine) are shown in green, bold text and negative controls (no drug and glycine) are shown in red, bold text. A positive result was defined as having an efflux rate greater than or equal to 0.35 fmol/minute.

	Efflux Rate (fmol/min)	Standard Deviation (fmol/min)
Gabapentin	**0.83**	**0.26**
Leucine	**0.64**	**0.43**
Glycine	**0.26**	**0.20**
No Drug	**0.26**	**0.02**
Ethosuximide	0.91	0.86
Vigabatrin	0.54	0.20
Clonazepam	0.49	0.13
Ropinirole	0.47	0.13
Methylphenidate	0.36	0.07
Atomoxetine	0.31	0.06
LAAM	0.29	0.05
Fluphenazine	0.26	0.14
Fentanyl	0.23	0.03
Venlafaxine	0.18	0.11
Tramadol	0.18	0.13
Clomipramine	0.17	0.06
Duloxetine	0.12	0.08
Acamprosate	0.11	0.20
Buprenorphine	0.11	0.06
Caffeine	0.11	0.11
Piracetam	0.10	0.08
Talipexole	0.09	0.17
Paroxetine	0.08	0.05
Hydrocodone	0.06	0.05
Valproic Acid	-0.12	0.04

Table 3.1B. Trans-stimulation assay of compounds dissolved in DMSO. The positive control (gabapentin) is shown in green, bold text and negative control (no drug) is shown in red, bold text. A positive result was identified as having an efflux rate greater than 0.15 fmol/minute.

	Efflux Rate (fmol/min)	Standard Deviation (fmol/min)
Gabapentin	**0.19**	**0.12**
No Drug	**0.11**	**0.06**
Lamotrigine	0.37	0.24
Varenicline	0.35	0.13
Mirtazepine	0.31	0.06
Vinpocetine	0.26	0.07
Zolpidem	0.21	0.17
Fluvoxamine	0.21	0.14
Topiramate	0.14	0.06
Ramelteon	0.14	0.03
Zaleplon	0.13	0.12
Escitalopram	0.07	0.07
Levetiracetam	0.06	0.07
Moclobemide	0.05	0.04
Brontizolam	0.04	0.09

LC/MS/MS Follow-Up Studies Did Not Identify Novel Substrates. A cellular uptake assay was performed on compounds that were found to be potential hits in the trans-stimulation assay, and intracellular concentrations of each of these compounds were quantified using LC/MS/MS. Gabapentin, our positive control, was the only compound found to be a substrate of LAT1, such that uptake was significantly reduced in EV cells as well as in the presence of the inhibitor (Figure 3.5). Concentrations of vigabatrin, valproic acid and ethosuximide were determined to be below the limit of quantitation for the instrument, thus it was not possible to definitively determine whether these three compounds are or are not LAT1 substrates.
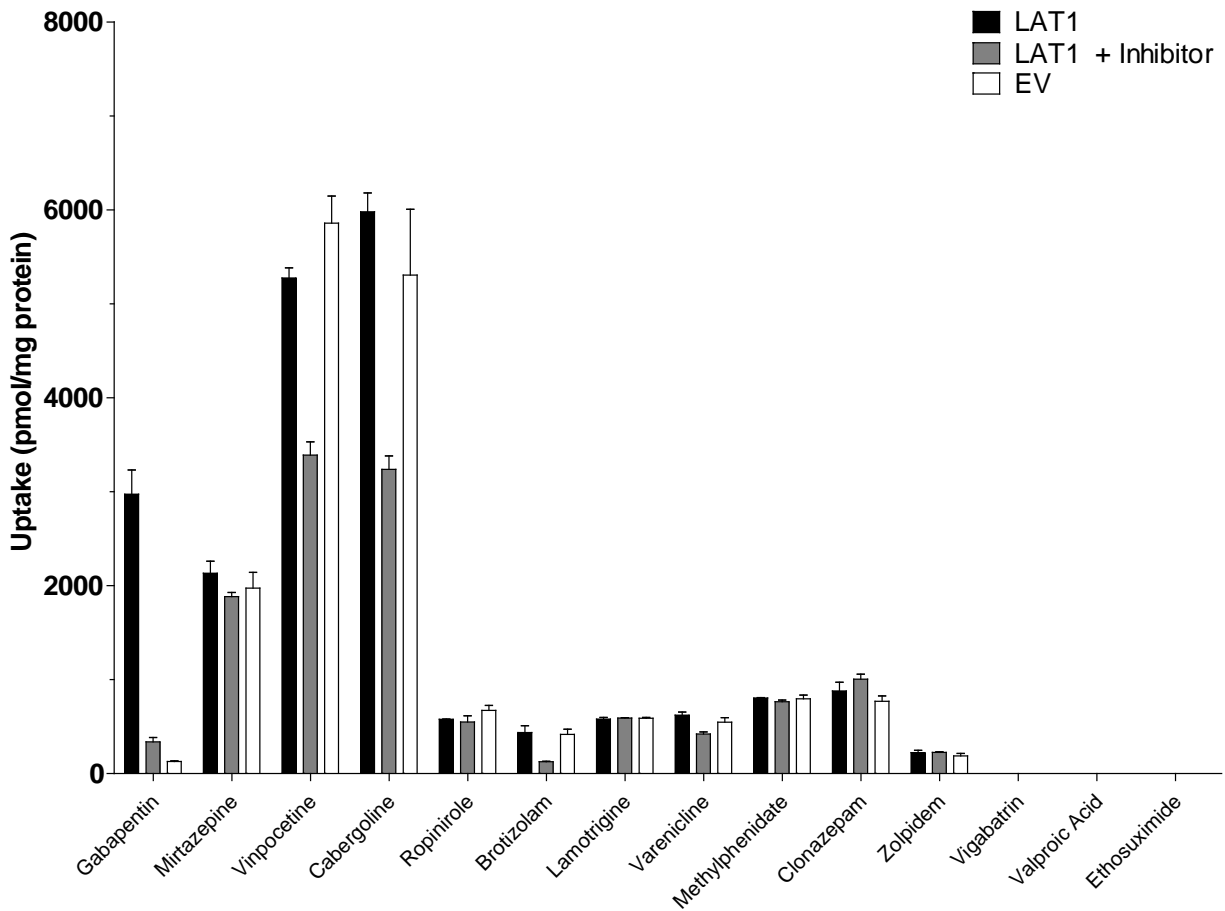
Figure 3.5. Uptake of potential substrates in LAT1-overexpressing cells. Gabapentin was the positive control and the only compound tested that is shown to be a LAT1 substrate. Vigabatrin, valproic acid and ethosuximide levels were below the limit of quantitation.

BCH is Able to Block Brain Uptake of Gabapentin in a Rat Perfusion Model. Our rat perfusion assay shows that gabapentin enters the brain and that this entry can be blocked with a LAT1-specific inhibitor, BCH (Figure 3.6). Diazepam is used as the diffusion control, and shows a high Brain Uptake Index (BUI) of 47.44. Gabapentin does not diffuse across the BBB but it still able to enter the CNS, therefore showing a lower BUI of 14. However, the BUI drops to 14% of control with the addition of BCH, indicating that brain uptake of gabapentin can be almost completely blocked with BCH. The novel LAT1 inhibitor clomipramine was also demonstrated to reduce BUI to 21% of control. Inulin is used as a vascular marker control and is normalized to a BUI of 1. This validates this *in situ* perfusion model as a way to study uptake of LAT1 substrates *in vivo*, and demonstrates that clomipramine is an inhibitor of LAT1 in the brain.

Figure 3.6. Development of a rat perfusion assay shows that BCH is able to block gabapentin brain uptake. Diazepam is used as the diffusion marker and inulin is used as the vascular marker. The Brain Uptake Index (BUI) reflects brain uptake, and drops from 14 to 1.9 for gabapentin in the presence of BCH. One of the novel LAT1 inhibitors, clomipramine, was also able to block brain uptake of gabapentin.

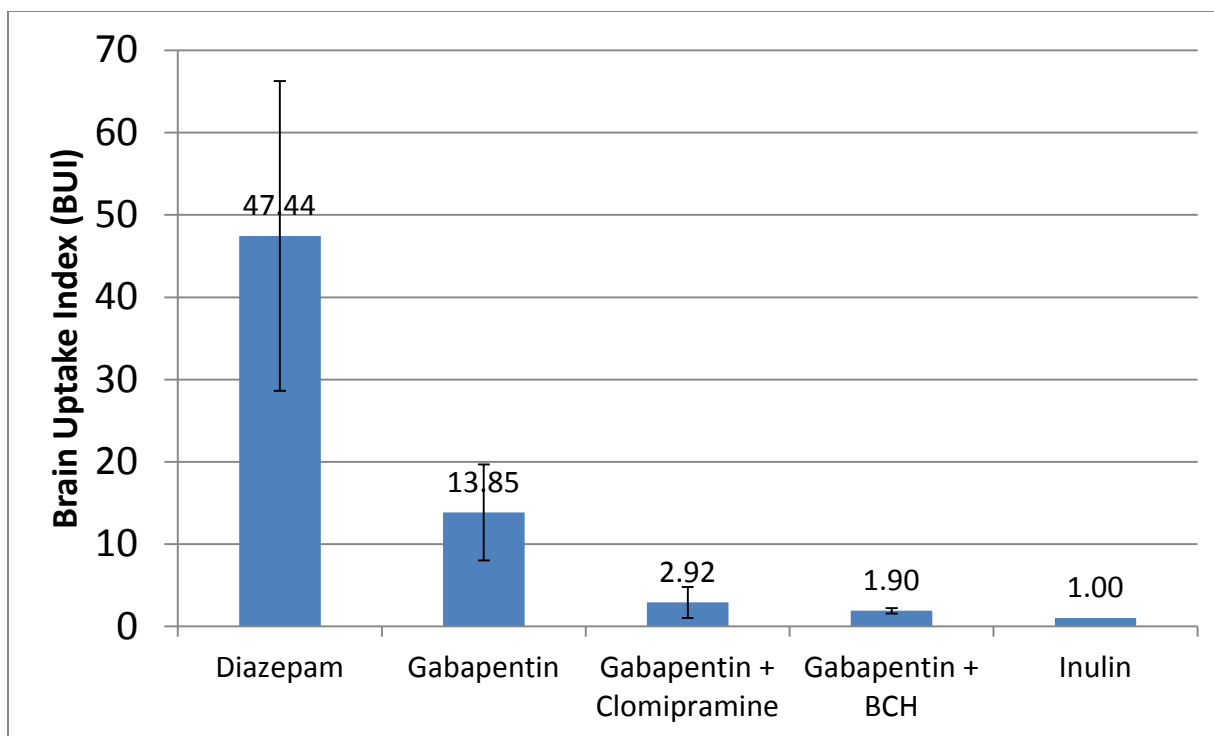**Discussion**

LAT1 is one of the most highly-expressed transporters at the blood-brain barrier, where it plays a role in the influx of amino acids and various drugs into the brain. Delivery of amino acid-conjugate drugs through LAT1 has been proposed as a strategy to enhance brain penetration for drugs with CNS targets. However, the lack of a published crystal structure for human LAT1 has limited the use of computational methods such as molecular docking to identify new ligands of this transporter. Studies are needed to gain a better understanding of LAT1's substrate specificity, as well as to identify inhibitors of LAT1 that may interfere with the use of drugs that utilize this transporter for absorption in the intestine or distribution to the site of action. Understanding transporters responsible for absorption or distribution at biological barriers could be relevant for treatment outcome.

In this study, inhibition assays, cellular uptake studies and trans-stimulation assays were used on a library of 115 CNS-active drugs to identify novel ligands of LAT1 with the goal of exploring the chemical space of this highly-expressed transporter. An *in situ* rat perfusion model was also developed that can be used to test the *in vivo* relevance of LAT1 substrates. The inhibition screen found that the majority of compounds in our library were unable to inhibit LAT1 (Figure 3.2). Our key finding is the discovery and characterization of four novel, structurally-diverse inhibitors of LAT1, which include the antidepressant drugs duloxetine (IC_{50} = 578 µM), paroxetine (IC_{50} = 481 µM) and clomipramine (IC_{50} = 383 µM), as well as the antipsychotic drug fluphenazine (IC_{50} = 725 µM) (Figure 3.3 and Figure 3.7).
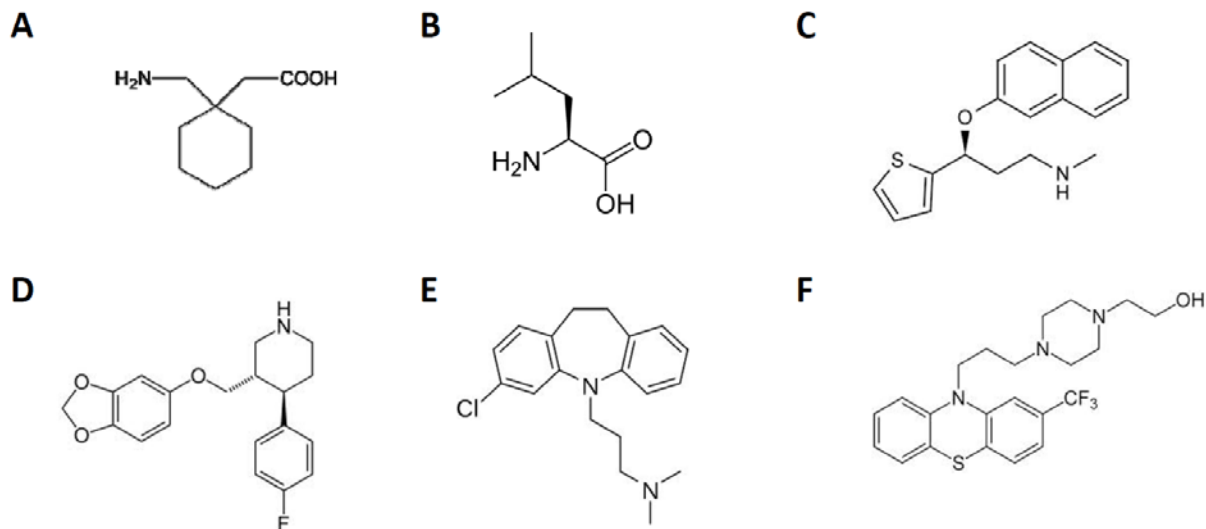
Figure 3.7. Substrates and inhibitors of LAT1. Gabapentin (A) and leucine (B) are both known substrates of LAT1. Duloxetine (C), paroxetine (D), clomipramine (E) and fluphenazine (F) were identified as novel LAT1 inhibitors.

The major drug currently known to be transported by LAT1 is levodopa, which is a dopamine prodrug used to treat Parkinson's disease. However, clinical response to levodopa has been reported to diminish immediately following ingestion of high-protein meals consisting of large neutral amino acids, resulting in inadequate control of Parkinson's symptoms[21]. A study in monkeys found that administration of a high-protein meal or intravenous infusion of large neutral amino acids prior to administration of levodopa reduced the transport of levodopa into the brain[22]. Inhibition of LAT1 by dietary factors or by other drugs may therefore result in clinically-significant pharmacokinetic changes that affect treatment efficacy. Of particular note, Parkinson's disease is frequently complicated by depression, with a reported prevalence of 31

percent in Parkinson's disease patients[23]. It is therefore possible that both levodopa as well as the antidepressant drugs identified as LAT1 inhibitors are dosed concomitantly in Parkinson's disease patients, resulting in impaired transport of either drug through LAT1. While not likely to occur at the blood-brain barrier due to the relatively high concentrations needed to inhibit LAT1, reduction in levodopa absorption in the gastrointestinal tract by concomitant administration of antidepressant drugs may occur. In particular, estimated intestinal concentrations (oral dose/250 mL intestinal fluid content) are considerably higher than plasma concentrations of these drugs. Further, LAT1 is expressed in the intestine and may contribute to the absorption of levodopa.

As LAT1 is frequently upregulated in cancer and enhances nutrient utilization, development of novel LAT1 inhibitors as antitumor agents is currently underway[24–26]. In contrast to standard chemotherapies that target all rapidly-dividing cells, molecular-targeted compounds may show increased selectivity for tumor cells, thus increasing efficacy and decreasing adverse effects. It has been previously reported that two selective LAT1 inhibitors were found to inhibit tumor cell growth in both cultured cells as well as in a xenograft mouse model[26]. Although the inhibitors identified in this study inhibited LAT1 at a higher concentration than the inhibitors previously tested as anticancer agents, a better understanding of the structure-activity relationship of LAT1 inhibitors will invariably be useful in the development of novel LAT1 inhibitors as anticancer agents.

Using a trans-stimulation assay, several compounds were identified that were potentially LAT1 substrates due to their ability to enhance the efflux of gabapentin in a LAT1-overexpressing cell line (Table 3.1). A subsequent cellular uptake assay using LC/MS/MS quantification failed to confirm any new substrates of LAT1 (Figure 3.5). However, the most promising compounds from the trans-stimulation assay (ethosuximide and vigabatrin) were not

detectable due to limitations of our instrumentation and assay. Additional studies to conclusively determine if these compounds are in fact LAT1 substrates are clearly warranted. Finally, an *in situ* rat perfusion model was developed that may be useful in testing LAT1 substrates for *in vivo* relevance; a LAT1 inhibitor was shown to be able to dramatically reduce uptake of a known LAT1 substrate into the brain (Figure 3.6).

References

1.   Fraga, S., Pinho, M. J. & Soares-Da-Silva, P. Expression of LAT1 and LAT2 amino acid transporters in human and rat intestinal epithelial cells. *Amino Acids* **29,** 229–233 (2005).

2.   Ritchie, J. W. & Taylor, P. M. Role of the System L permease LAT1 in amino acid and iodothyronine transport in placenta. *Biochem. J.* **356,** 719–725 (2001).

3.   Tomi, M., Mori, M., Tachikawa, M., Katayama, K., Terasaki, T. & Hosoya, K. I. L-type amino acid transporter 1-mediated L-leucine transport at the inner blood-retinal barrier. *Investig. Ophthalmol. Vis. Sci.* **46,** 2522–2530 (2005).

4.   Geier, E. G., Chen, E. C., Webb, A., Papp, A. C., Yee, S. W., Sadee, W. & Giacomini, K. M. Profiling solute carrier transporters in the human blood-brain barrier. *Clin. Pharmacol. Ther.* **94,** 636–639 (2013).

5.   Yanagida, O., Kanai, Y., Chairoungdua, a, Kim, D. K., Segawa, H., Nii, T., Cha, S. H., Matsuo, H., Fukushima, J., Fukasawa, Y., Tani, Y., Taketani, Y., Uchino, H., Kim, J. Y., Inatomi, J., Okayasu, I., Miyamoto, K., Takeda, E., Goya, T. & Endou, H. Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines. *Biochim. Biophys. Acta* **1514,** 291–302 (2001).

6.   Kaira, K., Oriuchi, N., Imai, H., Shimizu, K., Yanagitani, N., Sunaga, N., Hisada, T., Tanaka, S., Ishizuka, T., Kanai, Y., Endou, H., Nakajima, T. & Mori, M. Prognostic significance of L-type amino acid transporter 1 expression in resectable stage I-III nonsmall cell lung cancer. *Br. J. Cancer* **98,** 742–748 (2008).

7. Nakanishi, K., Ogata, S., Matsuo, H., Kanai, Y., Endou, H., Hiroi, S., Tominaga, S., Aida, S., Kasamatsu, H. & Kawai, T. Expression of LAT1 predicts risk of progression of transitional cell carcinoma of the upper urinary tract. *Virchows Arch.* **451,** 681–690 (2007).

8. Kaira, K., Sunose, Y., Ohshima, Y., Ishioka, N. S., Arakawa, K., Ogawa, T., Sunaga, N., Shimizu, K., Tominaga, H., Oriuchi, N., Itoh, H., Nagamori, S., Kanai, Y., Yamaguchi, A., Segawa, A., Ide, M., Mori, M., Oyama, T. & Takeyoshi, I. Clinical significance of L-type amino acid transporter 1 expression as a prognostic marker and potential of new targeting therapy in biliary tract cancer. *BMC Cancer* **13,** 482 (2013).

9. Sakata, T., Ferdous, G., Tsuruta, T., Satoh, T., Baba, S., Muto, T., Ueno, A., Kanai, Y., Endou, H. & Okayasu, I. L-type amino-acid transporter 1 as a novel biomarker for high-grade malignancy in prostate cancer. *Pathol. Int.* **59,** 7–18 (2009).

10. Sanchez del Pino, M. M., Peterson, D. R. & Hawkins, R. A. Neutral amino acid transport characterization of isolated: Luminal and abluminal membranes of the blood-brain barrier. *J. Biol. Chem.* **270,** 14913–14918 (1995).

11. Roberts, L. M., Black, D. S., Raman, C., Woodford, K., Zhou, M., Haggerty, J. E., Yan, A. T., Cwirla, S. E. & Grindstaff, K. K. Subcellular localization of transporters along the rat blood-brain barrier and blood-cerebral-spinal fluid barrier by in vivo biotinylation. *Neuroscience* **155,** 423–438 (2008).

12. Hawkins, R. A., O'Kane, R. L., Simpson, I. A. & Viña, J. R. Structure of the blood-brain barrier and its role in the transport of amino acids. *J. Nutr.* **136,** 218S–226S (2006).

13. Rautio, J., Gynther, M. & Laine, K. LAT1-mediated prodrug uptake: a way to breach the blood-brain barrier? *Ther. Deliv.* **4,** 281–4 (2013).

14. Peura, L., Malmioja, K., Huttunen, K., Leppänen, J., Hämäläinen, M., Forsberg, M. M., Rautio, J. & Laine, K. Design, synthesis and brain uptake of lat1-targeted amino acid prodrugs of dopamine. *Pharm. Res.* **30,** 2523–2537 (2013).

15. Dickens, D., Webb, S. D., Antonyuk, S., Giannoudis, A., Owen, A., Rädisch, S., Hasnain, S. S. & Pirmohamed, M. Transport of gabapentin by LAT1 (SLC7A5). *Biochem. Pharmacol.* **85,** 1672–1683 (2013).

16. Ylikangas, H., Malmioja, K., Peura, L., Gynther, M., Nwachukwu, E. O., Leppänen, J., Laine, K., Rautio, J., Lahtela-Kakkonen, M., Huttunen, K. M. & Poso, A. Quantitative insight into the design of compounds recognized by the L-type amino acid transporter 1 (LAT1). *Chem. Med. Chem.* **1,** 1–10 (2014).

17. Killian, D. M., Hermeling, S. & Chikhale, P. J. Targeting the cerebrovascular large neutral amino acid transporter (LAT1) isoform using a novel disulfide-based brain drug delivery system. *Drug Deliv.* **14,** 25–31 (2007).

18. Gynther, M., Jalkanen, A., Lehtonen, M., Forsberg, M., Laine, K., Ropponen, J., Leppänen, J., Knuuti, J. & Rautio, J. Brain uptake of ketoprofen-lysine prodrug in rats. *Int. J. Pharm.* **399,** 121–128 (2010).

19. Geier, E. G., Schlessinger, A., Fan, H., Gable, J. E., Irwin, J. J., Sali, A. & Giacomini, K. M. Structure-based ligand discovery for the large-neutral amino acid transporter 1, LAT-1. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 5480–5485 (2013).

20. Eskow, K. L., Gupta, V., Alam, S., Park, J. Y. & Bishop, C. The partial 5-HT1A agonist buspirone reduces the expression and development of l-DOPA-induced dyskinesia in rats and improves l-DOPA efficacy. *Pharmacol. Biochem. Behav.* **87,** 306–314 (2007).

21. Nutt, J. G., Woodward, W. R., Hammerstad, J. P., Carter, J. H. & Anderson, J. L. The 'on-off' phenomenon in Parkinson's disease. Relation to levodopa absorption and transport. *N. Engl. J. Med.* **310,** 483–488 (1984).

22. Alexander, G. M., Schwartzman, R. J., Grothusen, J. R. & Gordon, S. W. Effect of plasma levels of large neutral amino acids and degree of parkinsonism on the blood-to-brain transport of levodopa in naive and MPTP parkinsonian monkeys. *Neurology* **44,** 1491–1499 (1994).

23. Slaughter, J. R., Slaughter, K. A., Nichols, D., Holmes, S. E. & Martens, M. P. Prevalence, clinical manifestations, etiology, and treatment of depression in Parkinson's disease. *J. Neuropsychiatry Clin. Neurosci.* **13,** 187–196 (2001).

24. Imai, H., Kaira, K., Oriuchi, N., Shimizu, K., Tominaga, H., Yanagitani, N., Sunaga, N., Ishizuka, T., Nagamori, S., Promchan, K., Nakajima, T., Yamamoto, N., Mori, M. & Kanai, Y. Inhibition of L-type amino acid transporter 1 has antitumor activity in non-small cell lung cancer. *Anticancer Res.* **30,** 4819–4828 (2010).

25. Yun, D.-W., Lee, S. A., Park, M.-G., Kim, J.-S., Yu, S.-K., Park, M.-R., Kim, S.-G., Oh, J.-S., Kim, C. S., Kim, H.-J., Kim, J.-S., Chun, H. S., Kanai, Y., Endou, H., Wempe, M. F. & Kim, D. K. JPH203, an L-type amino acid transporter 1–selective compound, induces apoptosis of YD-38 human oral cancer cells. *J. Pharmacol. Sci.* **124,** 208–217 (2014).

26. Oda, K., Hosoda, N., Endo, H., Saito, K., Tsujihara, K., Yamamura, M., Sakata, T., Anzai, N., Wempe, M. F., Kanai, Y. & Endou, H. L-Type amino acid transporter 1 inhibitors inhibit tumor cell growth. *Cancer Sci.* **101,** 173–179 (2010).

CHAPTER FOUR

Identification of Novel Substrates of the Organic Cation Transporter, MATE1

Introduction

Many oral drugs are cationic or weak bases at physiological pH, and organic cation transporters often mediate their absorption, distribution and elimination in the body. In the kidney, organic cation transporters (OCTs) and multidrug and toxin extrusion proteins (MATEs) have been shown to act in concert with each other in the excretion of drugs from the body, as both families of transporters show overlapping substrate and inhibitor specificities[1,2]. These transporters are known to transport a wide variety of xenobiotics, including commonly used drugs such as the anti-diabetic drug metformin, anticancer platinum compounds, the anti-ulcer medication cimetidine, the antiviral agent lamivudine and others[3], and it is generally believed that apical MATEs mediate the efflux of substrates taken up by basolateral OCT1 and OCT2[4].

The MATE family of transporters transport substrates in a sodium-independent, pH-dependent manner, and consist of multidrug and toxin extrusion protein 1 (MATE1, SLC47A1) and multidrug and toxin extrusion protein 2 (MATE2, SLC47A2) that exist in multiple alternatively spliced variants, MATE2, MATE2-B and MATE2-K. MATE2-K is a functional variant, expressed almost exclusively in the kidney[3]. Both MATE1 and MATE2-K share similar substrate specificity[5]. The broad substrate specificity of these transporters can result in known drug-drug interactions. For example, the OCT/MATE inhibitor cimetidine has been found to decrease the renal tubular secretion of ranitidine[6], procainamide[7], dofetilide[8], varenicline[9], metformin[10] and fexofenadine. Cimetidine shows a higher affinity for the MATE transporters than OCT2, suggesting that drug-drug interactions involving cimetidine previously attributed to

inhibition of OCT2 may also be due to potent MATE1 inhibition[11]. However, because the studies are performed *in vivo* in humans, it is difficult to establish the precise proteins responsible for the interactions.

The 2010 white paper from the International Transporter Consortium recommended that seven transporters (including OCT2) be studied *in vitro* to determine the potential for clinical drug-drug interactions[12]. An update from the International Transporter Consortium in 2013 recommended that in addition to the seven transporters, new molecular entities be evaluated as potential substrates or inhibitors of the MATE transporters, which are known to participate in biliary and renal excretion of a number of drugs[13]. It has been previously reported that administration of the MATE inhibitor pyrimethamine significantly reduces the renal clearance of metformin in healthy subjects[14]. It has also been hypothesized that loss of function mutations in *SLC47A1* (MATE1) may induce lactic acidosis in patients treated with metformin, based on studies using MATE1 knockout mice[15]. It is clear that MATE1 can be an important factor in mediating the transport of drugs across biological membranes.

Our laboratory has previously performed a high-throughput screen using a prescription drug library for MATE1 inhibitors[16], and while the screen provides information about this transporter's inhibitor specificity, to our knowledge no large screen of MATE1 substrates has been reported. In this study, several novel inhibitors and substrates of MATE1 were identified. First, a library of 86 CNS-active small molecule drugs was screened to identify inhibitors of MATE1. Compounds that showed inhibition activity were further tested as substrates using cellular uptake studies with LC/MS/MS quantification. Multiple novel substrates of MATE1 were identified, including the anti-psychotic medication, sulpiride. Because of its expression in the liver, kidney, skeletal muscle, adrenal gland, testis and blood-brain barrier[3,17], these data

suggest that MATE1 plays a role in the elimination and tissue distribution of these novel substrates.

Materials and Methods

Chemicals. In the library of 86 CNS-active compounds, 47 compounds were obtained from Pfizer (Groton, CT). The remaining 39 compounds were purchased from various sources, including Sigma Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ), Santa Cruz Biotechnology (Santa Cruz, CA), Enzo Life Sciences (Farmingdale, NY), and TCI America (Portland, OR). Cell culture media (DMEM H-21), hygromycin B, Hank's Buffered Salt Solution (HBSS), phosphate buffered saline (PBS), 4-(4-Diethylaminostyryl)-1-methylpyridinium iodide (ASP$^+$) and the Pierce BCA Protein Assay Kit were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum and penicillin/streptomycin were obtained from University of California San Francisco Cell Culture Facility. Cimetidine was purchased from Sigma Aldrich (St. Louis, MO). [^{3}H]-sulpiride was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Cell Culture. Flp-In HEK cells stably transfected with human MATE1 (HEK-hMATE1) were previously established in our laboratory[16]. These cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) H-21 media supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, and 100 µg/mL hygromycin B. Cells were grown at 37 °C in a humidified incubator with 5% CO_2.

Inhibition Screen. HEK-hMATE1 cells were grown on poly-D-lysine coated 96-well plates in DMEM medium described above to at least 90% confluence at 48 hours post-seeding. Cells were washed once then preincubated for 30 minutes in HBSS buffer (pH 6.5) containing 30 mM ammonium chloride. Each of the tested compounds (20 µM to 500 µM) were added to HBSS containing 2 µM ASP[+], which was then added to the cells for 90 seconds. The reaction was terminated by washing the cells twice with ice-cold HBSS containing 500 µM cimetidine. The plate was dried overnight and read in a Tecan Genios Pro fluorescence microplate reader with an excitation filter at 485 nm and emission filter at 585 nm.

Uptake Studies Using LC/MS/MS Quantitation. HEK-hMATE1 cells were grown on poly-D-lysine coated 24-well plates in DMEM medium described above to at least 90% confluence at least 48 hours post-seeding. Cells were washed and preincubated for 30 minutes in HBSS buffer (pH 6.5) containing 30 mM ammonium chloride. The buffer was removed and replaced with uptake buffer (HBSS containing 5 µM of the test compounds). Uptake was performed at 37 °C for various time points up to 2 minutes, and then was terminated by washing the cells twice with ice-cold HBSS containing 500 µM cimetidine. The inhibitor used was 10 µM pyrimethamine. Plates containing cells were dried overnight at 4°C. Samples were extracted by adding 200 µL of ice-cold methanol for 10 minutes, followed by two 10-minute incubations with 200 µL of 70:30 methanol:water. The methanol:water solution was pooled in a 96-well plate and evaporated off in a Savant centrifugal evaporator overnight. Dried plates were sent to York Bioanalytical Solutions (York, UK) for quantitation by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Results were normalized by protein content as determined with a Pierce BCA Protein Assay Kit (Life Technologies) and analyzed using Graphpad Prism 5.0.

Sulpiride Uptake Studies. HEK-hMATE1 cells were grown on poly-D-lysine coated 24-well plates in DMEM medium described above to at least 90% confluence at 48 hours post-seeding. Cells were washed and preincubated for 30 minutes in HBSS buffer (pH 6.5) containing 30 mM ammonium chloride. The buffer was removed and replaced with uptake buffer (HBSS containing 6 nM of [^{3}H]-sulpiride). Uptake was performed at 37 °C for various time points up to 1 hour, and then was terminated by washing the cells twice with ice-cold HBSS. Cells were lysed by the addition of 0.1 N NaOH and 0.1% SDS, and intracellular radioactivity was determined by scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter) and normalized by protein content per well, as determined using a Pierce BCA Protein Assay Kit (Life Technologies). Results were analyzed using Graphpad Prism 5.0.

Rat Perfusion Studies. Adult Sprague-Dawley rats approximately 12-16 weeks of age were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. A catheter was inserted into the left interior carotid artery and the external carotid artery was ligated shut. Animals were then infused with the infusion solution, which consisted of Krebs-Henseleit buffer at pH 7.4, warmed to 37 °C and bubbled with a mixture of 5% CO_2 and 95% O_2. The infusion solution included 0.5 µCi/mL of [^{3}H]-sulpiride and 0.2 µCi/mL of [^{14}C]-inulin as a vascular marker, with or without the addition of 10 µM pyrimethamine as an inhibitor. The infusion pump was set to infuse 8 mL of the infusion solution in one minute. Animals were decapitated immediately following infusion and the brain was removed from the skull, bisected with a razor blade into the left and right hemispheres, and incubated in 2.5 mL Solvable tissue solubilizer (PerkinElmer) overnight at 50°C. The radioactivity in each sample was determined by scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter).

Rat Microvessel Isolation and Staining. Brain microvessels were isolated from rat brain as previously described[18,19]. Briefly, rat brain tissue was manually homogenized using a Potter-Elvehjem tissue homogenizer in 5 volumes of ice-cold HBSS buffer. After centrifugation for 5 minutes at 1,000 x g, the pellet was suspended in 10 mL of a 15% dextran solution and centrifuged for 15 minutes at 2,500 x g to remove neuronal contaminants. The resulting pellet was suspended in HBSS buffer and sequentially passed through a 100-micron nylon mesh and a 20-micron nylon mesh. The microvessels retained on the 20-micron mesh were collected, visualized on an EVOS XL Core microscope (Life Technologies) and fixed in 4% paraformaldehyde for 15 minutes on a glass slide. After several washes with PBS, slides were blocked with a solution of 10% normal donkey serum in PBS containing 0.05% Tween-20 for 1 hour, and incubated overnight in PBS containing 5% normal donkey serum and a rMATE1 antibody (Alomone Labs). Slides were washed with PBS and incubated with an Alexa Fluor 488 anti-rabbit secondary antibody for 1 hour, then mounted with ProLong Gold Antifade Mountant with DAPI (Life Technologies). Fluorescent imaging was performed on a Zeiss AxioImager M1 microscope with an AxioCam Mrm camera (Carl Zeiss Microscopy).

Results

Validation of HEK-hMATE1 Cell Line. ASP^+ is a known substrate of MATE1. Stably-transfected MATE1 cells (HEK-hMATE1) showed significantly greater uptake of ASP^+ compared with empty vector cells with linear kinetics at time points up to 1 minute (Figure 4.1A). Due to technical limitations, 90 seconds was selected as the time point to perform inhibition studies. The V_{max} of ASP^+ for MATE1 was determined to be 34.5 ± 2.7 pM/minute/µg protein, whereas the K_m of ASP^+ for MATE1 was determined to be 34.1 ± 6.4 µM (Figure 4.1B). Cimetidine was a strong inhibitor of MATE1-mediated ASP^+ uptake, with an IC_{50} of 1.2 ± 0.25 µM (Figure 4.1C). These results validate the HEK-hMATE1 cell line as an *in vitro* model for studying MATE1-mediated uptake.
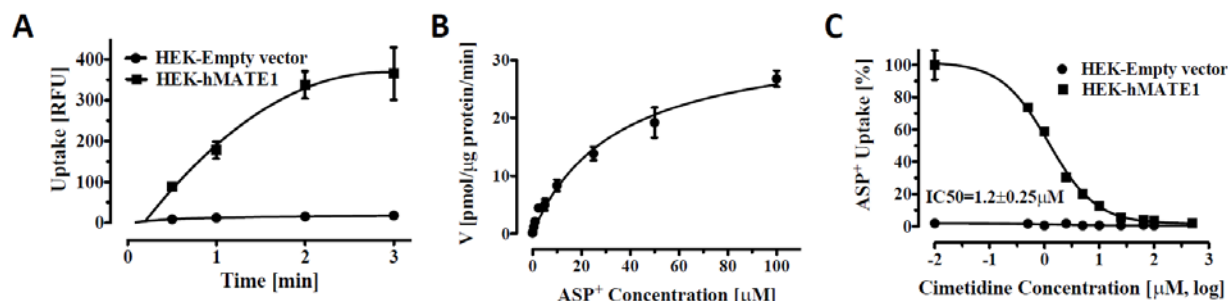
Figure 4.1. Validation of HEK-hMATE1 cell line. (A) MATE1-mediated uptake of ASP^+ at time points up to 3 minutes. (B) Uptake of ASP^+ by MATE1 (V_{max} = 34.5 ± 2.7 pM/minute/µg protein and K_m = 34.1 ± 6.4 µM). (C) Cimetidine is a good inhibitor of MATE1 (IC_{50} = 1.2 ± 0.25 µM).

Inhibition Screen for Novel Inhibitors of MATE1. Eighty-six compounds were screened at 200 µM for the ability to inhibit MATE1-mediated ASP$^+$ uptake (Figure 4.2A and Figure 4.2B). Data were normalized such that the uptake of ASP$^+$ without the addition of an inhibitor was determined to be 100%. The positive control used was 200 µM cimetidine, a known MATE1 inhibitor. The majority of the CNS active compounds were able to inhibit MATE1 greater than 50% at 200 µM, probably because the compound library is over-represented with hydrophobic basic drugs, which are known to be inhibitors of MATE1[16,20–23]. Subsequent studies screened most of these compounds at multiple additional concentrations, including 20, 50, 200 and 500 µM. Results are shown by categorizing compounds based on their inhibition potency (Figure 4.3).

A

Figure showing Percent Uptake of ASP⁺ (y-axis, 0–150) across various inhibitors on the x-axis: No Inhibitor, Positive Control, Buspirone, Risperidone, Quetiapine, Cidoxepin, Tacrine, Ropinirole, Mirtazapine, Fluoxetine, Minaprine, Varenicline, Amfebutamone, Trazodone, Talipexole, R-Apomorphine, Brontizolam, Atomoxetine, Donepezil, Paliperidone, Sertindole, Paroxetine, Olanzapine, Pramipexole, Rivastigmine, Tramadol, Rizatriptan, Riluzole, Memantine, Nimodipine, Nortriptyline, Escitalopram, Cabergoline, Clomipramine, Aprepitant, Eletriptan, Lamotrigine, Sumatriptan, Verapamil, Metoclopramide, Carbamazepine, Galantamine, Sulpiride, Triprolidine.
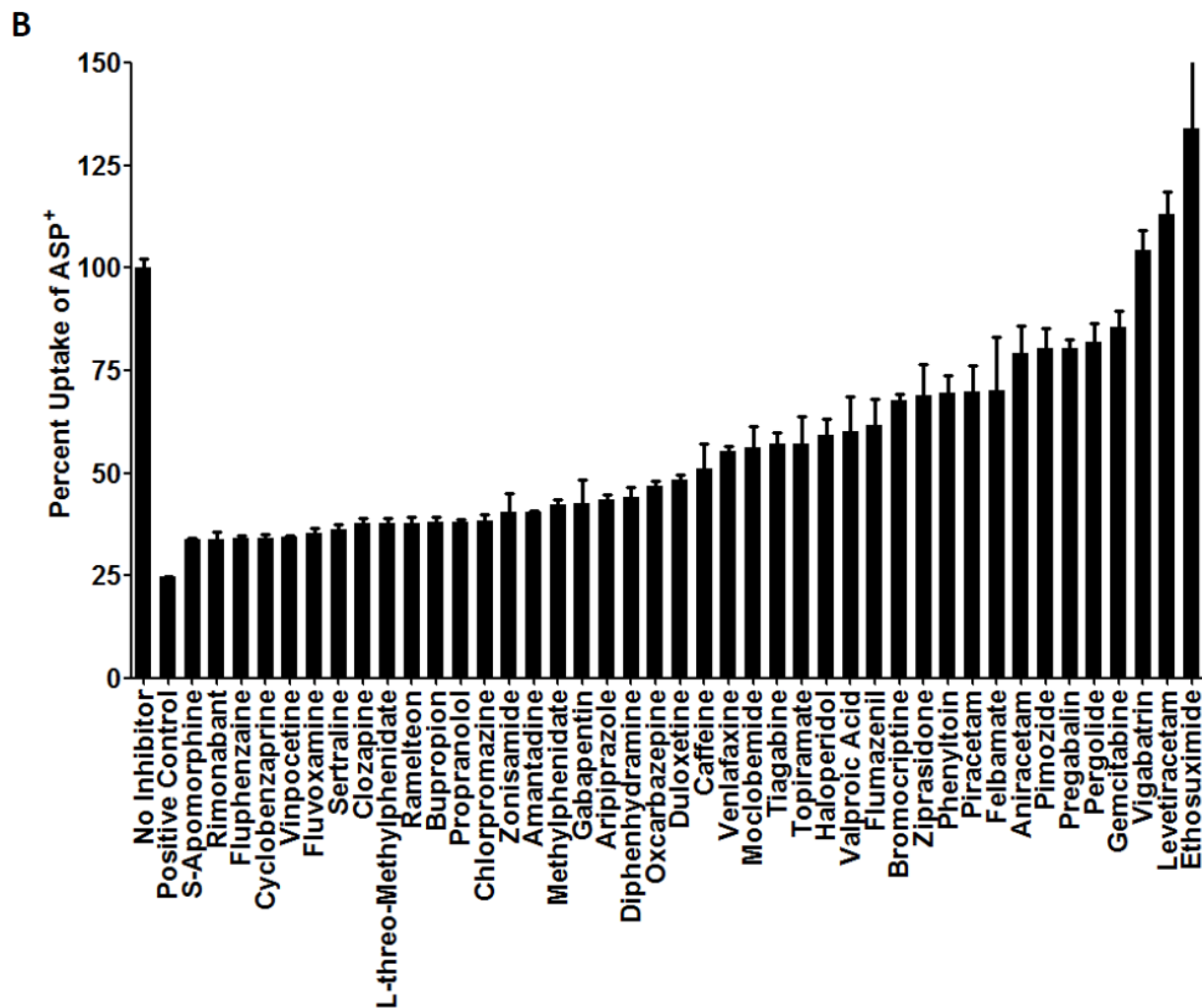
Figure 4.2. Inhibition screen for MATE1 inhibitors. Compounds are sorted by percent of inhibition of ASP$^+$ uptake from highest to lowest. The results were split into two graphs, A and B, for ease of viewing.

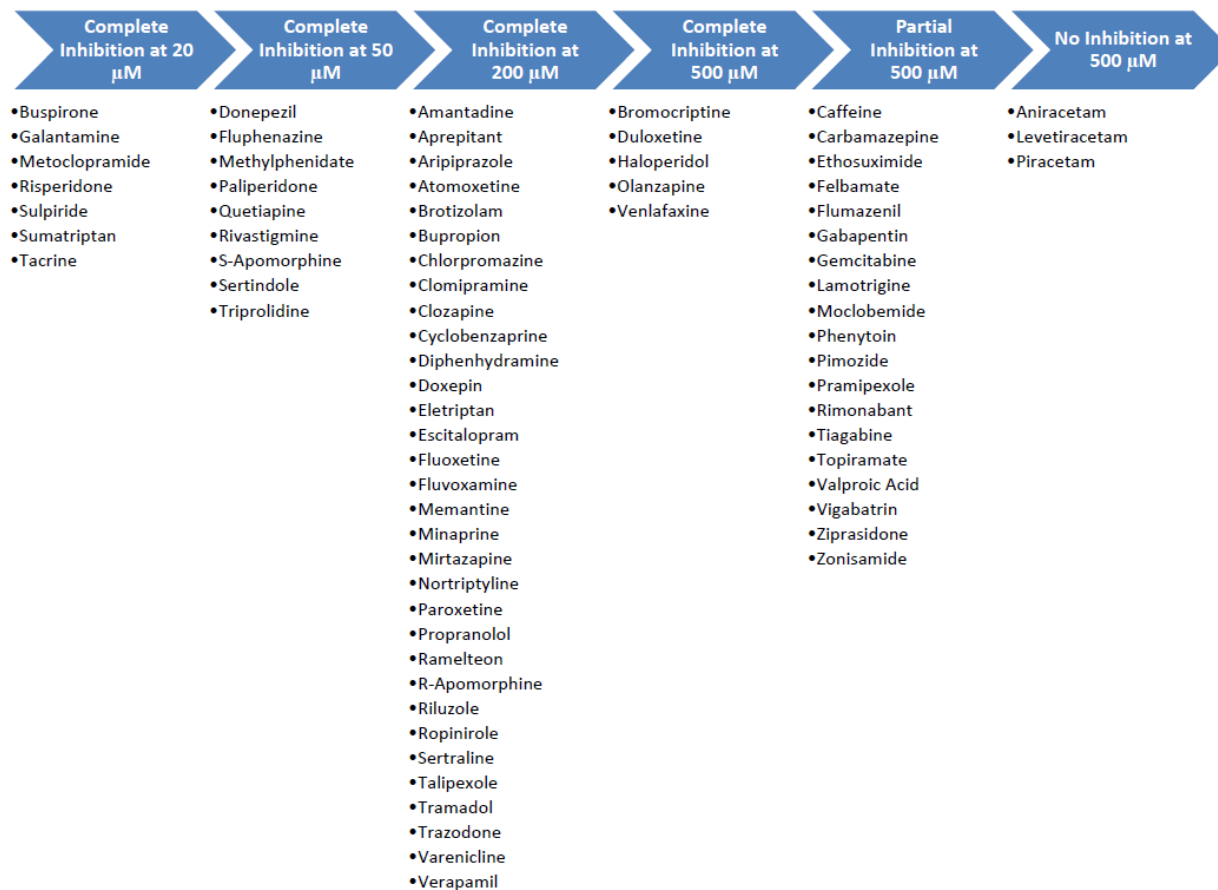| Complete Inhibition at 20 μM	Complete Inhibition at 50 μM	Complete Inhibition at 200 μM	Complete Inhibition at 500 μM	Partial Inhibition at 500 μM	No Inhibition at 500 μM
• Buspirone	• Donepezil	• Amantadine	• Bromocriptine	• Caffeine	• Aniracetam
• Galantamine	• Fluphenazine	• Aprepitant	• Duloxetine	• Carbamazepine	• Levetiracetam
• Metoclopramide	• Methylphenidate	• Aripiprazole	• Haloperidol	• Ethosuximide	• Piracetam
• Risperidone	• Paliperidone	• Atomoxetine	• Olanzapine	• Felbamate	
• Sulpiride	• Quetiapine	• Brotizolam	• Venlafaxine	• Flumazenil	
• Sumatriptan	• Rivastigmine	• Bupropion		• Gabapentin	
• Tacrine	• S-Apomorphine	• Chlorpromazine		• Gemcitabine	
	• Sertindole	• Clomipramine		• Lamotrigine	
	• Triprolidine	• Clozapine		• Moclobemide	
		• Cyclobenzaprine		• Phenytoin	
		• Diphenhydramine		• Pimozide	
		• Doxepin		• Pramipexole	
		• Eletriptan		• Rimonabant	
		• Escitalopram		• Tiagabine	
		• Fluoxetine		• Topiramate	
		• Fluvoxamine		• Valproic Acid	
		• Memantine		• Vigabatrin	
		• Minaprine		• Ziprasidone	
		• Mirtazapine		• Zonisamide	
		• Nortriptyline			
		• Paroxetine			
		• Propranolol			
		• Ramelteon			
		• R-Apomorphine			
		• Riluzole			
		• Ropinirole			
		• Sertraline			
		• Talipexole			
		• Tramadol			
		• Trazodone			
		• Varenicline			
		• Verapamil			

Figure 4.3. Follow-up inhibition screening for MATE1 inhibitors. Compounds were grouped into categories based on their ability to inhibit MATE1-mediated ASP$^+$ uptake.

Initial Identification of MATE1 Substrates. Following up on the inhibition screen results, each compound that was found to have MATE1 inhibitory activity was tested for uptake in HEK-hMATE1 and empty vector control cells. Eighteen novel substrates of MATE1 were identified, including bromocriptine (Figure 4.4A), buprenorphine (Figure 4.4B), caffeine (Figure 4.4C), chlorpromazine (Figure 4.4D), cyclobenzaprine (Figure 4.4E), diphenhydramine (Figure 4.4F), riluzole (Figure 4.4G), rizatriptan (Figure 4.4H), sulpiride (Figure 4.4I), talipexole (Figure 4.4J), tramadol (Figure 4.4K), frovatriptan (Figure 4.5A), levetiracetam (Figure 4.5B), rocuronium (Figure 4.5C), sumatriptan (Figure 4.5D), triprolidine (Figure 4.5E), valproic acid (Figure 4.5F) and zolmitriptan (Figure 4.5G). Uptake in the HEK-MATE1 cell line was inhibited in the presence of a MATE1 inhibitor, pyrimethamine, for several compounds (Figure 4.4A-K), providing additional evidence that these compounds are substrates of MATE1. For several substrates, the uptake was considerably lower in cells expressing MATE1 in the presence of pyrimethamine than in the EV cells. It is possible that pyrimethamine at 10 µM inhibited other transporters endogenously expressed in HEK cells. The K_i of pyrimethamine for MATE1 is around 100 nM, but at 10 µM pyrimethamine inhibits other transporters[24]. For some substrates, the uptake in the MATE1 cells in the presence of pyrimethamine was greater than the uptake in EV cells. In this case, pyrimethamine may not have fully inhibited the drug uptake by MATE1 at the concentrations of substrates used in the uptake studies.
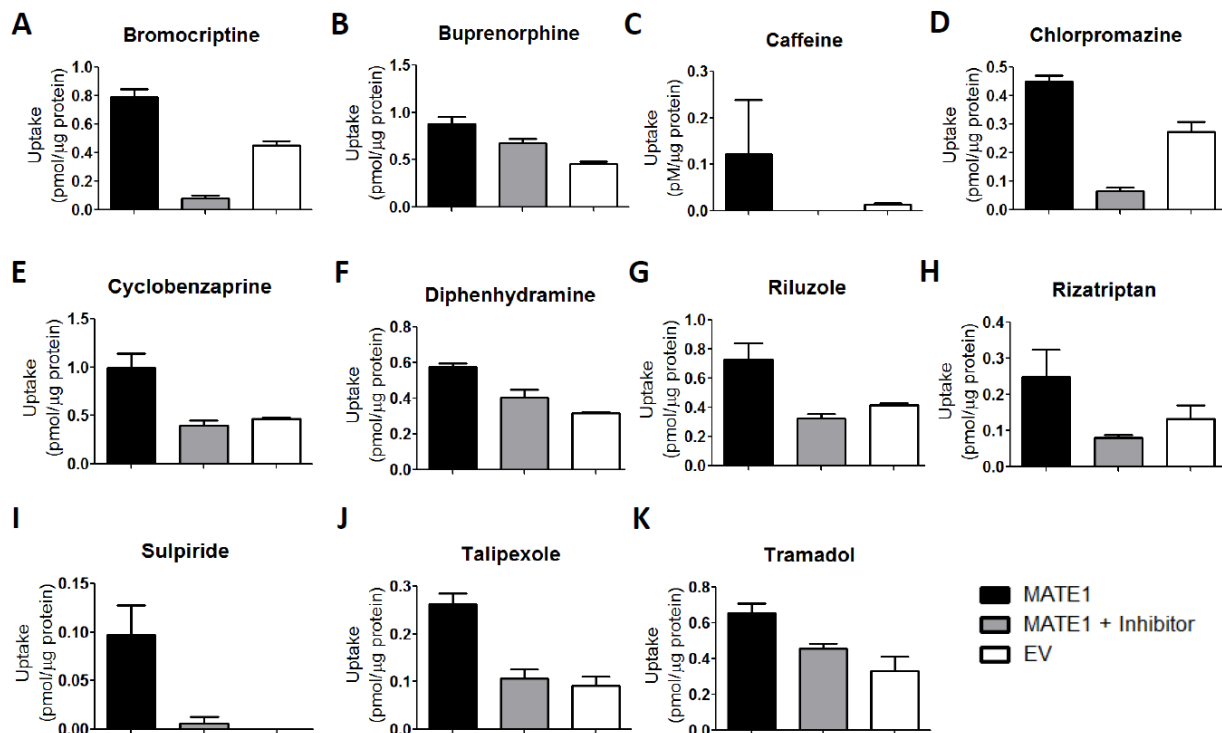
Figure 4.4. Compounds identified as substrates of MATE1 by uptake studies using LC/MS/MS quantitation. Uptake was found to be higher in HEK-MATE1 cells as opposed to empty vector cells for (A) bromocriptine, (B) buprenorphine, (C) caffeine, (D) chlorpromazine, (E) cyclobenzaprine, (F) diphenhydramine, (G) riluzole, (H) rizatriptan, (I) sulpiride, (J) talipexole and (K) tramadol. Uptake in the HEK-MATE1 cell line was inhibited in the presence of a MATE1 inhibitor, pyrimethamine.
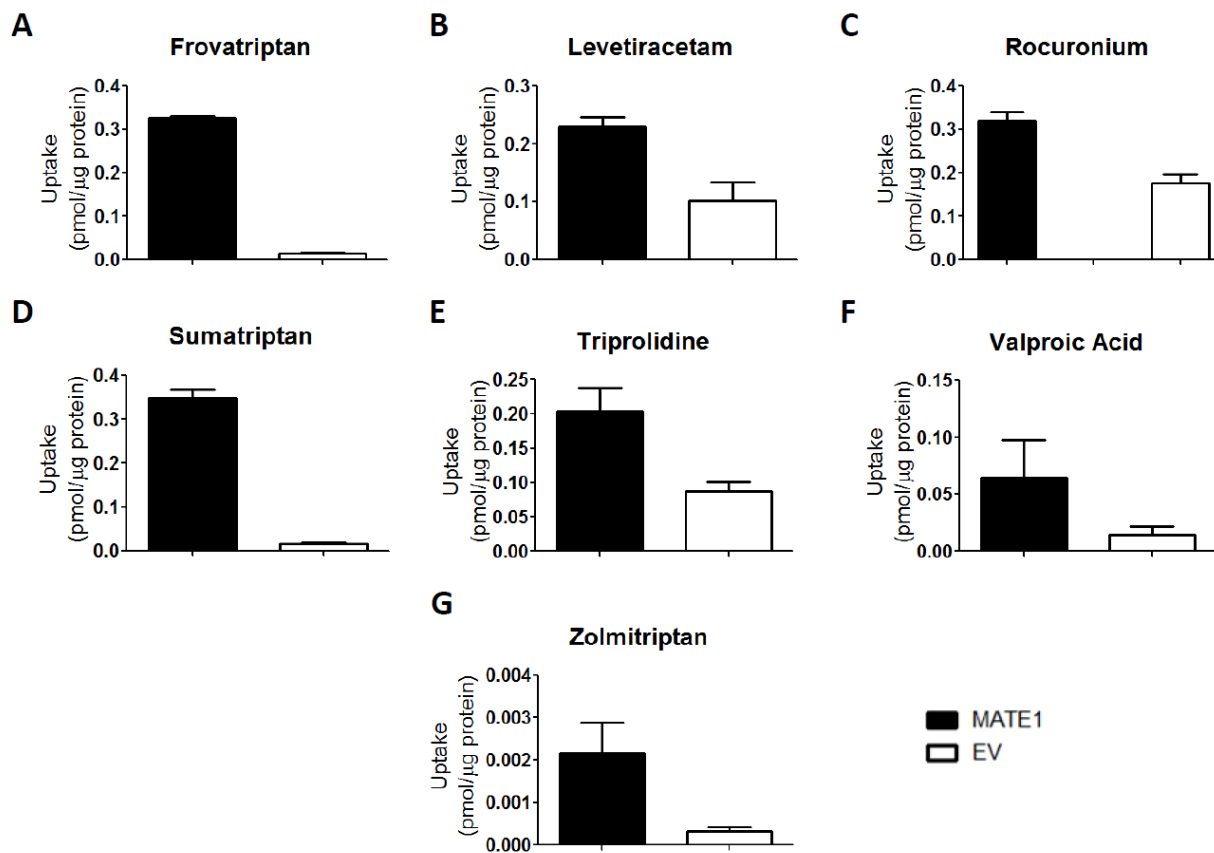
131

Figure 4.5. Compounds identified as substrates of MATE1 by uptake studies using LC/MS/MS quantitation. Uptake was found to be higher in HEK-MATE1 cells as opposed to empty vector cells for (A) frovatriptan, (B) levetiracetam, (C) rocuronium, (D) sumatriptan, (E) triprolidine, (F) valproic acid and (G) zolmitriptan.

Identification of Sulpiride as a MATE1 Substrate. From the set of novel substrates of MATE1 identified through substrate screening, sulpiride was selected for further characterization. Uptake studies were performed using [^{3}H]-sulpiride, and sulpiride uptake in HEK-hMATE1 cells was ablated in the presence of a MATE1 inhibitor or at 4 ºC (Figure 4.6A). In addition, concentration-dependent sulpiride uptake in HEK-MATE1 cells was observed and at low concentrations, transporter-mediated uptake surpasses passive diffusion, with a V_{max} of 0.213 µmol/min/µg protein (95% CI: 0.18-0.24 µmol/min/µg protein) and K_m of 7.6 µM (95% CI: 2.8-12.4 µM) (Figure 4.6B).
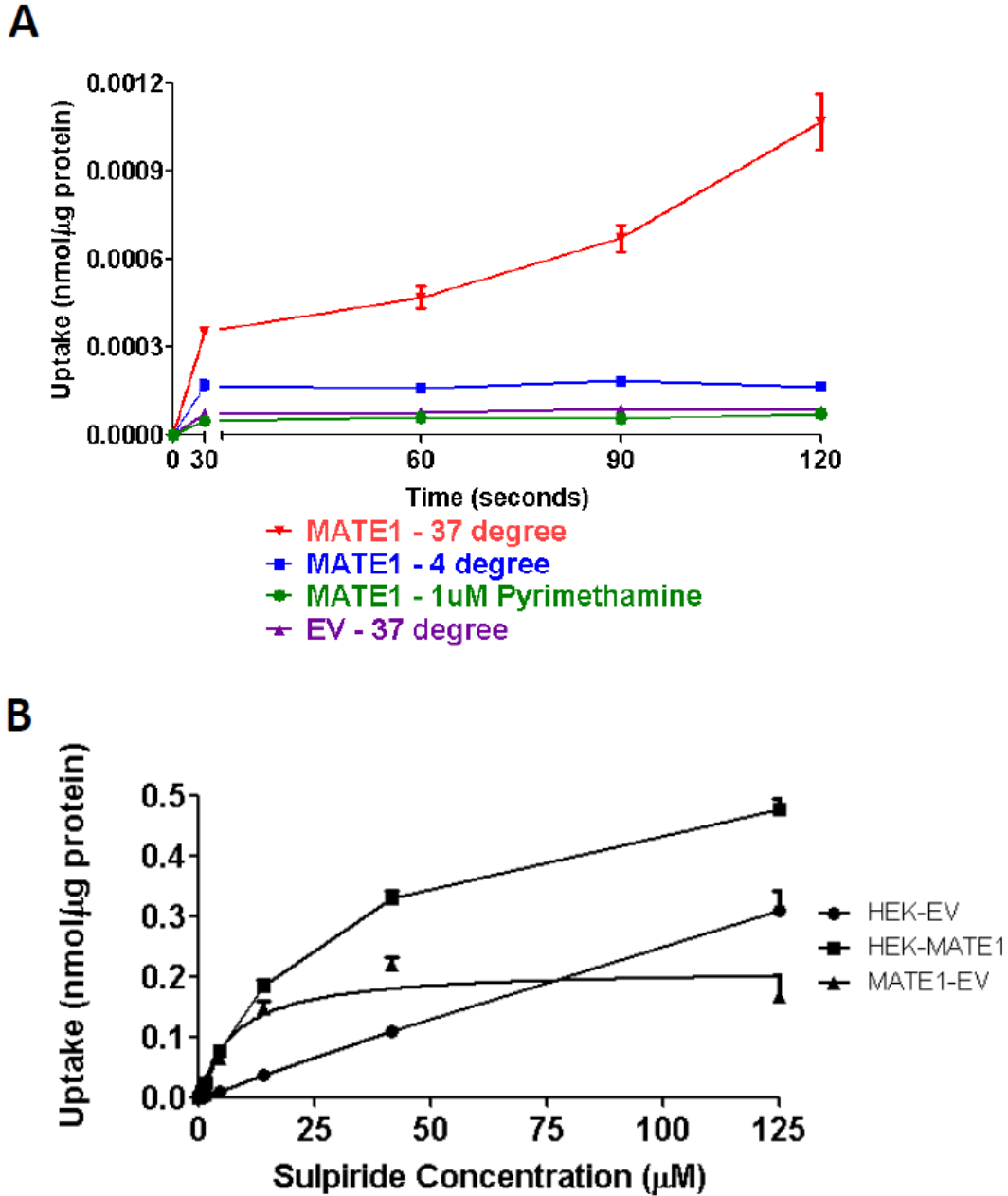
Figure 4.6. Sulpiride is a substrate of human MATE1. (A) [³H]-sulpiride uptake is significantly higher in stably-transfected MATE1 cells than in empty vector cells. Uptake in stably-transfected MATE1 cells can be ablated at 4 degrees or in the presence of the MATE1 inhibitor pyrimethamine. (B) Concentration curve of [³H]-sulpiride uptake shows that at low concentrations, transporter-mediated uptake surpasses passive diffusion.

Rat Perfusion Model Did Not Show Evidence of Facilitated Brain Uptake of Sulpiride by MATE1. Our rat perfusion assay shows that sulpiride brain penetration was not inhibited by the MATE1 inhibitor pyrimethamine (Figure 4.7). Diazepam is used as the diffusion control, and shows a high Brain Uptake Index (BUI). However, BUI values are low for sulpiride both in the presence and absence of the MATE1 inhibitor pyrimethamine, and there is no significant difference between the two groups. Inulin is the vascular marker control and is normalized to a BUI of 1.
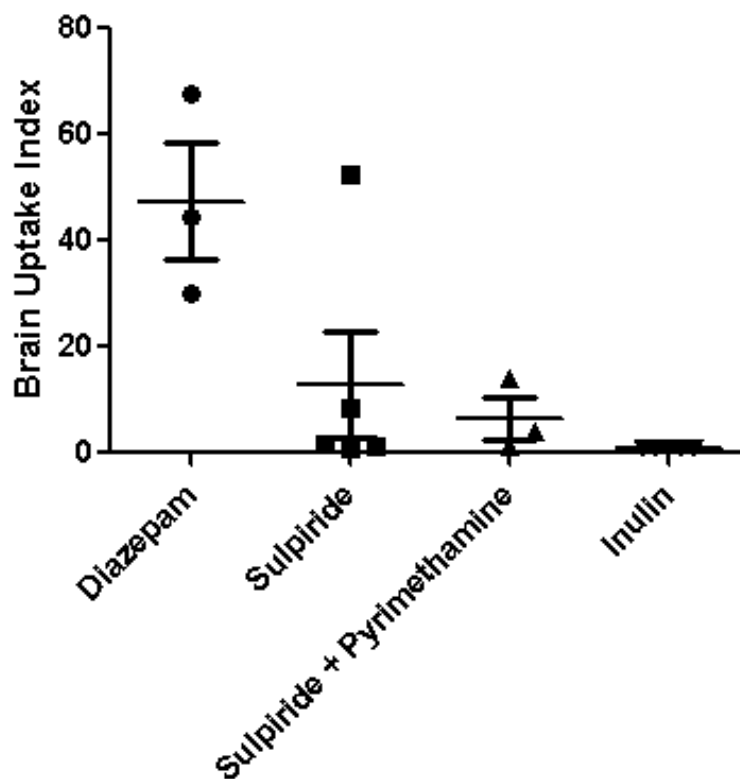

Figure 4.7. Sulpiride does not enter the brain in a rat perfusion model. Using diazepam as the diffusion marker and inulin as a vascular marker, sulpiride uptake is very low, with no significant difference in the presence and absence of the MATE1 inhibitor, pyrimethamine.

rMATE1 is Detected in Rat Brain Microvessels. As the rat perfusion model showed low brain penetration of sulpiride, rMATE1 was confirmed to be expressed in rat brain endothelial cells. Through a series of isolation and purification steps, rat brain microvessels were successfully isolated (Figure 4.8A). These vessels were then stained using an antibody for rMATE1 and protein was detected by immunofluorescence (Figure 4.8B). rMATE1 is stained in green and nuclei are stained in blue with DAPI. Notably, rather than a strong, consistent signal as expected for proteins expressed on the plasma membrane, a weak, punctate pattern was observed, perhaps consistent with an endosomal or sub-plasma membrane compartment for the transporter.
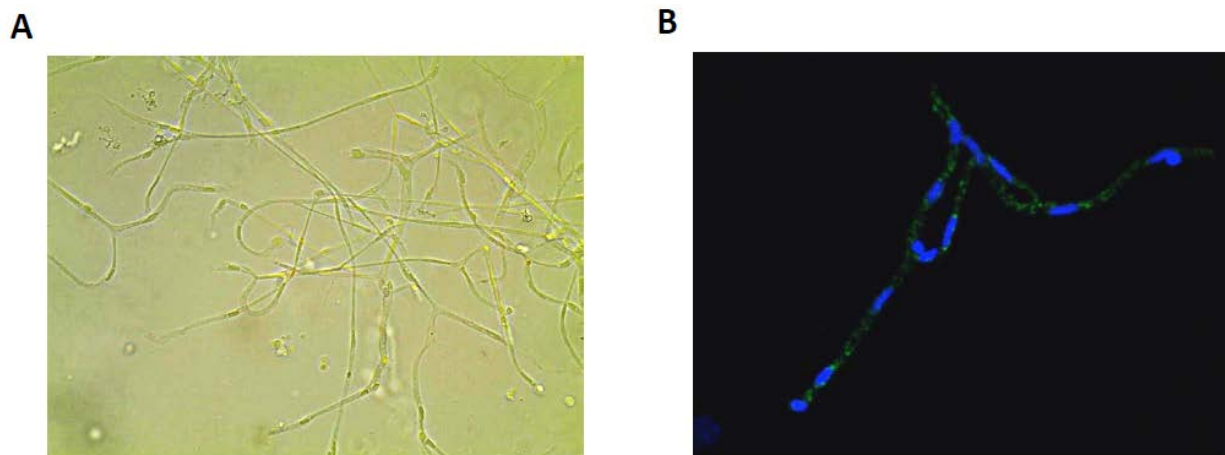

Figure 4.8. Imaging of isolated rat brain microvessels. (A) Bright field image (20X) of rat brain microvessels immediately after isolation from rat brain tissue. (B) Immunofluorescence microscopy (60X) showing positive MATE1 staining (green) in an isolated rat brain microvessel. Blue stain is DAPI.

Discussion

MATE1 has been found to be important in mediating drug transport in the kidney. The International Transporter Consortium recommends that new molecular entities be evaluated as potential substrates or inhibitors of the MATE transporters in order to identify potential drug-drug interactions. While inhibitors of MATE1 have been identified in high-throughput screening studies, only a limited number of studies have identified substrates of the transporter despite its known role in mediating drug-drug interactions. The broad tissue distribution of MATE1 suggests that in addition to mediating the elimination of drugs in the kidney, MATE1 may also participate in the tissue distribution of drugs.

In this study, inhibition assays and cellular uptake studies were used on a library of 86 CNS-active drugs to identify novel ligands of MATE1, especially novel substrates. Not surprisingly, the inhibition screen found that the majority of compounds in our library were inhibitors of MATE1 (Figure 4.2 and Figure 4.3). Previous studies have indicated that hydrophobicity and a positive charge are important contributors to MATE1 binding[20–22,25,26] and a proposed structural model of MATE1 features a large pore that contains hydrophobic and negatively charged residues, suggesting that hydrophobic and cationic molecules are favored. The composition of our CNS-active library is over-represented by compounds with these characteristics, with 60 percent of the molecules being basic compounds and half of the molecules having cLogP values > 3. In contrast, the ICONIX library contains 910 FDA-approved drugs, of which only a third are reported to be basic drugs[16]. The identification of potent inhibitors of MATE1 is significant, as inhibition of MATE1 may cause drug-drug interactions that lead to drug accumulation in the kidney, resulting in nephrotoxicity.

Cellular uptake studies further identified several novel substrates of MATE1, including bromocriptine, buprenorphine, caffeine, chlorpromazine, cyclobenzaprine, diphenhydramine, riluzole, sulpiride, talipexole, tramadol, levetiracetam, rocuronium, triprolidine and valproic acid (Figure 4.4 and Figure 4.5). Four triptan drugs were identified as novel substrates of MATE1: rizatriptan, frovatriptan, sumatriptan and zolmitriptan. Eletriptan and naratriptan were also tested but they did not appear to be substrates of MATE1. While all triptan drugs share the same mechanism of action, their pharmacokinetic properties can be very different: oral bioavailability ranges from 14% (sumatriptan) to 74% (naratriptan) and elimination half-life ranges from 2 hours (sumatriptan/rizatriptan) to 25 hours (frovatriptan)[27]. As MATE1 is known to be highly expressed on the luminal membrane of proximal tubular cells[3], these data suggest that MATE1 plays a role in active renal elimination of these triptan drugs. For most triptan drugs, renal clearance is greater than the product of the fraction of the drug unbound to protein and glomerular filtration rate, suggesting that active secretion is the predominant mechanism in the kidney (Table 4.1). Of the four MATE1 substrates, frovatriptan represents the only exception, with its net renal clearance being less than filtration clearance. However, renal clearance of frovatriptan occasionally exceeds GFR immediately post dose, suggesting that at high concentrations the drug may undergo net active secretion in the kidney[28]. It is possible that frovatriptan interacts with a reabsorptive transporter, which leads to its low renal clearance, and though the compound may be actively cleared by MATE1 in the kidney, reabsorption generally predominates.

Table 4.1. Pharmacokinetic properties of triptan drugs. Data obtained from MicroMedex Healthcare Series (Truven Health Analytics, Ann Arbor, MI).

	f_e^1	f_u^2	CL_R (mL/min)	Secretory Clearance (mL/min)
Rizatriptan	16%	86%	400	296
Frovatriptan	32%	85%	82	0
Sumatriptan	42%	79-86%	272	177
Zolmitriptan	8%	75%	367	277
Eletriptan	9%	15%	65	47
Naratriptan	50%	69-72%	220	133

^1f_e = fraction excreted unchanged in urine; ^2f_u = fraction unbound in plasma

Triptan drugs have been suggested to cross the blood-brain barrier through the organic anion transporting polypeptide 1A2 (OATP1A2, SLCO1A2)[29]. In a previous publication from our laboratory, we identified MATE1 at the human blood-brain barrier[17]. This suggests that MATE1 may also play a role in brain penetration of some triptans and other drugs. While it is unclear whether triptan drugs need to cross the blood-brain barrier to achieve their antinociceptive effects, studies suggest that CNS adverse effects are attributable to triptans crossing into the brain[30]. Another mechanism through which drugs get into the brain is by crossing the blood-cerebrospinal fluid barrier, BCSFB, through the choroid plexus. Much like the blood-brain barrier, cells of the choroid plexus exhibit tight junctions with enhanced expression of drug metabolizing enzymes and transporters. In a recent paper, medium-high MATE1 transcription was detected in primate choroid plexus that increased during gestation[31]. Therefore, even if MATE1 is found to play a minor role at the human blood-brain barrier, it could still play a role in brain drug penetration through the BCSFB and choroid plexus. Further

studies are required to explore the characteristics of MATE1-mediated triptan uptake, particularly into the brain.

Following identification of several novel substrates of MATE1, the selective dopamine receptor D2 blocker sulpiride was chosen for follow-up studies. Given its low membrane permeability ($K_{p,uu} < 0.1$), transporters are likely required for its uptake into the brain, the site of action. Sulpiride has been previously reported to be a substrate of the organic cation transporters OCT1 and OCT2, and the authors suggest that sulpiride enters the brain through organic cation transporters[32]. In this study, both time- and concentration-dependent uptake of sulpiride was observed through MATE1, with a V_{max} of 0.213 µmol/min/µg protein and K_m of 7.6 µM (Figure 4.6A-B). The uptake of sulpiride was inhibited by the addition of a known MATE1 inhibitor, pyrimethamine. Since the maximal plasma concentration after oral administration of 100 mg sulpiride is only 0.29 µM[33], transport *in vivo* by MATE1 will not be saturated. Sulpiride was not shown to enter the brain in a rat perfusion assay (Figure 4.7), but this may be attributable to interspecies differences. A recent study quantified hMATE1 protein expression in human choroid plexus but rMATE1 was below the limit of quantitation in rat choroid plexus, indicating at least 5.98-fold higher expression in human choroid plexus than in rats[34]. Furthermore, the signal in microvessels from rat brain was not as strong as expected for a membrane transporter but rather showed a punctate pattern, suggesting that rMATE1, while expressed, may not be functionally located at the plasma membrane (Figure 4.8).

In conclusion, we report eighteen novel substrates of MATE1 that are CNS-active drugs, and we believe that MATE1 plays a role in the renal disposition and tissue distribution of these compounds. Given that MATE1 has been reportedly expressed at both the human blood-brain and blood-cerebrospinal fluid barriers, MATE1 may also be involved in the brain penetration of

some of these CNS-active drugs. These findings enhance understanding of the substrate specificity of this clinically-important transporter.

References

1. König, J., Zolk, O., Singer, K., Hoffmann, C. & Fromm, M. F. Double-transfected MDCK cells expressing human OCT1/MATE1 or OCT2/MATE1: Determinants of uptake and transcellular translocation of organic cations. *Br. J. Pharmacol.* **163,** 546–555 (2011).

2. Meyer zu Schwabedissen, H. E., Verstuyft, C., Kroemer, H. K., Becquemont, L. & Kim, R. B. Human multidrug and toxin extrusion 1 (MATE1/SLC47A1) transporter: functional characterization, interaction with OCT2 (SLC22A2), and single nucleotide polymorphisms. *Am. J. Physiol. Renal Physiol.* **298,** F997–F1005 (2010).

3. Nies, A. T., Koepsell, H., Damme, K. & Schwab, M. Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy. *Handb. Exp. Pharmacol.* **201,** 105–167 (2011).

4. Motohashi, H. & Inui, K. I. Multidrug and toxin extrusion family SLC47: Physiological, pharmacokinetic and toxicokinetic importance of MATE1 and MATE2-K. *Mol. Aspects Med.* **34,** 661–668 (2013).

5. Tanihara, Y., Masuda, S., Sato, T., Katsura, T., Ogawa, O. & Inui, K.-I. Substrate specificity of MATE1 and MATE2-K, human multidrug and toxin extrusions/H(+)-organic cation antiporters. *Biochem. Pharmacol.* **74,** 359–371 (2007).

6. Van Crugten, J., Bochner, F., Keal, J. & Somogyi, A. Selectivity of the cimetidine-induced alterations in the renal handling of organic substrates in humans. Studies with anionic, cationic and zwitterionic drugs. *J. Pharmacol. Exp. Ther.* **236,** 481–487 (1986).

7. Lai, M. Y., Jiang, F. M., Chung, C. H., Chen, H. C. & Chao, P. D. Dose dependent effect of cimetidine on procainamide disposition in man. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **26,** 118–121 (1988).

8. Abel, S., Nichols, D. J., Brearley, C. J. & Eve, M. D. Effect of cimetidine and ranitidine on pharmacokinetics and pharmacodynamics of a single dose of dofetilide. *Br. J. Clin. Pharmacol.* **49,** 64–71 (2000).

9. Feng, B., Obach, R. S., Burstein, A. H., Clark, D. J., de Morais, S. M. & Faessel, H. M. Effect of human renal cationic transporter inhibition on the pharmacokinetics of varenicline, a new therapy for smoking cessation: an in vitro-in vivo study. *Clin. Pharmacol. Ther.* **83,** 567–576 (2008).

10. Somogyi, A., Stockley, C., Keal, J., Rolan, P. & Bochner, F. Reduction of metformin renal tubular secretion by cimetidine in man. *Br. J. Clin. Pharmacol.* **23,** 545–551 (1987).

11. Maeda, K. & Sugiyama, Y. Transporter biology in drug approval: Regulatory aspects. *Mol. Aspects Med.* **34,** 711–718 (2013).

12. Giacomini, K. M., Huang, S. M., Tweedie, D. J., Benet, L. Z., Brouwer, K. L., Chu, X., Dahlin, A., Evers, R., Fischer, V., Hillgren, K. M., Hoffmaster, K. A., Ishikawa, T., Keppler, D., Kim, R. B., Lee, C. A., Niemi, M., Polli, J. W., Sugiyama, Y., Swaan, P. W., Ware, J. A., Wright, S. H., Yee, S. W., Zamek-Gliszczynski, M. J. & Zhang, L. Membrane transporters in drug development. *Nat. Rev. Drug Discov.* **9,** 215–236 (2010).

13. Hillgren, K. M., Keppler, D., Zur, A. A., Giacomini, K. M., Stieger, B., Cass, C. E. & Zhang, L. Emerging transporters of clinical importance: an update from the International Transporter Consortium. *Clin. Pharmacol. Ther.* **94,** 52–63 (2013).

14. Kusuhara, H., Ito, S., Kumagai, Y., Jiang, M., Shiroshita, T., Moriyama, Y., Inoue, K., Yuasa, H. & Sugiyama, Y. Effects of a MATE protein inhibitor, pyrimethamine, on the renal elimination of metformin at oral microdose and at therapeutic dose in healthy subjects. *Clin. Pharmacol. Ther.* **89,** 837–844 (2011).

15. Toyama, K., Yonezawa, A., Masuda, S., Osawa, R., Hosokawa, M., Fujimoto, S., Inagaki, N., Inui, K. & Katsura, T. Loss of multidrug and toxin extrusion 1 (MATE1) is associated with metformin-induced lactic acidosis. *Br. J. Pharmacol.* **166,** 1183–1191 (2012).

16. Wittwer, M. B., Zur, A. A., Khuri, N., Kido, Y., Kosaka, A., Zhang, X., Morrissey, K. M., Sali, A., Huang, Y. & Giacomini, K. M. Discovery of potent, selective multidrug and toxin extrusion transporter 1 (MATE1, SLC47A1) inhibitors through prescription drug profiling and computational modeling. *J. Med. Chem.* **56,** 781–795 (2013).

17. Geier, E. G., Chen, E. C., Webb, A., Papp, A. C., Yee, S. W., Sadee, W. & Giacomini, K. M. Profiling solute carrier transporters in the human blood-brain barrier. *Clin. Pharmacol. Ther.* **94,** 636–639 (2013).

18. Dallaire, L., Tremblay, L. & Béliveau, R. Purification and characterization of metabolically active capillaries of the blood-brain barrier. *Biochem. J.* **276,** 745–752 (1991).

19. Bressler, J., Clark, K. & O'Driscoll, C. Assessing blood-brain barrier function using in vitro assays. *Methods Mol. Biol.* **1066,** 67–79 (2013).

20. Ullrich, K. J., Papavassiliou, F., David, C., Rumrich, G. & Fritzsch, G. Contraluminal transport of organic cations in the proximal tubule of the rat kidney. I. Kinetics of N1-methylnicotinamide and tetraethylammonium, influence of K+, HCO3-, pH; inhibition by

aliphatic primary, secondary and tertiary amines and mono- and bisq. *Pflugers Arch.* **419,** 84–92 (1991).

21. Ullrich, K. J., Rumrich, G., Neiteler, K. & Fritzsch, G. Contraluminal transport of organic cations in the proximal tubule of the rat kidney. II. Specificity: anilines, phenylalkylamines (catecholamines), heterocyclic compounds (pyridines, quinolines, acridines). *Pflugers Arch.* **420,** 29–38 (1992).

22. David, C., Rumrich, G. & Ullrich, K. J. Luminal transport system for H+/organic cations in the rat proximal tubule - Kinetics, dependence on pH; specificity as compared with the contraluminal organic cation-transport system. *Pflugers Arch.* **430,** 477–492 (1995).

23. Ullrich, K. J. & Rumrich, G. Luminal transport system for choline+ in relation to the other organic cation transport systems in the rat proximal tubule. Kinetics, specificity: alkyl/arylamines, alkylamines with OH, O, SH, NH2, ROCO, RSCO and H2PO4-groups, methylaminostyryl, rhodamine. *Pflugers Arch.* **432,** 471–485 (1996).

24. Ito, S., Kusuhara, H., Kuroiwa, Y., Wu, C., Moriyama, Y., Inoue, K., Kondo, T., Yuasa, H., Nakayama, H., Horita, S. & Sugiyama, Y. Potent and specific inhibition of mMate1-mediated efflux of type I organic cations in the liver and kidney by pyrimethamine. *J. Pharmacol. Exp. Ther.* **333,** 341–350 (2010).

25. Wright, S. H., Wunz, T. M. & Wunz, T. P. Structure and interaction of inhibitors with the TEA/H+ exchanger of rabbit renal brush border membranes. *Pflugers Arch.* **429,** 313–324 (1995).

26. Wright, S. H. & Wunz, T. M. Influence of substrate structure on turnover of the organic cation/H+ exchanger of the renal luminal membrane. *Pflugers Arch.* **436,** 469–477 (1998).

27. Jhee, S. S., Shiovitz, T., Crawford, A. W. & Cutler, N. R. Pharmacokinetics and pharmacodynamics of the triptan antimigraine agents: a comparative review. *Clin. Pharmacokinet.* **40,** 189–205 (2001).

28. Buchan, P., Keywood, C., Wade, A. & Ward, C. Clinical pharmacokinetics of frovatriptan. *Headache* **42 Suppl 2,** S54–S62 (2002).

29. Cheng, Z., Liu, H., Yu, N., Wang, F., An, G., Xu, Y., Liu, Q., Guan, C. & Ayrton, A. Hydrophilic anti-migraine triptans are substrates for OATP1A2, a transporter expressed at human blood-brain barrier. *Xenobiotica* **42,** 880–890 (2012).

30. Tfelt-Hansen, P. C. Does sumatriptan cross the blood-brain barrier in animals and man? *J. Headache Pain* **11,** 5–12 (2010).

31. Ek, C. J., D'Angelo, B., Lehner, C., Nathanielsz, P., Li, C. & Mallard, C. Expression of tight junction proteins and transporters for xenobiotic metabolism at the blood-CSF barrier during development in the nonhuman primate (P. hamadryas). *Reprod. Toxicol.* **56,** 32–44 (2015).

32. Dos Santos Pereira, J. N., Tadjerpisheh, S., Abu Abed, M., Saadatmand, A. R., Weksler, B., Romero, I. A., Couraud, P.-O., Brockmöller, J. & Tzvetkov, M. V. The poorly membrane permeable antipsychotic drugs amisulpride and sulpiride are substrates of the organic cation transporters from the SLC22 family. *AAPS J.* **16,** 1247–1258 (2014).

33. Wiesel, F. A., Alfredsson, G., Ehrnebo, M. & Sedvall, G. The pharmacokinetics of intravenous and oral sulpiride in healthy human subjects. *Eur. J. Clin. Pharmacol.* **17,** 385–391 (1980).

34.   Uchida, Y., Zhang, Z., Tachikawa, M. & Terasaki, T. Quantitative targeted absolute proteomics of rat blood-cerebrospinal fluid barrier transporters: comparison with a human specimen. *J. Neurochem.* (2015). doi:10.1111/jnc.13147

CHAPTER FIVE

Prediction and Validation of Enzyme and Transporter Off-Targets for Metformin

Introduction

Organic cation transporters are known to transport a variety of drugs and endogenous molecules such as serotonin and histamine[1–5]. Organic cation transporter 1 (OCT1, SLC22A1) is primarily expressed in the liver, organic cation transporter 2 (OCT2, SLC22A2) is primarily expressed in the kidney and organic cation transporter 3 (OCT3, SLC22A3) is expressed ubiquitously in various tissues[6]. All three transporters have also been detected at the blood-brain barrier using antibodies against these transporters in isolated human brain microvessel endothelial cells[7,8]. Substrates of organic cation transporters have also been found to inhibit the serotonin reuptake transporter (SERT, SLC6A4)[9], and SERT knockout mice show upregulation of OCT transporters[10], suggesting that OCTs and SERT may work in concert in the transport of organic cations[11]. In this chapter, we explore the role that organic cation transporters detected at the blood-brain barrier can play in other barrier tissues, namely, the intestine.

Metformin, a biguanide, is used worldwide as first-line therapy for the treatment of type 2 diabetes, and acts primarily in the liver to reduce gluconeogenesis[12,13]. However, 30-50% of patients on metformin report gastrointestinal (GI) side effects, such as diarrhea and bloating[14–17]. These GI side effects are generally dose-dependent, and for the majority of patients, subside after several doses. In approximately 5% of patients, these side effects are severe enough to warrant discontinuation of the drug, and these patients switch to other anti-diabetic drugs. Several hypotheses as to the causes of these metformin-induced GI side effects have been proposed, including malabsorption of bile salts in the ileum[18], increased serotonin release from human gut

mucosa[19], increased gastrointestinal hormone levels and increased gastric acid secretion[20]. A recent study showed that patients on metformin with concomitant use of other medications known to inhibit OCT1 were more likely to be metformin-intolerant, and that people carrying reduced-function mutations in OCT1 variants were also more likely to be intolerant to metformin[21].

Signaling molecules such as histamine and serotonin can bind to receptors in the GI tract to regulate normal physiologic function. Histamine receptors are highly expressed in the gastrointestinal tract. Interactions of histamine with H1 receptors regulate intestinal smooth muscle contraction and with H2 receptors stimulate gastric acid secretion. In contrast, histamine interactions with H3 receptors may inhibit gastric acid secretion, whereas histamine interactions with H4 receptors have been implicated in immune-mediated responses in gut inflammation[22–24]. Given the important roles that histamine plays in the gastrointestinal tract, it is not surprising that when histamine levels increase due to impaired histamine degradation or ingestion, GI effects such as nausea, vomiting and diarrhea can occur[25].

Most of the serotonin in the body is produced in the gastrointestinal tract, where the monoamine plays an important role in the regulation of gastrointestinal physiology[26]. Primarily stored in enterochromaffin (EC) cells of the mucosal epithelium, serotonin release activates intrinsic and extrinsic sensory GI neurons, leading to gut motility, secretion and sensation[26,27]. Defective serotonergic signaling has been implicated in a number of GI disorders, and small molecules that interact with serotonin receptors have been developed to treat GI symptoms[28]. The primary serotonin transporter, SERT, is responsible for the reuptake of extracellular serotonin in order to terminate serotonergic signaling. SERT knockout mice were observed to have increased stool water and colon motility when compared to wildtype mice[10]. Further, many

drugs, including many antidepressants that inhibit SERT cause GI adverse effects, suggesting that inhibition of the transporter may produce undesired GI effects[29].

The goal of this study was to determine the effect of metformin on modulating the uptake of histamine and serotonin through amine transporters expressed in intestinal cells. Inhibition studies were performed using metformin to determine its effect on the uptake of serotonin or histamine through OCT1, OCT3 and SERT. In addition, the Similarity Ensemble Approach (SEA)[30–34], a chemoinformatic method, predicted that metformin interacts with another protein involved in intestinal histamine disposition, diamine oxidase (DAO, ABP1). Collectively, these data suggest that metformin interacts with proteins involved in the intestinal disposition of serotonin and histamine at clinically relevant intestinal concentrations and that such interaction may ultimately contribute to the GI side effects associated with the drug.

Materials and Methods

Chemicals. All compounds (metformin, aminoguanidine, histamine, 5-hydroxytryptamine (5-HT, serotonin), putrescine, dianisidine, Tris, 2-(*N*-morpholino)ethanesulfonic Acid (MES), acetic acid, porcine diamine oxidase and peroxidase) were purchased from Sigma Aldrich (St. Louis, MO). Cell culture media (DMEM H-21), fetal bovine serum, penicillin/streptomycin, hygromycin B and Hank's Balanced Salt Solution (HBSS) were obtained from the University of California San Francisco Cell Culture Facility. [^{14}C]-Metformin (MC 2043) was purchased from Moravek Biochemicals (Brea, CA); [^{3}H]-histamine (ART 1432) and [^{3}H]-5-hydroxytryptamine (5-HT) (ART 0350) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Cell Culture. Human embryonic kidney (HEK-293) Flp-In-293 cells stably transfected with the full-length reference human OCT1 cDNA (HEK-hOCT1), OCT3 cDNA (HEK-hOCT3), and the empty vector (HEK-EV) were established previously in our laboratory[3,35,36]. Stably transfected HEK-293 cells were maintained in Dulbecco's modified eagle medium (DMEM) H-21 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, and 150 µg/mL hygromycin B. HEK-293 cells transiently transfected with the full-length reference SERT cDNA (HEK-hSERT) was established by transfecting 500 ng/well of pCMV6-XL4 vector containing the full-length transporter cDNA (NM_001045.2) from Origene (Rockville, MD) into HEK-293 cells using Lipofectamine LTX from Life Technologies (Grand Island, NY) per manufacturer instructions. The expression level of each transporter mRNA in each transfected HEK-293 cell line was verified by quantitative RT-PCR. HEK-293 cells that were used for transient transfection were cultured in the above medium but without the selection antibiotic, hygromycin B. All cell lines were grown at 37 °C in a humidified atmosphere with 5% CO_2.

Transporter Inhibition Studies. Transporter inhibition studies were performed using HEK-293 cells expressing the vector only (HEK-EV), *SLC22A1* (OCT1), *SLC22A3* (OCT3) or *SLC6A4* (SERT). Briefly, transfected cell lines were grown on poly-D-lysine coated 24-well plates in DMEM H-21 medium supplemented with 10% fetal bovine serum to at least 90% confluence (16-48 hours post seeding). For uptake studies, HEK-EV, HEK-hOCT1, HEK-hOCT3 and HEK-hSERT were preincubated in Hank's balanced salt solution (HBSS; 5.4 mmol/L potassium chloride, 0.44 mmol/L monobasic potassium phosphate, 4.2 mmol/L sodium bicarbonate, 137 mmol/L sodium chloride, 0.34 mmol/L dibasic sodium phosphate, 5.6 mmol/L D-glucose, 1.3

mmol/L calcium chloride, 0.49 mmol/L magnesium chloride, 0.41 mmol/L magnesium sulfate, pH 7.4) for 15-20 minutes. The buffer was removed and replaced with uptake buffer (HBSS containing 10 nM unlabeled histamine and a trace amount of [^{3}H]-histamine or 10 nM unlabeled serotonin and trace amount of [^{3}H]-5-hydroxytryptamine). Uptake was performed at 37 °C for a designated period of time for which linear uptake was observed, then cells were washed twice with ice-cold HBSS. To determine the inhibition of the uptake, the cells were simultaneously exposed to the substrate (histamine or serotonin) and metformin at various concentrations (0, 1, 5, 10, 15 and 30 mM). Cells were lysed by the addition of 0.1 N NaOH and 0.1% SDS, and intracellular radioactivity was determined by scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter) and normalized by protein content per well, as determined using a Pierce BCA Protein Assay Kit (Life Technologies). The transport activity of metformin and serotonin in transfected cell lines were compared to the HEK-EV cell line in every transport assay. Results were analyzed using Graphpad Prism 5.0.

Similarity Ensemble Approach (SEA). SEA (http://sea.bkslab.org/) predictions were calculated as previously described[34,37] using RDKit (http://rdkit.org) ECFP4 (Morgan) fingerprints. For the SEA target panel, subsets of ChEMBL-14 and ChEMBL-16 were used, extracted as previously described[31], and ligand structures were prepared using the ChEMBL standardiser (https://github.com/flatkinson/standardiser). Small SEA p-values (for ChEMBL-14 predictions) and expectation values (E-values; for ChEMBL-16 predictions) denote relationships between drugs and ligand sets that were stronger than would be expected by random chance alone. High maximum Tanimoto coefficients (Max Tc) reflect high pair-wise chemical structural similarities between the drug and its closest ligand neighbor annotated to the predicted target. A maximum

Tc of 1.00 means that a drug has already been reported within the ChEMBL database to bind to the predicted target.

Diamine Oxidase Assay. Metformin was tested for inhibition of porcine kidney diamine oxidase (E.C. number 1.4.3.6, Sigma-Aldrich). Stocks of the compounds were prepared in purified water or in 50 mM phosphate buffer and subsequently diluted into a three-component, constant ionic strength buffer[38] (50 mM acetic acid, 50 mM MES and 100 mM Tris final concentrations) to yield a final reaction volume of 1 mL. This buffer helped to alleviate the previously reported ionic strength effects on DAO activity[39]. In addition to the buffer, assays for diamine oxidase contained 0.15 mg/mL *O*-dianisidine, 100 pyrogallol units/mL peroxidase, 24 µUnits/mL diamine oxidase, and 0-100 mM putrescine and 0-100 mM metformin. All reagents and water were added to the cuvette and the reactions were incubated at 37 °C for 5 minutes and the reaction was initiated by the addition of diamine oxidase. Assays were performed at 37 °C, pH 7.0. Data acquisition was performed by a Hewlett Packard HP8453a spectrophotometer using kinetic mode with UV−Vis ChemStation software (Agilent Technologies). The rate of reaction was monitored spectrophotometrically at 440 nm. Activity was measured in triplicate for six different concentrations of metformin. The assay was repeated on two different days. Dose−response curves were plotted, and K_i values were calculated using Graphpad Prism 6 (Graphpad, San Diego, CA), using a sigmoidal dose−response curve analysis with variable slope. During the curve fitting process, the inhibition constant was defined as a global parameter. The K_i values reported for metformin represent the best-fit values for the respective data sets.

Results

Metformin-mediated Inhibition of Histamine and Serotonin Uptake by OCT1, OCT3 and SERT. Various concentrations of metformin were used to determine its potency in inhibiting OCT1, OCT3 and SERT in overexpressing cell lines. Figure 5.1 shows the effects of various concentrations of metformin on inhibition of transporter-mediated serotonin uptake (Figure 5.1a, c and e) and histamine uptake (Figure 5.1b and d). Among the three transporters tested, metformin inhibited OCT1 most potently, with IC_{50} of 1.46 ± 0.14 mM and 1.46 ± 0.06 mM for serotonin and histamine, respectively (Figure 5.1f). In contrast, metformin had a lower potency (<50% at 10 mM) in inhibiting monoamine uptake by OCT3 and SERT (Figure 5.1, c-e). Verapamil was used as a positive control of inhibition of OCT1, corticosterone was used as the positive control of OCT3 inhibition, and desipramine was the positive control for inhibition of SERT uptake.
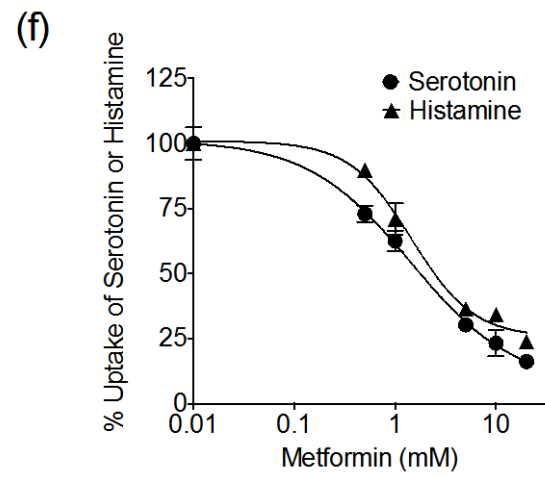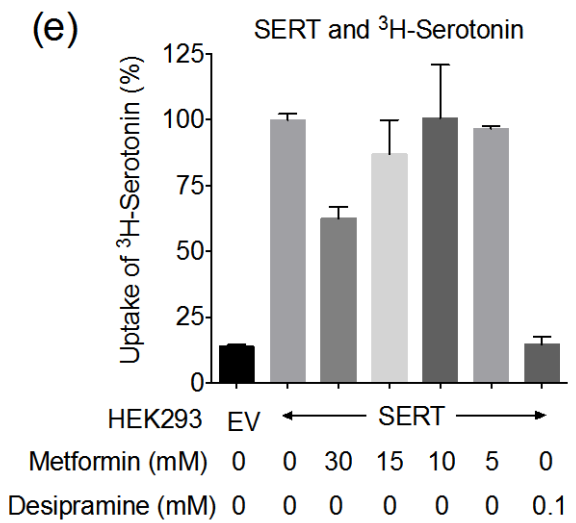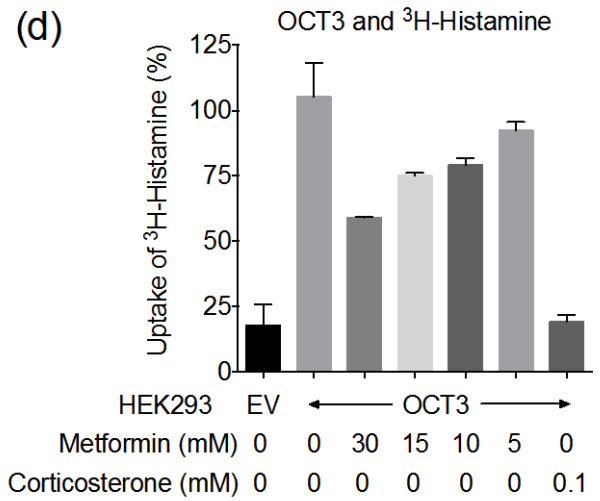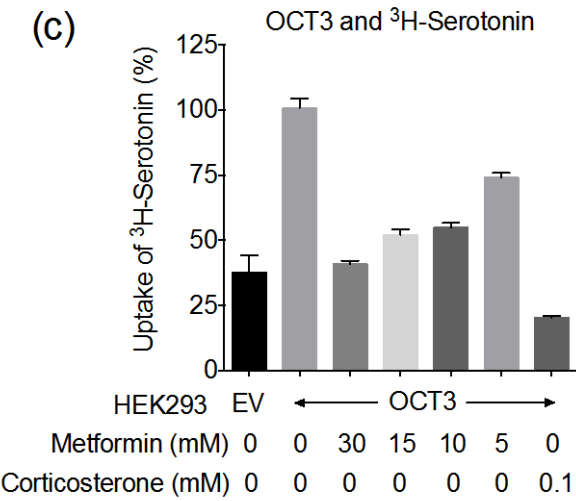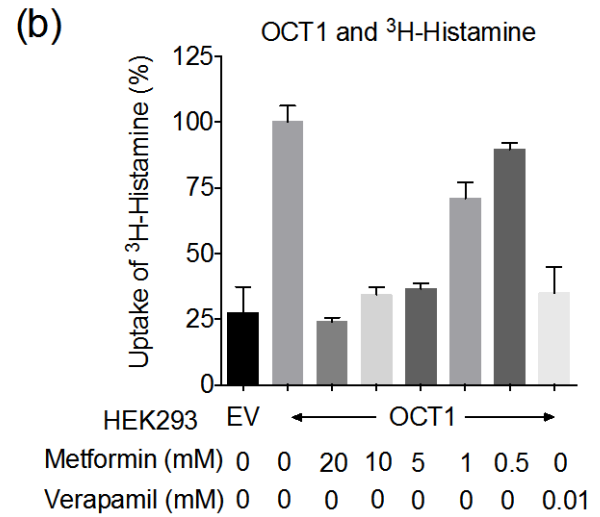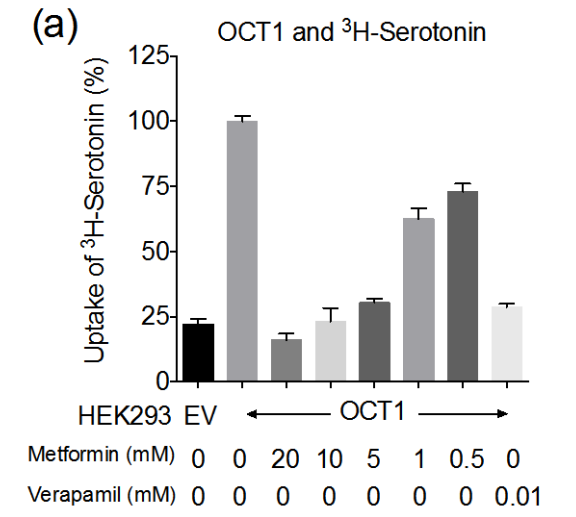
Figure 5.1. Inhibition of serotonin- and histamine-mediated uptake through OCT1, OCT3 and SERT by metformin. HEK-293 cells stably expressing OCT1 (a, b, f) or OCT3 (c, d) were used for serotonin and histamine uptake. HEK293 cells transiently expressing SERT (e) were used for serotonin uptake. Various concentrations of metformin were used for the inhibition studies and validated inhibitors of the transporter were used as controls.

Using SEA to Predict Metformin Off-targets. SEA was used to predict whether metformin might bind to any unreported molecular targets. Of the approximately 2,100 protein targets present in ChEMBL-16, SEA predicted that metformin would bind to only one: diamine oxidase (DAO, also known as ABP1). Notably, aminoguanidine has a similar structure to metformin and is a potent inhibitor of diamine oxidase (Table 5.1), but inhibition of DAO activity by metformin has not been previously reported. Diamine oxidase is highly expressed in the gut[40,41] and metabolizes polyamines such as putrescine and histamine; for the latter, it is one of only two enzymes to do so. Diamine oxidase plays an important role in degrading exogenous polyamines, and reduced levels of DAO have been associated with histamine intolerance and allergy[42–45]. The top prescription drugs predicted to target DAO were identified (Table 5.2).

Table 5.1. Metformin off-target prediction. Tc: Tanimoto coefficient. The Tc calculates the number of on bits in common between fingerprints divided by the total number of non-overlapping on bits between fingerprints.
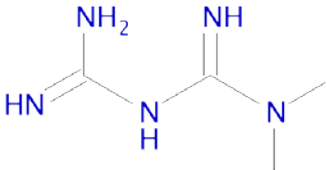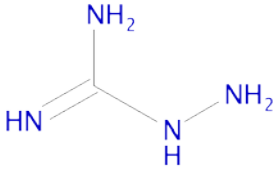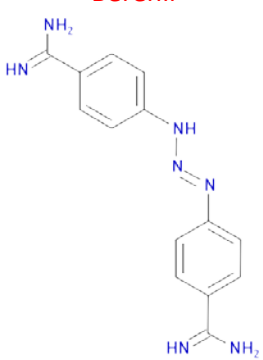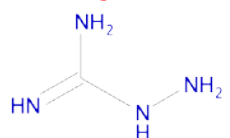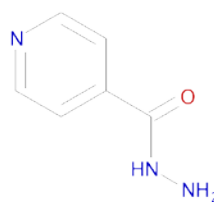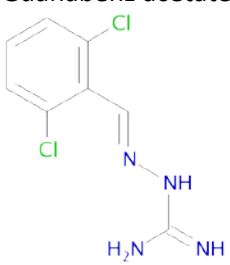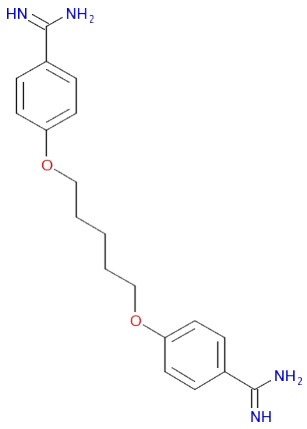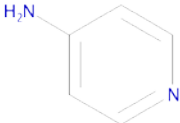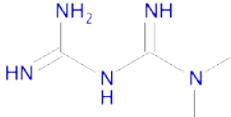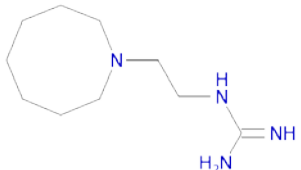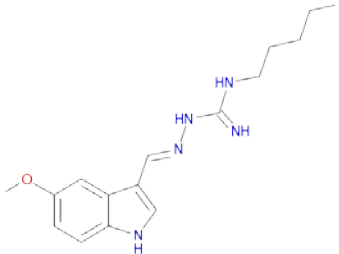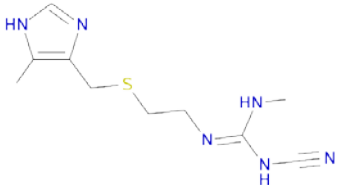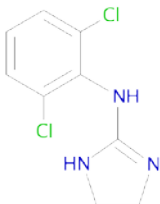
Drug	Closest Ligand for Predicted Target	Predicted Target	SEA E-value	Closest (Max) Tc
Metformin	Aminoguanidine	DAO (ABP1)	7.4×10^{-5}	0.35 Tc

Table 5.2. Top prescription drugs predicted to target DAO. Drug names in red are reported to bind DAO in the literature.

	Drug	DAO SEA E-value[1]	Affinity Bin[2] (µM)	Max Tc[3]	Literature IC$_{50}$
1	Berenil	4.4×10^{-30} (rat)[a]	10	0.36 Tc	13 ± 1 nM[b]
2	Aminoguanidine	1.8×10^{-19}	1	1.00 Tc (known in ChEMBL)	10 - 150 nM[c]
3	Isoniazid	1.1×10^{-16} [a]	1	0.28 Tc	970 ± 50 µM[b]
4	Guanabenz acetate	6.8×10^{-14} (rat)	10	0.38 Tc	5.1 ± 0.8 µM[c]

5	Pentamidine	2.2×10^{-8} (rat)[a]	10	0.29 Tc	0.29 - 3 μM[b]
6	4-Aminopyridine	4.4×10^{-5}	1	0.36 Tc	-
7	Metformin	7.4×10^{-5}	1	0.35 Tc	-
8	Guanethidine sulfate	3.5×10^{-4}	1	0.32 Tc	-
9	Tegaserod	4.8×10^{-4} (rat)	10	0.44 Tc;	-
10	Cimetidine	Not predicted	-	-	90 ± 14 μM[b]

11	Clonidine	Not predicted	-	-	100 ± 8 μM[c]

[a]Predicted using ChEMBL-14 (p-value) instead of ChEMBL-16 (E-value). [b]IC_{50} values (concentrations that inhibit DAO by 50%) were obtained from literature[46]. [c]IC_{50} values were obtained from the literature[47–52]. [1]<u>E-value</u>: This is derived from a statistical model that presents the probability of observing this raw score by random chance alone. The model is similar to that underlying BLAST[34]. The smaller the E-value, the stronger it is, and this indicates strong overall chemical structural similarity between two sets of compounds. [2]<u>Affinity Bin</u>: The ligands for each protein target that is used as a reference (e.g., DAO) are subdivided ("binned") by the log of their affinities, and a SEA calculation is run independently against each bin. Thus, when asking whether a particular compound has a strong SEA E-value for DAO, we first compute the E-value for the compound against DAO at 1 nM, DAO at 10 nM, ..., DAO at 10 μM, and finally report the affinity bin at which DAO achieves its strongest E-value for the compound of interest. This may be thought of as an approximate prediction of the compound's expected binding affinity at the target, but this interpretation has not yet been tested at scale. [3]<u>Tc</u>: Tanimoto coefficient. The Tc calculates the number of "on" bits in common between two fingerprints, divided by the total number of non-overlapping "on" bits between them. It is an overall measure of the similarity between any two molecular fingerprints, on a 0.0 (completely dissimilar) to 1.0 (completely similar) scale.

In vitro **Analysis of Diamine Oxidase Inhibition.** In an enzymatic assay, metformin inhibited porcine DAO with mixed-type inhibition, with a K_i value of 8.6 ± 3.1 mM (Figure 5.2). Meanwhile, aminoguanidine, a known inhibitor of DAO, inhibited porcine DAO >80% at 100 µM in the presence of putrescine (100 µM) as the substrate. It has been previously reported that phenformin, a biguanide and a predecessor of metformin, inhibits diamine oxidase with K_i of 4 mM[51]. Both this result and the activity of aminoguanidine support the reliability of the assay. Whereas 9 mM is a high concentration, metformin is a small molecule (MW 129.16 g/mol) and a polar drug (i.e. bearing at least one and possibly two positive charges), and concentrations higher than this are thought to be reached in the human intestine upon standard dosing[53,54].
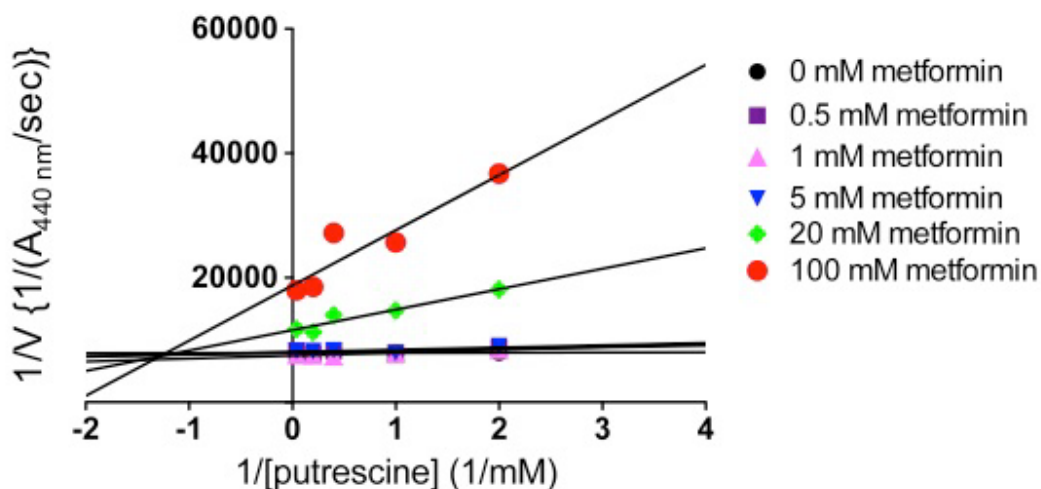

Figure 5.2. Lineweaver-Burk plot showing mixed inhibition of porcine diamine oxidase by metformin at 37 °C. A three-component, constant ionic strength buffer (MES, Tris and acetic acid, pH 7.0) was used. The K_i of metformin is 8.6 ± 3.1 mM.

Discussion

Metformin is one of the most widely prescribed drugs worldwide due to its efficacy in treating patients with type 2 diabetes. Its major adverse effects are gastrointestinal and include diarrhea, nausea, vomiting and bloating. Several studies have reported that up to 50% of patients on metformin have one or two of these symptoms[14–17], and in one recent study, Dujic *et al.* reported that approximately 5% of patients treated with metformin exhibit intolerance due to gastrointestinal symptoms that leads to discontinuation of the drug[21].

Our group and others have shown that the organic cation transporters, OCT1 and OCT3, as well as the serotonin transporter, SERT, play a role in the uptake of serotonin and histamine as well as metformin[1,3,11]. In the intestine, these transporters appear to be involved in the influx of their substrates from the lumen into the intestinal epithelial cells or the transport between the blood and intestinal epithelial cells. Thus, the transporters are thought to be important determinants of the disposition of signaling molecules (e.g., histamine and serotonin) and as such play a role in gastrointestinal physiology. Notably, both histamine and serotonin have important roles in gastrointestinal physiology and pathophysiology[25,26]. For example, extrinsic sensory neurons activated by serotonin initiate sensations from the bowel, which may lead to nausea and bloating[27,28,55]. Similarly, histamine also plays important roles in gut motility and gastrointestinal symptoms[25]. In this study, it is hypothesized that metformin inhibits intestinal transporters and enzymes known to function in the disposition of these important monoamines.

Our inhibition studies of serotonin and histamine uptake via OCT1, OCT3 and SERT showed that metformin inhibits OCT1 more potently than the other two transporters (Figure 5.1). Previously, we showed that metformin can competitively inhibit the uptake of thiamine through OCT1 (IC_{50} ~ 1.4 mM), which may lead to alterations in thiamine disposition[1]. Since the

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primary mechanisms by which serotonin and histamine are cleared are through cellular uptake and subsequent intracellular degradation, inhibition of these transporters by metformin may result in higher extracellular concentrations, particularly in the intestinal lumen, thereby enhancing or prolonging serotonergic or histaminergic signaling in the intestine. These effects may result in gastrointestinal side effects. Because metformin doses are high, its predicted concentrations in the intestinal lumen are about 10 to 20 mM; thus the drug would not have to interact potently with intestinal targets to exhibit pharmacologic effects in the intestinal tract *in vivo*[56]. Our data clearly show that at concentrations achievable in the GI tract, metformin can inhibit OCT1-mediated transport of both serotonin and histamine (Figure 5.1). Thus, though speculative, it is possible that metformin modulates levels of these monoamines in the GI tract, contributing to its adverse effects in the gut. Histamine from the diet or synthesized from L-histidine is metabolized by DAO to N-methylhistamine, which is an inactive histamine metabolite. In addition, serotonin, which is synthesized from tryptophan, is stored in enterochromaffin cells and released into the blood via the serotonin transporter. A recent study suggests that SERT is localized on the apical and basolateral membrane of enterocytes and plays a role in metformin and serotonin uptake[57]. Diamine oxidase (DAO) plays an important role in enterocytes to remove histamine and putrescine from diet. Other transporters not shown may also contribute to serotonin or histamine disposition (Figure 5.3).
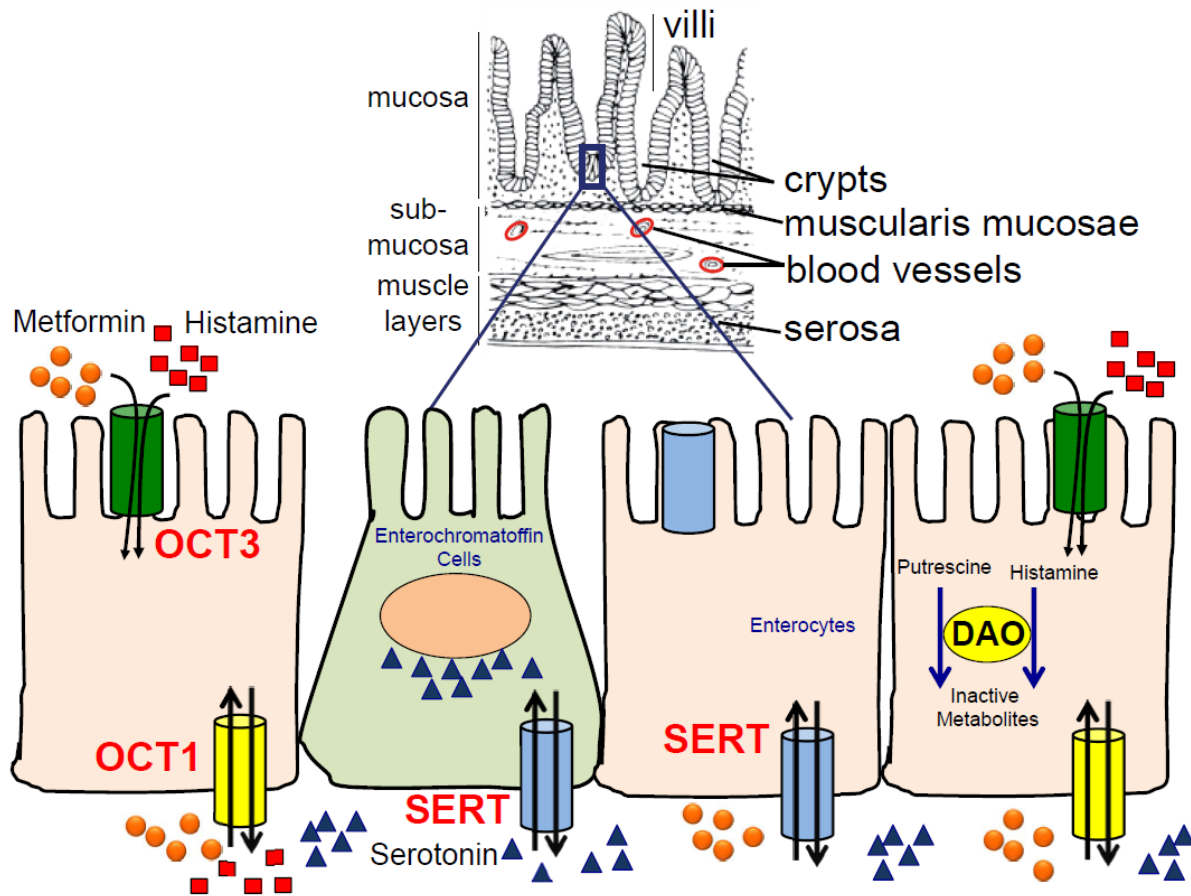
Figure 5.3. Localization of organic cation transporters (OCT1 and OCT3) and the serotonin transporter (SERT) in enterocytes and enterochromaffin cells. Histamine (red squares) from diet and metformin (orange circles) are transported into cells via OCT3. Transport of histamine, serotonin (blue triangles) or metformin across the basolateral membrane is likely mediated by OCT1 or SERT. The bidirectional arrows show that the molecules could be transported into or out of the cells depending on the concentrations of the molecules inside and outside the cells. The green cylinder represents OCT3, the yellow cylinder represents OCT1 and the blue cylinder represents SERT.

This idea is supported by a recent publication, in which patients with reduced function non-synonymous variants (R61C, G401S, 420del, G465R) in OCT1 were found to be more likely to have metformin-induced gastrointestinal side effects. Furthermore, in a large cohort of type 2 diabetic patients on metformin, it was reported that patients with concomitant use of medications known to inhibit OCT1 activity (e.g. calcium channel blockers, proton pump inhibitors, and alpha-adrenergic blockers) were more likely to be metformin intolerant[21]. Thus, it is possible that OCT1 genetic variants or concomitant use of prescription drugs that are OCT1 inhibitors may exacerbate metformin gastrointestinal side effects through effects on histamine or serotonin. Recent genomewide association studies of the human metabolome suggest an important link between OCT1 and serotonin. In particular, Shin *et al.*, observed a strong association between the reduced-function OCT1 variant, rs683369, with serotonin levels, suggesting that OCT1 is involved in the systemic disposition of serotonin[58]. Serotonin and other 5-HT$_3$ agonists through interactions with 5-HT$_3$ receptors are associated with vomiting and diarrhea in rodents[59]. Thus, through inhibition of OCT1, metformin may modulate intestinal or systemic levels of serotonin, which may contribute to the GI side effects of the drug. Metformin also inhibited serotonin uptake by OCT3 and SERT but at much higher concentrations (>30 mM to inhibit 50% of serotonin or histamine) (Figure 5.1). These transporters may also play a role in serotonin (and histamine) disposition and actions in the body. Other organic cation transporters in intestinal epithelial cells, for example, PMAT (SLC29A4), which transports monoamines[4], and MATE1 (SLC47A1) were not examined in this study.

Metformin was tested as an inhibitor of other proteins involved in histamine disposition. Beginning with a chemoinformatic approach (SEA), we predicted one target for metformin with E-value $< 10^{-4}$, the enzyme diamine oxidase (Table 5.1). It is known that DAO is involved in

metabolism of putrescine and histamine to their inactive metabolites (Figure 5.3). Previous studies have demonstrated that genetic polymorphisms in DAO and serum DAO activity are associated with food intolerance and polyamine levels[43,44,58]. Inhibition of DAO by metformin could therefore lead to aberrant histamine levels in the intestine and cause metformin-induced GI side effects. Though metformin was not as potent of an inhibitor of DAO as compared with OCT1 (IC_{50}= 8.6 mM for DAO *vs* 1.5 mM for OCT1), it is still possible that inhibition of DAO occurs *in vivo* based on its estimated intestinal concentrations (10 to 20 mM). Further studies are needed to assess the *in vivo* effects of metformin on proteins involved in the disposition of histamine and serotonin.

References

1.   Chen, L., Shu, Y., Liang, X., Chen, E. C., Yee, S. W., Zur, A. A., Li, S., Xu, L., Keshari, K. R., Lin, M. J., Chien, H. C., Zhang, Y., Morrissey, K. M., Liu, J., Ostrem, J., Younger, N. S., Kurhanewicz, J., Shokat, K. M., Ashrafi, K. & Giacomini, K. M. OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 9983–9988 (2014).

2.   Boxberger, K. H., Hagenbuch, B. & Lampe, J. N. Common drugs inhibit human organic cation transporter 1 (OCT1)-mediated neurotransmitter uptake. *Drug Metab. Dispos.* **42,** 990–995 (2014).

3.   Chen, L., Pawlikowski, B., Schlessinger, A., More, S. S., Stryke, D., Johns, S. J., Portman, M. A., Chen, E., Ferrin, T. E., Sali, A. & Giacomini, K. M. Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. *Pharmacogenet. Genomics* **20,** 687–699 (2010).

4.   Duan, H. & Wang, J. Selective transport of monoamine neurotransmitters by human plasma membrane monoamine transporter and organic cation transporter 3. *J. Pharmacol. Exp. Ther.* **335,** 743–753 (2010).

5.   Sakata, T., Anzai, N., Kimura, T., Miura, D., Fukutomi, T., Takeda, M., Sakurai, H. & Endou, H. Functional analysis of human organic cation transporter OCT3 (SLC22A3) polymorphisms. *J .Pharmacol. Sci.* **113,** 263–266 (2010).

6. Koepsell, H., Lips, K. & Volk, C. Polyspecific organic cation transporters: Structure, function, physiological roles, and biopharmaceutical implications. *Pharm. Res.* **24,** 1227–1251 (2007).

7. Lin, C.-J. J., Tai, Y., Huang, M.-T. T., Tsai, Y.-F. F., Hsu, H.-J. J., Tzen, K.-Y. Y. & Liou, H.-H. H. Cellular localization of the organic cation transporters, OCT1 and OCT2, in brain microvessel endothelial cells and its implication for MPTP transport across the blood-brain barrier and MPTP-induced dopaminergic toxicity in rodents. *J. Neurochem.* **114,** 717–727 (2010).

8. Geier, E. G., Chen, E. C., Webb, A., Papp, A. C., Yee, S. W., Sadee, W. & Giacomini, K. M. Profiling solute carrier transporters in the human blood-brain barrier. *Clin. Pharmacol. Ther.* **94,** 636–639 (2013).

9. Keating, E., Lemos, C., Monteiro, R., Azevedo, I. & Martel, F. The effect of a series of organic cations upon the plasmalemmal serotonin transporter, SERT. *Life Sci.* **76,** 103–119 (2004).

10. Chen, J. J., Li, Z., Pan, H., Murphy, D. L., Tamir, H., Koepsell, H. & Gershon, M. D. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. *J. Neurosci.* **21,** 6348–6361 (2001).

11. Han, T. K., Proctor, W. R., Costales, C. L., Cai, H., Everett, R. S. & Thakker, D. R. Four cation-selective transporters contribute to apical uptake and accumulation of metformin in Caco-2 cell monolayers. *J. Pharmacol. Exp. Ther.* **352,** 519–28 (2015).

12. Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J., Foretz, M. & Andreelli, F. Cellular and molecular mechanisms of metformin: an overview. *Clinical Science* **122,** 253–270 (2011).

13. Viollet, B. & Foretz, M. Revisiting the mechanisms of metformin action in the liver. *Ann. Endocrinol.* **74,** 123–129 (2013).

14. Dandona, P., Fonseca, V., Mier, A. & Beckett, A. G. Diarrhea and metformin in a diabetic clinic. *Diabetes Care* **6,** 472–474 (1983).

15. Krentz, A. J., Ferner, R. E. & Bailey, C. J. Comparative tolerability profiles of oral antidiabetic agents. *Drug Saf.* **11,** 223–241 (1994).

16. Diabetes Prevention Program Research Group. Long-term safety, tolerability, and weight loss associated with metformin in the diabetes prevention program outcomes study. *Diabetes Care* **35,** 731–737 (2012).

17. Bouchoucha, M., Uzzan, B. & Cohen, R. Metformin and digestive disorders. *Diabetes Metab.* **37,** 90–96 (2011).

18. Carter, D., Howlett, H. C., Wiernsperger, N. F. & Bailey, C. Effects of metformin on bile salt transport by monolayers of human intestinal Caco-2 cells. *Diabetes Obes. Metab.* **4,** 424–427 (2002).

19. Cubeddu, L. X., Bönisch, H., Göthert, M., Molderings, G., Racké, K., Ramadori, G., Miller, K. J. & Schwörer, H. Effects of metformin on intestinal 5-hydroxytryptamine (5-

HT) release and on 5-HT3 receptors. *Naunyn. Schmiedebergs Arch. Pharmacol.* **361,** 85–91 (2000).

20.   Molloy, A. M., Ardill, J. & Tomkin, G. H. The effect of metformin treatment on gastric acid secretion and gastrointestinal hormone levels in normal subjects. *Diabetologia* **19,** 93–96 (1980).

21.   Dujic, T., Zhou, K., Donnelly, L. A., Tavendale, R., Palmer, C. N. & Pearson, E. R. Association of organic cation transporter 1 with intolerance to metformin in type 2 diabetes: A GoDARTS study. *Diabetes* **64,** 1786–1793 (2015).

22.   Deiteren, A., De Man, J. G., Pelckmans, P. A. & De Winter, B. Y. Histamine H(4) receptors in the gastrointestinal tract. *Br. J. Pharmacol.* **172,** 1165–1178 (2015).

23.   Bertaccini, G. & Coruzzi, G. An update on histamine H3 receptors and gastrointestinal functions. *Dig. Dis. Sci.* **40,** 2052–2063 (1995).

24.   Leurs, R., Brozius, M. M., Smit, M. J., Bast, A. & Timmerman, H. Effects of histamine H1-, H2- and H3-receptor selective drugs on the mechanical activity of guinea-pig small and large intestine. *Br. J. Pharmacol.* **102,** 179–185 (1991).

25.   Maintz, L. & Novak, N. Histamine and histamine intolerance. *Am. J. Clin. Nutr.* **85,** 1185–1196 (2007).

26.   Mawe, G. M. & Hoffman, J. M. Serotonin signalling in the gut--functions, dysfunctions and therapeutic targets. *Nat. Rev. Gastroenterol. Hepatol.* **10,** 473–486 (2013).

27. Gershon, M. D. Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. *Aliment. Pharmacol. Ther.* **20 Suppl 7,** 3–14 (2004).

28. Gershon, M. D. & Tack, J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* **132,** 397–414 (2007).

29. Beasley Jr., C. M., Koke, S. C., Nilsson, M. E. & Gonzales, J. S. Adverse events and treatment discontinuations in clinical trials of fluoxetine in major depressive disorder: an updated meta-analysis. *Clin. Ther.* **22,** 1319–1330 (2000).

30. Cameron, R. T., Coleman, R. G., Day, J. P., Yalla, K. C., Houslay, M. D., Adams, D. R., Shoichet, B. K. & Baillie, G. S. Chemical informatics uncovers a new role for moexipril as a novel inhibitor of cAMP phosphodiesterase-4 (PDE4). *Biochem. Pharmacol.* **85,** 1297–1305 (2013).

31. Lounkine, E., Keiser, M. J., Whitebread, S., Mikhailov, D., Hamon, J., Jenkins, J. L., Lavan, P., Weber, E., Doak, A. K., Cote, S., Shoichet, B. K. & Urban, L. Large-scale prediction and testing of drug activity on side-effect targets. *Nature* **486,** 361–367 (2012).

32. Gregori-Puigjane, E., Setola, V., Hert, J., Crews, B. A., Irwin, J. J., Lounkine, E., Marnett, L., Roth, B. L. & Shoichet, B. K. Identifying mechanism-of-action targets for drugs and probes. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 11178–11183 (2012).

33. Hert, J., Keiser, M. J., Irwin, J. J., Oprea, T. I. & Shoichet, B. K. Quantifying the relationships among drug classes. *J. Chem. Inf. Model* **48,** 755–765 (2008).

34. Keiser, M. J., Roth, B. L., Armbruster, B. N., Ernsberger, P., Irwin, J. J. & Shoichet, B. K. Relating protein pharmacology by ligand chemistry. *Nat. Biotechnol.* **25,** 197–206 (2007).

35. Wittwer, M. B., Zur, A. A., Khuri, N., Kido, Y., Kosaka, A., Zhang, X., Morrissey, K. M., Sali, A., Huang, Y. & Giacomini, K. M. Discovery of potent, selective multidrug and toxin extrusion transporter 1 (MATE1, SLC47A1) inhibitors through prescription drug profiling and computational modeling. *J. Med. Chem.* **56,** 781–795 (2013).

36. Minematsu, T. & Giacomini, K. M. Interactions of tyrosine kinase inhibitors with organic cation transporters and multidrug and toxic compound extrusion proteins. *Mol. Cancer Ther.* **10,** 531–539 (2011).

37. Keiser, M. J., Setola, V., Irwin, J. J., Laggner, C., Abbas, A. I., Hufeisen, S. J., Jensen, N. H., Kuijer, M. B., Matos, R. C., Tran, T. B., Whaley, R., Glennon, R. A., Hert, J., Thomas, K. L., Edwards, D. D., Shoichet, B. K. & Roth, B. L. Predicting new molecular targets for known drugs. *Nature* **462,** 175–181 (2009).

38. Ellis, K. J. & Morrison, J. F. Buffers of constant ionic strength for studying pH-dependent processes. *Methods Enzym.* **87,** 405–426 (1982).

39. Bardsley, W. G., Crabbe, M. J. & Shindler, J. S. Kinetics of the diamine oxidase reaction. *Biochem. J.* **131,** 459–469 (1973).

40. Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A., Kampf, C., Sjostedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm,

T., Edqvist, P.-H. P., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J. M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., Ponten, F., Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm, T., Edqvist, P.-H. P., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J. M., Hamsten, M., Feilitzen, K. Von, Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., Heijne, G. Von, Nielsen, J. & Pontén, F. Tissue-based map of the human proteome. *Science (80-.).* **347,** 1260419–1260419 (2015).

41.   Schwelberger, H. G., Feurle, J. & Houen, G. New tools for studying old questions: antibodies for human diamine oxidase. *J. Neural. Transm.* **120,** 1019–1026 (2013).

42.   Shamji, M. H., Layhadi, J. A., Scadding, G. W., Cheung, D. K. M., Calderon, M. A., Turka, L. A., Phippard, D. & Durham, S. R. Basophil expression of diamine oxidase: a novel biomarker of allergen immunotherapy response. *J. Allergy Clin. Immunol.* **135,** 913–921 (2015).

43.   Music, E., Korosec, P., Silar, M., Adamic, K., Kosnik, M. & Rijavec, M. Serum diamine oxidase activity as a diagnostic test for histamine intolerance. *Wien Klin. Wochenschr.* **125,** 239–243 (2013).

44.   Maintz, L., Yu, C. F., Rodriguez, E., Baurecht, H., Bieber, T., Illig, T., Weidinger, S. & Novak, N. Association of single nucleotide polymorphisms in the diamine oxidase gene with diamine oxidase serum activities. *Allergy* **66,** 893–902 (2011).

45.   Wantke, F., Gotz, M. & Jarisch, R. Histamine-free diet: treatment of choice for histamine-induced food intolerance and supporting treatment for chronic headaches. *Clin. Exp. Allergy* **23,** 982–985 (1993).

46.   Danhof, M., Hisaoka, M. & Levy, G. Kinetics of drug action in disease states. VI. Effect of experimental diabetes on phenobarbital concentrations in rats at onset of loss of righting reflex. *J. Pharmacol. Exp. Ther.* **232,** 435–438 (1985).

47.   Hisaoka, M., Danhof, M. & Levy, G. Kinetics of drug action in disease states. VII. Effect of experimental renal dysfunction on the pharmacodynamics of ethanol in rats. *J. Pharmacol. Exp. Ther.* **232,** 717–721 (1985).

48.   Stapley, E. O., Birnbaum, J., Miller, A. K., Wallick, H., Hendlin, D. & Woodruff, H. B. Cefoxitin and cephamycins: microbiological studies. *Rev. Infect. Dis.* **1,** 73–89 (1979).

49.   Giacomini, K. M., Giacomini, J. C., Gibson, T. P. & Levy, G. Propoxyphene and norpropoxyphene plasma concentrations after oral propoxyphene in cirrhotic patients with and without surgically constructed portacaval shunt. *Clin. Pharmacol. Ther.* **28,** 417–424 (1980).

50. Gibson, T. P., Giacomini, K. M., Briggs, W. A., Whitman, W. & Levy, G. Propoxyphene and norpropoxyphene plasma concentrations in the anephric patient. *Clin. Pharmacol. Ther.* **27,** 665–670 (1980).

51. Cubria, J. C., Ordonez, D., Alvarez-Bujidos, M. L., Negro, A. & Ortiz, A. I. Inhibition of diamine oxidase from porcine kidney by pentamidine and other aminoguanidine compounds. *Comp. Biochem. Physiol. B* **100,** 543–546 (1991).

52. Pearson, W. R. An introduction to sequence similarity ('homology') searching. *Curr. Protoc. Bioinforma.* (2013). doi:10.1002/0471250953.bi0301s42

53. Wilcock, C. & Bailey, C. J. Accumulation of metformin by tissues of the normal and diabetic mouse. *Xenobiotica* **24,** 49–57 (1994).

54. Bailey, C. J., Wilcock, C. & Day, C. Effect of metformin on glucose metabolism in the splanchnic bed. *Br. J. Pharmacol.* **105,** 1009–1013 (1992).

55. Gershon, M. D. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Aliment. Pharmacol. Ther.* **13 Suppl 2,** 15–30 (1999).

56. Proctor, W. R., Bourdet, D. L. & Thakker, D. R. Mechanisms underlying saturable intestinal absorption of metformin. *Drug Metab. Dispos.* **36,** 1650–1658 (2008).

57. Han, T. K., Everett, R. S., Proctor, W. R., Ng, C. M., Costales, C. L., Brouwer, K. L. & Thakker, D. R. Organic cation transporter 1 (OCT1/mOct1) is localized in the apical membrane of Caco-2 cell monolayers and enterocytes. *Mol. Pharmacol.* **84,** 182–189 (2013).

58. Shin, S.-Y. Y., Fauman, E. B., Petersen, A.-K. K., Krumsiek, J., Santos, R., Huang, J., Arnold, M., Erte, I., Forgetta, V., Yang, T.-P. P., Walter, K., Menni, C., Chen, L., Vasquez, L., Valdes, A. M., Hyde, C. L., Wang, V., Ziemek, D., Roberts, P., Xi, L., Grundberg, E., Waldenberger, M., Richards, J. B., Mohney, R. P., Milburn, M. V, John, S. L., Trimmer, J., Theis, F. J., Overington, J. P., Suhre, K., Brosnan, M. J., Gieger, C., Kastenmüller, G., Spector, T. D., Soranzo, N., Kastenmuller, G., Spector, T. D. & Soranzo, N. An atlas of genetic influences on human blood metabolites. *Nat. Genet.* **46,** 543–550 (2014).

59. Miller, A. D. & Nonaka, S. Mechanisms of vomiting induced by serotonin-3 receptor agonists in the cat: effect of vagotomy, splanchnicectomy or area postrema lesion. *J. Pharmacol. Exp. Ther.* **260,** 509–517 (1992).

CHAPTER SIX

Identification of Novel Substrates of the Organic Anion Transporting Polypeptide 1A2

(OATP1A2)

Introduction

The blood-brain barrier (BBB) represents a major impediment for most compounds to enter the brain. The presence of tight junctions limits paracellular movement of most molecules, and the high expression levels of drug efflux transporters limit the penetration of lipophilic compounds that would ordinarily diffuse across the lipid bilayer. However, in order for the brain to obtain the nutrients it needs, uptake transporters are also highly expressed at the BBB. One family of transporters found to be expressed at the BBB is the organic anion transporting polypeptides (OATPs). OATPs have been implicated in the disposition of endogenous substrates such as bilirubin, steroid hormones and bile salts, as well as xenobiotics including fexofenadine, methotrexate[1], statins[2], triptans[3] and opioid analgesic peptides. OATPs transport a broad range of compounds, though most tend to be anionic. Their broad substrate specificity and importance in hepatic drug uptake is reflected by the fact that regulatory agencies require screening of new molecular entities against two of the transporters in this family, OATP1B1 and OATP1B3. While OATP transporters are known to be ATP- and sodium-independent, the driving force for transport is still unknown[4].

In a previous publication from our laboratory, OATP1A2 and OATP2B1 were found to be highly enriched in isolated human brain microvessels, and were the most prevalent OATPs expressed at the human BBB[5]. Both OATP1A2 and OATP2B1 have been localized to the luminal membrane of the BBB[6,7]. In a previous report, substrates of Oatp2 (Oatp1a4), a rodent

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ortholog of human OATP1A2, inhibited the brain influx of D-Penicillamine(2,5)-enkephalin (DPDPE) in mice, suggesting that Oatp2 functions as an influx transporter at the BBB[8]. Given the extent of its broad substrate specificity and high expression at the BBB, OATP1A2 and OATP2B1 may play an important role in the uptake of drugs to the brain.

In this study, a library of 103 CNS-active small molecule drugs (Table 6.1) was screened to identify novel substrates of OATP1A2 and OATP2B1, two of the most highly expressed anion transporters at the human blood-brain barrier. Twenty-four novel substrates of OATP1A2 were identified, and kinetic studies were performed to further understand their interactions with this transporter. Interestingly, none of the compounds in this library were found to be substrates of OATP2B1. As there is not a single clear rodent ortholog for each of the human OATPs, each of the 24 newly-identified substrates of OATP1A2 were tested in a rat Oatp2 cell line, and approximately one-third were also found to be substrates of Oatp2. As OATP1A2 has been previously reported to participate in the brain influx of some drugs, these data suggest that some of the novel substrates identified here enter the brain through OATP1A2.

Table 6.1. List of 103 compounds tested from CNS-active library.

Acamprosate	Diphenhydramine	Lofexidine	Phenytoin	Tadalafil
Amantadine	Donepezil	L-T-Methylphenidate	Pimozide	Talipexole
Amfebutamone	Duloxetine	Memantine	Piracetam	Thiopental
Aniracetam	Eletriptan	Meperidine	Pramipexole	Tiagabine
Aprepitant	Escitalopram	Methylphenidate	Pregabalin	Tianeptine
Aripiprazole	Ethosuximide	Metoclopramide	Propranolol	Topiramate
Atomoxetine	Felbamate	Milnacipran	Quetiapine	Tramadol
BCH	Fentanyl	Minaprine	Ramelteon	Trazodone
Bromocriptine	Flumazenil	Mirtazapine	R-Apomorphine	Triprolidine
Brotizolam	Fluoxetine	Moclobemide	Riluzole	Tropisetron
Buprenorphine	Fluphenazine	Modafinil	Rimonabant	Valproic Acid
Bupropion	Fluvoxamine	Naratriptan	Risperidone	Varenicline
Buspirone	Frovatriptan	Nemonapride	Rivastigmine	Venlafaxine
Cabergoline	Gabapentin	Nimodipine	Rizatriptan	Verapamil
Caffeine	Galantamine	Nortriptyline	Ropinirole	Vigabatrin
Carbamazepine	Gemcitabine	Olanzapine	S-Apomorphine	Vinpocetine
Chlorpromazine	Haloperidol	Oxcarbazepine	Sertindole	Zaleplon
Cidoxepin	Hydrocodone	Oxycodone	Sertraline	Ziprasidone
Clomipramine	LAAM	Paliperidone	Sulpiride	Zolmitriptan
Clozapine	Lamotrigine	Paroxetine	Sumatriptan	Zonisamide
Cyclobenzaprine	Levetiracetam	Pergolide	Tacrine	

Materials and Methods

Chemicals. In the library of 103 CNS-active compounds, 47 compounds were obtained from Pfizer (Groton, CT). The remaining 56 compounds were purchased from various sources, including Sigma Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ), Santa Cruz Biotechnology (Santa Cruz, CA), Enzo Life Sciences (Farmingdale, NY) and TCI America (Portland, OR). Cell culture media (DMEM H-21), blasticidin, GlutaMAX and the Pierce BCA Protein Assay Kit were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum, penicillin/streptomycin, non-essential amino acids (NEAA), sodium pyruvate, hygromycin B and HEPES were obtained from the University of California San Francisco Cell Culture Facility. [^{3}H]-estrone sulfate and [^{3}H]-Enkephalin (DPDPE) and was purchased from PerkinElmer (Waltham, MA).

Cell Culture. Stably-transfected and inducible HEK-hOATP1A2 and HEK-rOatp2 cells were generously provided by Pfizer, and stably-transfected CHO-OATP2B1 cells were obtained from Optivia Biotechnology (Menlo Park, CA). These cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) H-21 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, 2 mM GlutaMAX, 1 mM sodium pyruvate, 25 mM HEPES and 1X NEAA. The selection agent used was 3 µg/mL blasticidin for HEK-hOATP1A2 and HEK-rOatp2 and 100 µg/mL hygromycin B for CHO-OATP2B1. Cells were grown at 37 °C in a humidified incubator with 5% CO_2.

Substrate Screen Using LC/MS/MS Quantitation. HEK-hOATP1A2, CHO-OATP2B1 or HEK-rOatp2 cells were grown on poly-D-lysine coated 24-well plates in DMEM medium described above to at least 90% confluence at least 48 hours post-seeding. For the HEK-hOATP1A2 and HEK-rOatp2 cells, 24 hours before the experiment, cells were treated with 1 µg/mL doxycycline and 2 mM sodium butyrate to induce expression of hOATP1A2 or rOatp2. Cells were washed and preincubated for 30 minutes in HBSS buffer, pH 7.4. The buffer was removed and replaced with uptake buffer (HBSS containing 5 µM of the test compounds). Uptake was performed at 37 °C for 3 minutes, and then was terminated by washing the cells twice with ice-cold HBSS. The inhibitor used was 100 µM verapamil for OATP1A2 and OATP2B1, and 200 µM digoxin for rOatp2. Plates containing cells were dried overnight at 4°C. Samples were extracted by adding 200 µL of ice-cold methanol for 10 minutes, followed by scraping the cells with a plastic cell lifter and two 10-minute incubations with 200 µL of 70:30 methanol:water. The methanol:water solution were pooled in a 96-well plate and evaporated off in a Savant centrifugal evaporator overnight. Dried plates were sent to York Bioanalytical Solutions (York, UK) for quantitation by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Results were normalized by protein content as determined with a Pierce BCA Protein Assay Kit (Life Technologies) and analyzed using Graphpad Prism 5.0.

Determination of Kinetic Parameters Using LC/MS/MS Quantitation. HEK-hOATP1A2 cells were grown on poly-D-lysine coated 24-well plates in DMEM medium described above to at least 90% confluence at least 48 hours post-seeding. Twenty-four hours before the experiment, cells were treated with 1 µg/mL doxycycline and 2 mM sodium butyrate to induce expression of hOATP1A2. Cells were washed and preincubated for 30 minutes in HBSS buffer,

pH 7.4. The buffer was removed and replaced with uptake buffer (HBSS containing 0.7 to 1500 µM of the test compounds). Uptake was performed at 37°C for 3 minutes, and then was terminated by washing the cells twice with ice-cold HBSS. Plates containing cells were dried overnight at 4°C. Samples were extracted by adding 200 µL of ice-cold methanol for 10 minutes, followed by scraping the cells with a plastic cell lifter and two 10-minute incubations with 200 µL of 70:30 methanol:water. The methanol:water solution was pooled in a 96-well plate and evaporated off in a Savant centrifugal evaporator overnight. Dried plates were sent to York Bioanalytical Solutions (York, UK) for quantitation by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Results were normalized by protein content as determined with a Pierce BCA Protein Assay Kit (Life Technologies) and analyzed using Graphpad Prism 5.0.

Results

Validation of HEK-hOATP1A2 and CHO-OATP2B1 Cell Lines. Estrone sulfate is a known substrate of OATP transporters, and in particular, of both OATP1A2 and OATP2B1. We previously determined that uptake of [³H]-estrone sulfate in both cell lines was linear at 3 minutes and selected that as the uptake time (data not shown). In Figure 6.1A, cells treated with doxycycline and sodium butyrate to induce expression of OATP1A2 showed significantly higher uptake of [³H]-estrone sulfate, compared with cells treated with sodium butyrate only (marked as EV). Similarly, CHO-OATP2B1 cells take up more [³H]-estrone sulfate than CHO-EV cells. Treatment with 100 µM verapamil was sufficient to inhibit both OATP1A2 (Figure 6.1A) and

OATP2B1 (data not shown). These results validate use of the HEK-hOATP1A2 and CHO-OATP2B1 cell lines.
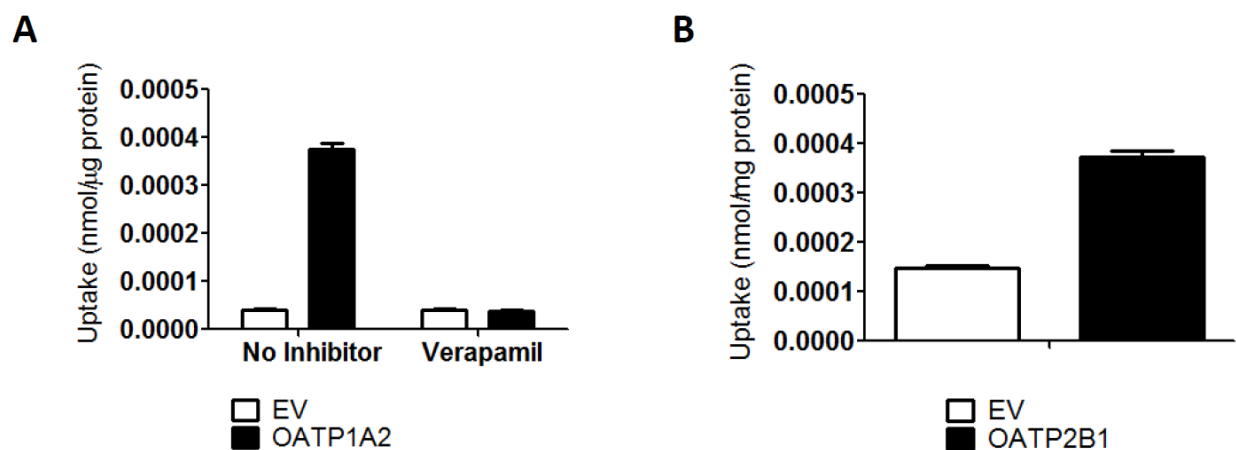

Figure 6.1. Validation of HEK-hOATP1A2 and CHO-OATP2B1 cell lines. (A) Uptake of [^{3}H]-estrone sulfate in induced HEK-hOATP1A2 cells and control (not induced) cells (EV). Verapamil (100 µM) inhibits uptake of [^{3}H]-estrone sulfate. (B) Uptake of [^{3}H]-estrone sulfate in CHO-OATP2B1 cells and control CHO-EV cells. All uptakes were performed at 3 minutes.

Identification of Novel Substrates of OATP1A2. A cellular uptake assay using HEK-hOATP1A2 cells was performed on all 103 compounds in the CNS-active library. From this library, frovatriptan, naratriptan, sumatriptan, verapamil and zolmitriptan were all previously known to be substrates of OATP1A2 and therefore served as positive controls in this screen.

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Significant uptake of all five compounds was observed in induced HEK-hOATP1A2 cells, and this uptake was inhibited by verapamil (100 μM) (Figure 6.2). In addition, twenty-four additional novel substrates of OATP1A2 were identified, including amfebutamone, R-apomorphine, buprenorphine, donepezil, escitalopram, fluvoxamine, galantamine, haloperidol, lamotrigine, lofexidine, methylphenidate, L-threo-methylphenidate, minaprine, mirtazapine, moclobemide, olanzapine, pimozide, propranolol, rivastigmine, ropinirole, sulpiride, tramadol, trazodone and varenicline (Figures 6.3, A-X).
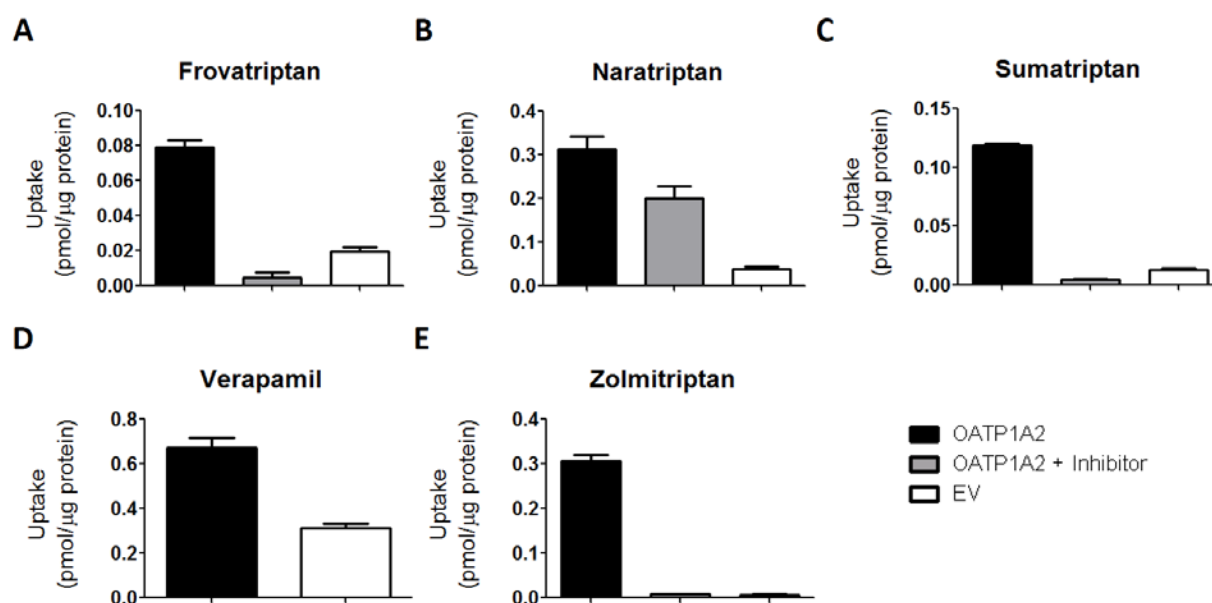
Figure 6.2. Positive controls in the OATP1A2 uptake screen. (A) Frovatriptan, (B) naratriptan, (C) sumatriptan, (D) verapamil and (E) zolmitriptan have all been previously reported to be substrates of OATP1A2. Uptake was found to be higher in induced HEK-hOATP1A2 cells as opposed to not induced control cells (EV). Addition of the OATP inhibitor verapamil resulted in significant inhibition of uptake.

A Amfebutamone
B R-Apomorphine
C Buprenorphine
D Donepezil
E Escitalopram
F Fluvoxamine
G Galantamine
H Haloperidol
I Lamotrigine
J Lofexidine
K Methylphenidate
L L-T-Methylphenidate
M Minaprine
N Mirtazepine
O Moclobemide
P Olanzapine
Q Pimozide
R Propranolol
S Rivastigmine
T Ropinirole
U Sulpiride
V Tramadol
W Trazodone
X Varenicline
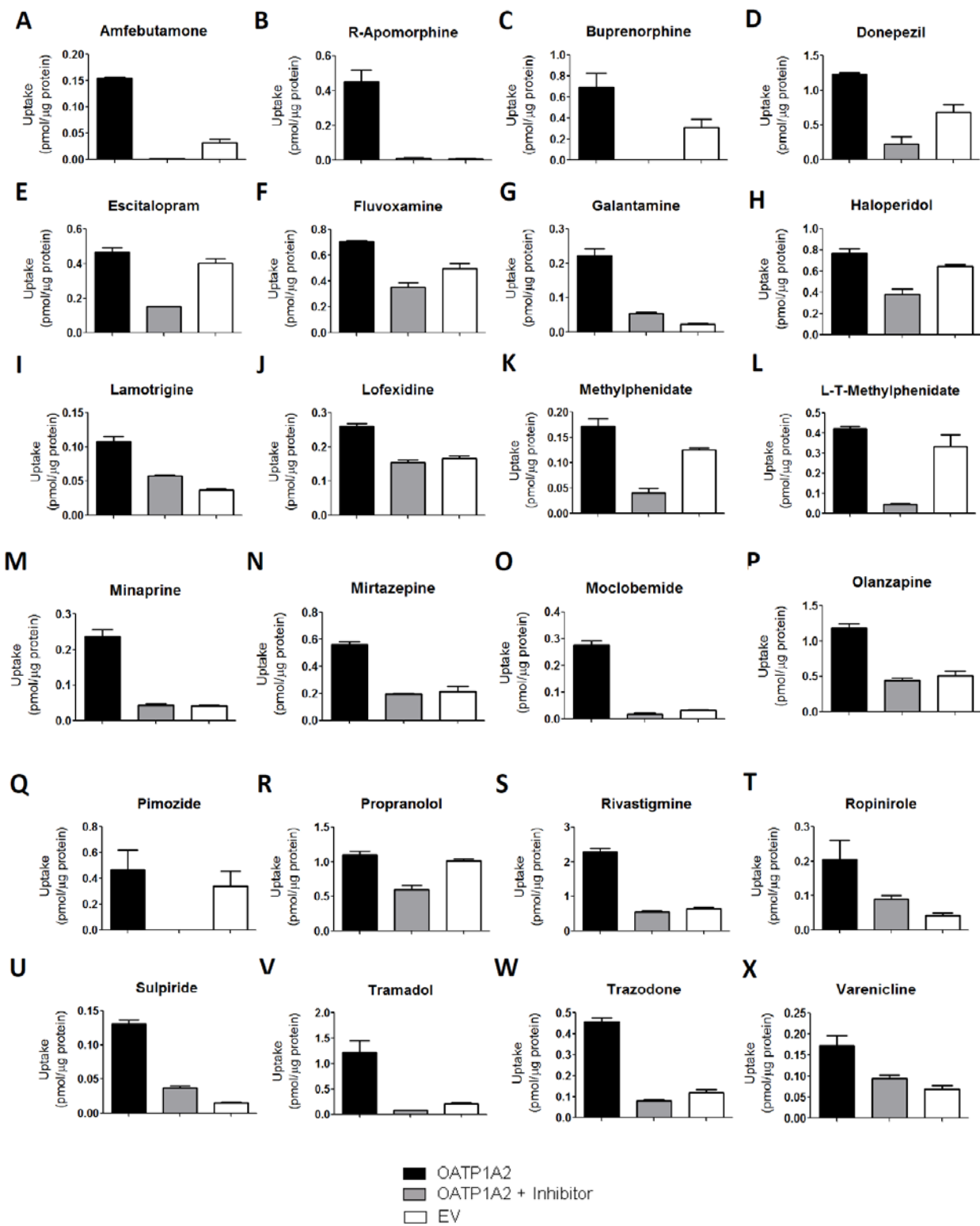
■ OATP1A2
▨ OATP1A2 + Inhibitor
□ EV

Figure 6.3. Potential substrates of OATP1A2 identified using a substrate screen. Test compounds were added to the cells at 5 µM and intracellular concentrations were determined by LC/MS/MS quantitation. Cellular uptake was found to be higher in induced OATP1A2 cells as opposed to uninduced empty vector control cells for (A) amfebutamone, (B) R-apomorphine, (C) buprenorphine, (D) donepezil, (E) escitalopram, (F) fluvoxamine, (G) galantamine, (H) haloperidol, (I) lamotrigine, (J) lofexidine, (K) methylphenidate, (L) L-threo-methylphenidate, (M) minaprine, (N) mirtazapine, (O) moclobemide, (P) olanzapine, (Q) pimozide, (R) propranolol, (S) rivastigmine, (T) ropinirole, (U) sulpiride, (V) tramadol, (W) trazodone and (X) varenicline. Addition of a known OATP1A2 inhibitor, verapamil, reduced uptake in the HEK-hOATP1A2 cell line. All uptakes were performed at 3 minutes.

Kinetic Determination of OATP1A2 Substrates. Kinetic parameters were determined for many of the compounds identified as substrates in the OATP1A2 substrate uptake screen. Figure 6.4 shows the fitted curves using the Michaelis-Menten equation and depicts both the passive and OATP1A2-mediated components of uptake for buprenorphine (Figure 6.4A), fluvoxamine (Figure 6.4B), haloperidol (Figure 6.4C), lamotrigine (Figure 6.4D), minaprine (Figure 6.4E), mirtazapine (Figure 6.5F), moclobemide (Figure 6.4G), olanzapine (Figure 6.4H), rivastigmine (Figure 6.4I), ropinirole (Figure 6.4J), tramadol (Figure 6.4K), sulpiride (Figure 6.4L), trazodone (Figure 6.4M) and varenicline (Figure 6.4N). The V_{max} and K_m for each compound for the OATP1A2 transporter are shown in Table 6.2.
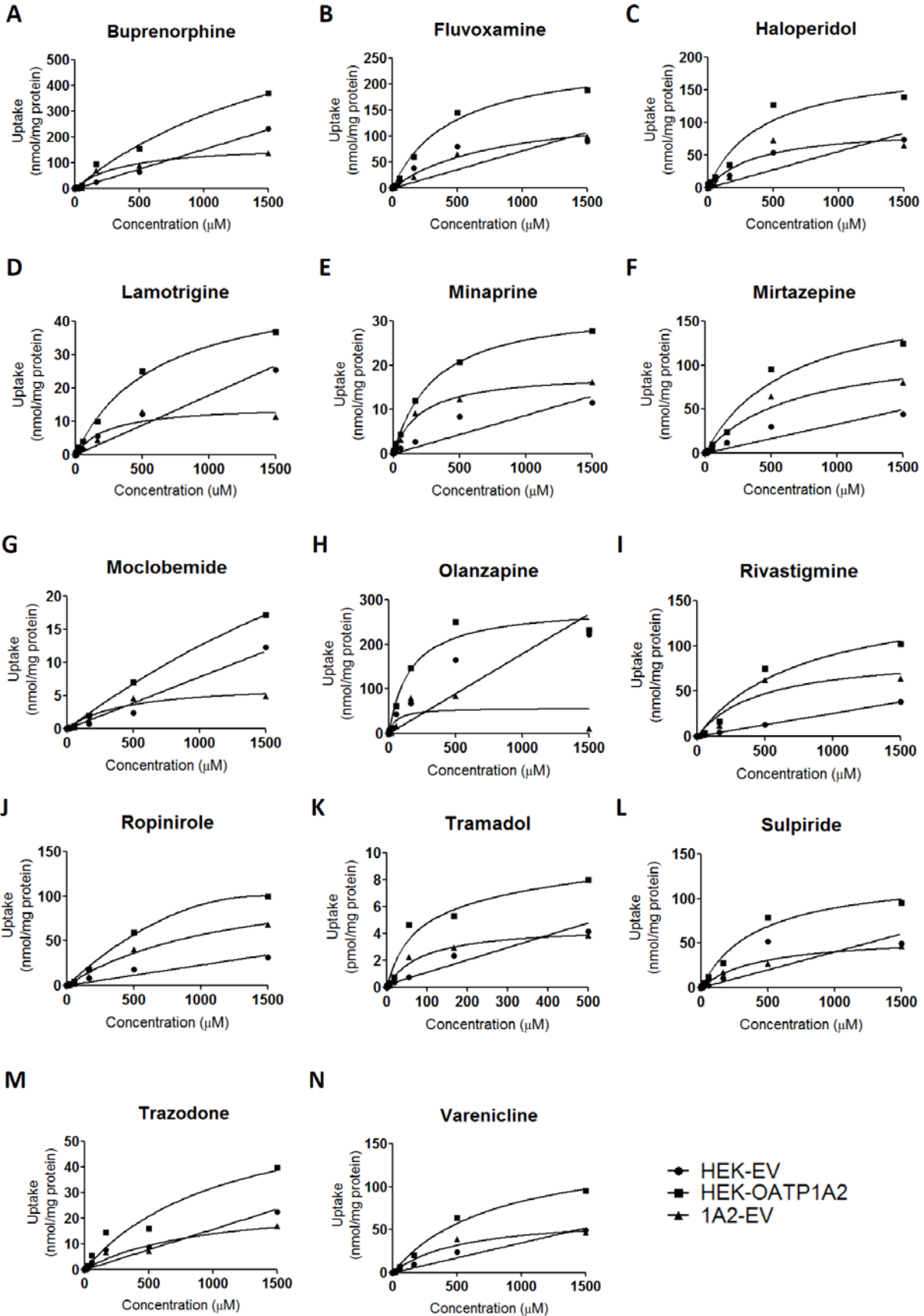
Figure 6.4. Kinetic determination of OATP1A2-mediated uptake for selected compounds. Each data point represents the average of three replicates. The Michaelis-Menten equation was used to fit linear and nonlinear regression lines representing the passive and OATP1A2-mediated uptake. Transport kinetic graphs are shown for (A) buprenorphine, (B) fluvoxamine, (C) haloperidol, (D) lamotrigine, (E) minaprine, (F) mirtazapine, (G) moclobemide, (H) olanzapine, (I) rivastigmine, (J) ropinirole, (K) tramadol, (L) sulpiride, (M) trazodone and (N) varenicline. Lines with the circle correspond to the uninduced cell line, lines with the square correspond to the total uptake in the induced HEK-hOATP1A2 cell line, and the line with the triangle correspond to the uptake in the difference between the induced HEK-hOATP1A2 and the uninduced cell line. All uptakes were performed at 3 minutes.

Table 6.2. Kinetic determination of OATP1A2-mediated uptake for selected compounds. Uptake data was fit to the Michaelis-Menten equation to determine the V_{max} and K_m of OATP1A2-mediated uptake for each compound. Uptake was performed at 3 minutes but results shown are normalized on a per minute basis.

	V_{max} (nmol/mg protein/min)	K_m (µM)
Buprenorphine	56.7 (95% CI: 136.7-203)	370 (95% CI: 173-567)
Fluvoxamine	51.7 (95% CI: 110-200)	799 (95% CI: 302-1297)
Haloperidol	30.6 (95% CI: 57.8-126)	370 (0-747)
Lamotrigine	4.9 (95% CI: 11.9-17.6)	238 (95% CI: 98-378)
Minaprine	6.1 (95% CI: 16.6-19.8)	204 (95% CI: 147-261)
Mirtazapine	40.7 (95% CI: 57.5-187)	673 (95% CI: 0-1483)
Moclobemide	2.3 (95% CI: 4.1-9.5)	432 (95% CI: 0-878)
Olanzapine	19.2 (95% CI: 25-90)	52.1 (95% CI: 0-166)
Rivastigmine	30.2 (95% CI: 54.4-127)	458 (95% CI: 0-933)
Ropinirole	42 (95% CI: 75.5-177)	1227 (95% CI: 310-2144)
Sulpiride	19.2 (95% CI: 41.2-73.8)	434 (95% CI: 116-753)
Tramadol	1.5 (95% CI: 1.6-7.2)	85.2 (95% CI: 0-245)
Trazodone	8.8 (95% CI: 15.6-40)	886 (95% CI: 140.7-1631)
Varenicline	22 (95% CI: 51.2-80.5)	529 (95% CI: 240.7-818)

Screen for Novel OATP2B1 Substrates. A cellular uptake assay was performed using CHO-OATP2B1 cells on all 103 compounds in the CNS-active library. Though one of the positive controls, rosuvastatin uptake was below the limit of quantitation of our assay in both CHO-OATP2B1 and CHO-EV cells; in contrast, increased uptake of pitavastatin in CHO-OATP2B1 cells compared with CHO-EV cells was observed (Figure 6.5). For all other compounds in this library, there was no difference in uptake in CHO-OATP2B1 cells as compared with CHO-EV cells, suggesting that none of the other compounds tested were substrates of OATP2B1.
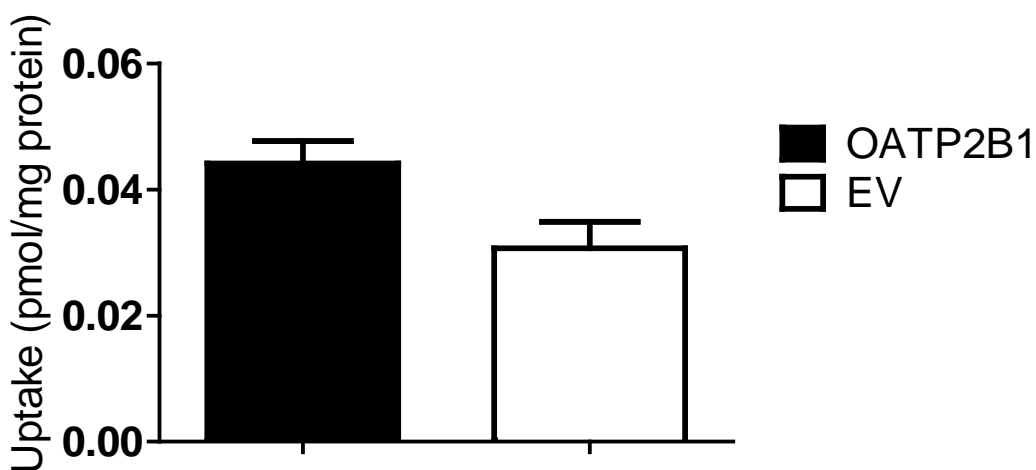

Figure 6.5. Testing of the CHO-OATP2B1 cell line. Uptake of pitavastatin was determined in the CHO-OATP2B1 and CHO-EV cell lines using LC/MS/MS. OATP2B1 cells were found to uptake more pitavastatin than CHO-EV cells, as expected.

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Human OATP1A2 Shares Some Substrate Similarity with Rodent Oatp2. To better understand the rodent orthologs of human OATP transporters, each of the 24 novel substrates identified in the OATP1A2 substrate screen was tested in a cell line overexpressing rat Oatp2. The function of the rOatp2 cell line was validated by testing uptake of a known Oatp2 substrate, DPDPE, at 5 minutes and cells that expressed Oatp2 showed increased uptake of [^{3}H]-DPDPE (Figure 6.6A). Lofexidine (Figure 6.6B), mirtazapine (Figure 6.6C), pimozide (Figure 6.6D), pyrilamine (Figure 6.6E), rivastigmine (Figure 6.6F), rocuronium (Figure 6.6G), tramadol (Figure 6.6H) and vinpocetine (Figure 6.6I) were all identified as substrates of rat Oatp2. Rocuronium (Figure 6.6G) has been previously reported to be a rOatp2 substrate and was therefore used as a positive control[9]. Furthermore, digoxin (200 µM) was able to inhibit the uptake of these compounds through rOatp2.
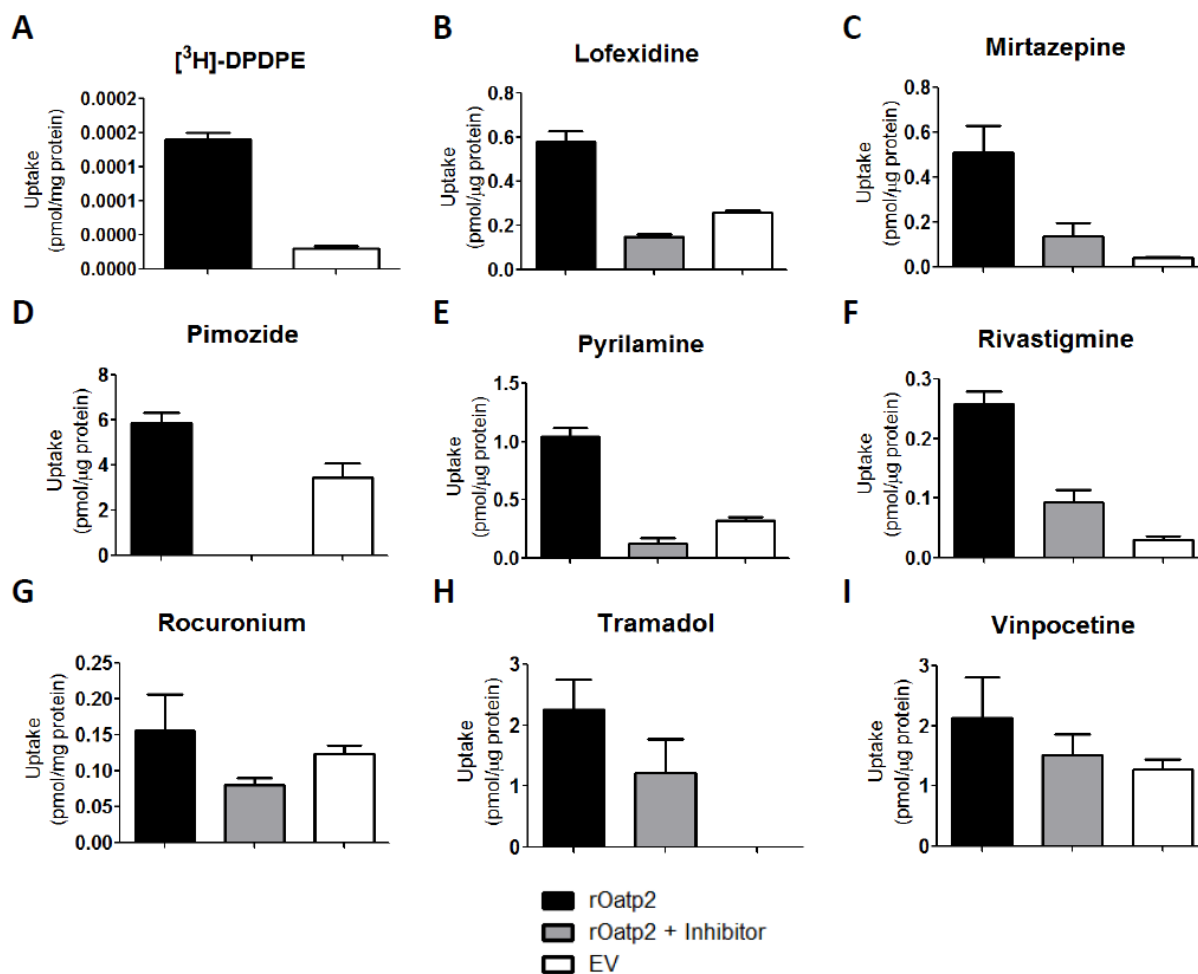
Figure 6.6. Identification of novel substrates of rOatp2. Uptake of each of the novel OATP1A2 substrates was tested in a rat Oatp2 cell line using LC/MS/MS. (A) The function of the rOatp2 cell line was validated by testing uptake of a known substrate, [3H]-DPDPE, at 5 minutes. Increased uptake of (B) lofexidine, (C) mirtazapine, (D) pimozide, (E) pyrilamine, (F) rivastigmine, (G) rocuronium, (H) tramadol and (I) vinpocetine demonstrates that these compounds are likely substrates of rOatp2. The known inhibitor of rOatp2 used was digoxin at 200 µM, which was sufficient to inhibit uptake of these nine compounds.

Discussion

Organic anion transporting polypeptides have been implicated in the transport of many drugs in different body tissues, including the brain, kidney and liver. As OATP1A2 and OATP2B1 are the most highly expressed transporters at the BBB, a library of 103 CNS-active compounds was tested to determine if some of these drugs could be taken up into the brain by either OATP1A2 or OATP2B1. The goal of this study was to identify a role for OATPs in the brain penetration of drugs and expand the chemical space of these transporters.

Consistent with a previous report[3], triptan drugs were found to be substrates of OATP1A2 (Figure 6.2). Furthermore, a surprisingly large number of other compounds in this library, 24 out of 103, were substrates of OATP1A2. These drugs included the antidepressants amfebutamone, escitalopram, fluvoxamine, minaprine, mirtazapine, moclobemide and trazodone; the Parkinson's drugs R-apomorphine, rivastigmine and ropinirole; the opioid analgesics buprenorphine and tramadol; the Alzheimer's drugs donepezil and galantamine; the antipsychotics haloperidol, olanzapine, pimozide and sulpiride; the anticonvulsant lamotrigine; the anti-addictive drugs lofexidine and varenicline; the stimulant methylphenidate and its enantiomer L-threo-methylphenidate; and the beta-blocker propranolol (Figure 6.3). Reflecting the composition of this library (61% basic, 31% neutral, 5% zwitterionic), it was observed that of the 24 novel substrates, most were basic drugs with the exception of the neutral drugs amfebutamone, lamotrigine, minaprine and moclobemide. Though a recognized transporter for anionic drugs, OATP1A2 has been previously found to transport some basic drugs[9].

The opioid analgesic tramadol has been reported to have wide variability in its pharmacokinetic and pharmacodynamics properties, even within subgroups categorized by CYP2D6 activity, suggesting the involvement of other genes in explaining this effect. As

tramadol is almost completely protonated at physiological pH (pKa 9.6), its uptake into the brain is likely dependent on uptake transporters at the BBB; however, passive diffusion of the unionized species may also contribute to its brain uptake. Furthermore, tramadol has been found to accumulate in the brain with a $K_{p,uu}$ value of 2.25[10], and is not a substrate of P-glycoprotein (P-gp)[11]. However, previous attempts to identify the transporter(s) responsible for its uptake have not been successful; a recent literature review found no studies that reported a correlation between tramadol's pharmacologic effect and polymorphisms in the transporters P-gp, OCT1, SERT, or NET[12]. One study found a significant efflux ratio of the two tramadol enantiomers (2.2 and 1.9) at pH 6.8, but this effect was abolished at pH 7.4[11]. We previously reported that OATP1A2-mediated uptake of organic anions is pH-dependent, with increased activity at lower pH[1]. Therefore, OATP1A2 may play a role in tramadol's brain penetration. In this study, tramadol was found to be one of the best substrates of OATP1A2 of all 24 compounds, with a K_m of 85.2 µM and V_{max} of 1.5 nmol/mg protein/minute.

The entire CNS-active drug library was also screened against OATP2B1, another OATP transporter highly expressed at the BBB. No new substrates of OATP2B1 were identified from this CNS-active drug library. A previous report found that OATP2B1 is a low-affinity, highly selective, low pH transporter of antifolate compounds[13]. However, the authors note that had OATP2B1 been screened at neutral pH, no transport activity would have been detected for any of the folate compounds tested. This may explain, in part, why no novel substrates of OATP2B1 were identified in this screen. That is, this screen was intended to identify substrates of the transporter that function at pH 7.4. Furthermore, OATP2B1-mediated BSP transport is entirely independent of extracellular pH over a range of 5.5 to 7.4, and BSP has the highest affinity for

OATP2B1[13]. These data suggest that screening the transporter will yield different results depending on the pH used in the screen.

A significant amount of gene duplication and divergence has occurred in the OATP family such that the human OATPs do not always have a direct ortholog in rodents. This is especially true for human OATP1A2, which has five rodent orthologs- Oatp1a1, Oatp1a3 (rat only), Oatp1a4 and Oatp1a5[4]. The closest rat ortholog, Oatp2 (Oatp1a4) shares only 72% gene homology with OATP1A2[14]. Additional studies have shown that for transporters of this family, the transport properties cannot be predicted from amino acid sequence similarities between human and rodent[15]. Therefore, each of the novel substrates of OATP1A2 was tested in a rOatp2 cell line to determine the species-dependent differences between human OATP1A2 and rat Oatp2 (Figure 6.6). Some species-specific differences between OATP1A2 and rOatp2 were observed with only 33% overlap between substrates of the two transporters; lofexidine, tramadol, rivastigmine, mirtazapine, rocuronium, pimozide, pyrilamine and vinpocetine were also identified as substrates of rOatp2 whereas the remaining 16 novel substrates of OATP1A2 were not found to be substrates of rOatp2. Further testing is necessary to determine the differences in the substrate specificity and transport activity of the OATP1A2 and rOatp2 transporters.

In conclusion, twenty-four novel substrates of OATP1A2 that are CNS-active drugs have been identified, and OATP1A2 may be the mechanism by which these drugs enter the brain. Kinetic values for several of these drugs for OATP1A2 are reported here, and each drug was also tested in a cell line that overexpresses rat Oatp2, the closest rodent ortholog. These findings suggest a role for OATP1A2 in mediating the influx of CNS-active drugs into the brain, and enhance our understanding of the substrate specificity of several members of the organic anion transporting polypeptide family.

References

1.   Badagnani, I., Castro, R. A., Taylor, T. R., Brett, C. M., Huang, C. C., Stryke, D., Kawamoto, M., Johns, S. J., Ferrin, T. E., Carlson, E. J., Burchard, E. G. & Giacomini, K. M. Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J. Pharmacol. Exp. Ther.* **318,** 521–529 (2006).

2.   Hagenbuch, B. & Stieger, B. The SLCO (former SLC21) superfamily of transporters. *Mol. Aspects Med.* **34,** 396–412 (2013).

3.   Cheng, Z., Liu, H., Yu, N., Wang, F., An, G., Xu, Y., Liu, Q., Guan, C. & Ayrton, A. Hydrophilic anti-migraine triptans are substrates for OATP1A2, a transporter expressed at human blood-brain barrier. *Xenobiotica* **42,** 880–890 (2012).

4.   Roth, M., Obaidat, A. & Hagenbuch, B. OATPs, OATs and OCTs: The organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br. J. Pharmacol.* **165,** 1260–1287 (2012).

5.   Geier, E. G., Chen, E. C., Webb, A., Papp, A. C., Yee, S. W., Sadee, W. & Giacomini, K. M. Profiling solute carrier transporters in the human blood-brain barrier. *Clin. Pharmacol. Ther.* **94,** 636–639 (2013).

6.   Gao, B., Hagenbuch, B., Kullak-Ublick, G. A., Benke, D., Aguzzi, A. & Meier, P. J. Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *J. Pharmacol. Exp. Ther.* **294,** 73–79 (2000).

7. Bronger, H., König, J., Kopplow, K., Steiner, H.-H. H., Ahmadi, R., Herold-Mende, C., Keppler, D. & Nies, A. T. ABCC drug efflux pumps and organic anion uptake transporters in human gliomas and the blood-tumor barrier. *Cancer Res.* **65,** 11419–11428 (2005).

8. Dagenais, C., Ducharme, J. & Pollack, G. M. Uptake and efflux of the peptidic delta-opioid receptor agonist [D-penicillamine2,5]-enkephalin at the murine blood-brain barrier by in situ perfusion. *Neurosci. Lett.* **301,** 155–158 (2001).

9. Van Montfoort, J. E., Hagenbuch, B., Fattinger, K. E., Müller, M., Groothuis, G. M., Meijer, D. K. & Meier, P. J. Polyspecific organic anion transporting polypeptides mediate hepatic uptake of amphipathic type II organic cations. *J. Pharmacol. Exp. Ther.* **291,** 147–152 (1999).

10. Fridén, M., Bergström, F., Wan, H., Rehngren, M., Ahlin, G., Hammarlund-Udenaes, M. & Bredberg, U. Measurement of unbound drug exposure in brain: Modeling of pH partitioning explains diverging results between the brain slice and brain homogenate methods. *Drug Metab. Dispos.* **39,** 353–362 (2011).

11. Kanaan, M., Daali, Y., Dayer, P. & Desmeules, J. Uptake/efflux transport of tramadol enantiomers and O-desmethyl-tramadol: Focus on P-glycoprotein. *Basic Clin. Pharmacol. Toxicol.* **105,** 199–206 (2009).

12. Lassen, D., Damkier, P. & Brøsen, K. The pharmacogenetics of tramadol. *Clin. Pharmacokinet.* (2015). doi:10.1007/s40262-015-0268-0

13. Visentin, M., Chang, M.-H., Romero, M. F., Zhao, R. & Goldman, I. D. Substrate- and pH-specific antifolate transport mediated by organic anion-transporting polypeptide 2B1 (OATP2B1-SLCO2B1). *Mol. Pharmacol.* **81,** 134–142 (2012).

14. Liu, H., Yu, N., Lu, S., Ito, S., Zhang, X., Prasad, B., He, E., Lu, X., Li, Y., Wang, F., Xu, H., An, G., Unadkat, J. D., Kusuhara, H., Sugiyama, Y. & Sahi, J. Solute carrier family of the organic anion-transporting polypeptides 1A2- Madin-Darby canine kidney II: A promising in vitro system to understand the role of organic anion-transporting polypeptide 1A2 in blood-brain barrier drug penetration. *Drug Metab. Dispos.* **43,** 1008–1018 (2015).

15. Kullak-Ublick, G. A., Ismair, M. G., Stieger, B., Landmann, L., Huber, R., Pizzagalli, F., Fattinger, K., Meier, P. J. & Hagenbuch, B. Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120,** 525–533 (2001).

CHAPTER SEVEN

Conclusions

Membrane transporters play critical roles in human physiology and pathology. In addition, transporters play important pharmacological roles, and for the last two decades, there has been substantial interest in determining the role of transporters in the absorption, distribution and elimination of drugs and other xenobiotics. Regulatory agencies have been increasingly interested in transporter-mediated drug-drug interactions (DDIs), and have released guidance documents that require *in vitro* DDI studies for new molecular entities that are likely to interact with hepatic and renal transporters- starting with the US Food and Drug Administration in 2012, the European Medicines Agency in 2013, and the Japanese Ministry of Health, Labour, and Welfare in 2014. It is therefore essential that current knowledge of transporter biology be expanded as transporters are identified as increasingly important in drug development.

Both the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) limit access of drugs to the brain, and feature tight junctions with enhanced expression of drug metabolizing enzymes and transporters. Therefore, compounds generally can only transverse the barrier by passive diffusion in the case of small lipophilic molecules, or carrier-mediated transport. While ATP-binding cassette (ABC) transporters usually participate in the active efflux of compounds from the brain, solute carrier (SLC) transporters often play important roles in the influx of molecules into the brain. In this dissertation, we focused on the role of SLC transporters in the influx of compounds into the brain and other tissues.

SLC transporters at the BBB and BCSFB have been previously demonstrated to participate in the brain influx of levodopa[1], gabapentin[2], valproic acid[3], triptans[4], statins[5], H1

antagonists[6], and various acidic drugs[7]. Furthermore, previous work in our laboratory identified novel transporters at the blood-brain barrier[8]. However, many questions regarding the role of SLC transporters at the blood-brain barrier remain unanswered. The exact mechanism through which many central nervous system (CNS)-active drugs cross the BBB is still unknown, and some drugs such as fentanyl[9] and pentazocine[10] show high brain penetration despite active efflux by P-glycoprotein, suggesting the presence of a high-efficiency influx transporter yet to be identified.

The study of membrane transporter proteins has historically been challenging due to its membrane localization, amphipathic characteristics, and relatively low abundance in various tissues. However, the development of molecular cloning techniques has helped to establish essential tools for the study of these transporters. Whereas there have been several studies that have used high-throughput screening techniques to identify novel inhibitors of key transporters, few research groups have attempted screening for novel substrates of these transporters, likely due to technical and cost limitations. The primary goal of this research was to identify new substrates and inhibitors of SLC transporters previously identified at the blood-brain barrier. Below a summary of the key findings in each of the chapters is presented together with a short perspective of future research.

Chapter 2: Solute Carrier Transporters as Therapeutic Targets

Chapter 2 focused on targeting solute carrier (SLC) transporters to treat human disease. Though much of the literature in transporter pharmacology has centered on the role of transporters in pharmacokinetics and drug-drug interactions, recent studies suggest that transporters represent an exciting group of proteins that can be targeted by pharmacologic agents

to treat human disease. In particular, studies of Mendelian diseases caused by defects in SLC transporters have revealed new information about the function of many of these transporters, and genome-wide association studies have implicated transporters in various diseases. At present, several classes of marketed drugs target SLC transporters as their primary mechanism of action, including loop and thiazide diuretics, serotonin and/or noradrenaline selective reuptake inhibitor antidepressants and recently approved SGLT2 inhibitors for the treatment of type 2 diabetes. Clearly, there are major opportunities in developing drugs that target transporters for a wide variety of indications, such as gout, neurological disorders, liver disease and cancer. Imaging agents that utilize SLC transporters may also have clinical utility in the diagnosis of Parkinson's disease and Alzheimer's disease. Drugs that target SLC transporters may inhibit the transporter, enhance the transport function, circumvent the defective transporter, or restore function of a defective transporter through gene therapy. Future research focused on targeting SLCs is needed to broaden the therapeutic armamentarium for the prevention and treatment of human disease.

Chapter 3: Identification of Novel Ligands of the L-Type Amino Acid Transporter 1 (LAT1)

Amino acids are essential to the function of the human body, and one of the major transport systems for large neutral amino acids is LAT1. In addition to being overexpressed in various cancers, LAT1 expression has also been found to be enriched at the blood-brain barrier. We screened a library of 115 CNS-active drugs using a LAT1-overexpressing cell line to identify novel ligands of this transporter. We identified four new, structurally-diverse inhibitors of LAT1 and performed kinetic studies to determine their potency of LAT1 inhibition. The antidepressant drugs duloxetine, paroxetine and clomipramine were found to inhibit LAT1 with an IC_{50} of 578

µM, 481 µM and 383 µM, respectively, and the antipsychotic drug fluphenazine was found to inhibit LAT1 with an IC$_{50}$ of 725 µM. Though these concentrations are too high to produce inhibition of LAT1 at the BBB *in vivo*, the finding that these drugs inhibit LAT1 suggests that the transporter has a much greater chemical space than previously anticipated. Clearly, future research to discover new ligands for LAT1 may greatly enhance treatment of CNS disorders.

Chapter 4: Identification of novel substrates of the organic cation transporter, MATE1

Organic cation transporters are known to participate in the absorption, distribution and elimination of various drugs in a variety of tissues, notably in the kidney and liver. MATE1 has been shown to play an important role in the kidney in mediating the elimination of various drugs, and drug-drug interactions have been demonstrated clinically when multiple drugs that inhibit MATE1 are taken concurrently. In this study, we screened a library of 86 CNS-active drugs and identified multiple novel substrates of MATE1, including bromocriptine, buprenorphine, caffeine, chlorpromazine, cyclobenzaprine, diphenhydramine, frovatriptan, levetiracetam, riluzole, rizatriptan, rocuronium, sulpiride, sumatriptan, talipexole, tramadol, triprolidine, valproic acid and zolmitriptan. We also show that the atypical antipsychotic drug sulpiride is taken up by MATE1 with a V$_{max}$ of 0.213 µmol/min/µg protein and K$_m$ of 7.6 µM. These results suggest that MATE1 may be involved in the renal clearance and drug distribution of many of these drugs in tissues where MATE1 is expressed, including the blood-brain and blood-cerebrospinal fluid barriers. Importantly, our study demonstrates that the substrate space for MATE1 is larger than previously anticipated and that targeting the transporter with a variety of ligands including anions, cations and zwitterions is achievable. Future studies should focus on

identifying the precise role of MATE1 in both the BBB and the BCSFB so that the transporter may be exploited in the development of new therapeutic agents.

Chapter 5: Prediction and Validation of Enzyme and Transporter Off-Targets for Metformin

Metformin is an established first-line treatment for patients with type 2 diabetes. Despite its beneficial effects, metformin causes gastrointestinal (GI) side effects that limit the use of the drug. Histamine and serotonin have potent effects in the GI tract. Using chemoinformatic and experimental methods, we show that metformin interacts with amine transporters that transport histamine and serotonin (OCT1, OCT3 and SERT) and intestinal diamine oxidase (DAO), which plays a role in histamine disposition. We found that metformin inhibited histamine and serotonin uptake by OCT1, OCT3 and SERT in a dose-dependent manner, with inhibition of OCT1-mediated histamine uptake being most potent (K_i = 1.5 mM). Using Similarity Ensemble Approach, we predicted that metformin interacts with diamine oxidase, which was validated experimentally *in vitro*. The K_i of metformin for DAO (8.6 ± 3.1 mM) was within the expected range of intestinal concentrations of metformin after therapeutic doses. These results demonstrate that metformin inhibits intestinal amine transporters and DAO, and suggest a possible mechanism for metformin-associated side effects. Clearly, future research should focus on determining whether the inhibition of organic cation transporters or DAO in the intestinal tract is important in the gastrointestinal side effects of the drug clinically.

Chapter 6: Identification of Novel Substrates of the Organic Anion Transporting Polypeptide 1A2 (OATP1A2)

The blood-brain barrier (BBB) represents a significant barrier for drugs to cross from systemic circulation into the brain. However, uptake transporters including members of the organic anion transporting polypeptide family have been localized at the BBB and are known to transport drugs and other xenobiotics. Screening a library of 103 CNS-active drugs against both OATP1A2 and OATP2B1, we identified 24 novel substrates of OATP1A2, including amfebutamone, R-apomorphine, buprenorphine, donepezil, escitalopram, fluvoxamine, galantamine, haloperidol, lamotrigine, lofexidine, methylphenidate, L-threo-methylphenidate, minaprine, mirtazapine, moclobemide, olanzapine, pimozide, propranolol, rivastigmine, ropinirole, sulpiride, tramadol, trazodone and varenicline. Many of these novel substrates are cationic drugs, even though OATP1A2 is known primarily as an anion transporter. We also determined kinetic parameters (K_m and V_{max}) of several of these drugs for OATP1A2. Surprisingly, none of the compounds in our library were substrates of OATP2B1. All new OATP1A2 substrates were also tested as substrates of the closest rodent ortholog, Oatp2, and roughly a third were substrates of both transporters. These results provide further insight into the substrate specificity of OATP transporters and implicate OATP1A2 in the brain uptake of several drugs.

Challenges and Future Directions

The work performed in this dissertation has dramatically expanded knowledge of solute carrier transporters, particularly by identifying many novel substrates of transporters already known to be important in the body. We have observed that identification of inhibitors using

high-throughput screening methodologies, as previously reported, is not sufficient in understanding the full chemical space of the transporter, as there is often not a clear overlap between compounds identified as inhibitors and compounds that are substrates. Therefore, new methodologies need to be developed that allow for the fast and efficient identification of novel substrates of these transporters.

We have identified multiple new substrates for several SLC transporters, and expanded their known chemical space. As most of the studies that we have performed utilized transporter-overexpressing cell lines, we are unable to fully comment on the *in vivo* relevance of these findings, especially as it is known that in many tissues multiple transporters often work together, as in the case of OCTs and MATEs[11]. We propose new mechanisms of tissue distribution and elimination for a number of CNS-active drugs, but additional studies are still needed to validate our findings.

Within the past 10 years, nine new SLC families have been identified and nearly 100 new human SLC genes have been recognized. Presently, crystal structures of transporters represent less than one percent of available 3D-resolved structures in the Protein Data Bank[12], but crystal structures and molecular models of SLC transporters are increasingly available[13,14]. We believe that the number of substrates identified for various transporters will continue to grow in the coming years, especially as computational methodologies improve. Furthermore, as our knowledge of transporters increases, we expect that regulatory agencies will require additional transporter studies of new molecular entities, and that the pharmaceutical companies will begin to develop novel, first-in-class drugs that target transporters for the treatment of human disease.

References

1. Uchino, H., Kanai, Y., Kim, D. K., Wempe, M. F., Chairoungdua, A., Morimoto, E., Anders, M. W. & Endou, H. Transport of amino acid-related compounds mediated by L-type amino acid transporter 1 (LAT1): insights into the mechanisms of substrate recognition. *Mol. Pharmacol.* **61,** 729–737 (2002).

2. Dickens, D., Webb, S. D., Antonyuk, S., Giannoudis, A., Owen, A., Rädisch, S., Hasnain, S. S. & Pirmohamed, M. Transport of gabapentin by LAT1 (SLC7A5). *Biochem. Pharmacol.* **85,** 1672–1683 (2013).

3. Adkison, K. D. & Shen, D. D. Uptake of valproic acid into rat brain is mediated by a medium-chain fatty acid transporter. *J. Pharmacol. Exp. Ther.* **276,** 1189–1200 (1996).

4. Cheng, Z., Liu, H., Yu, N., Wang, F., An, G., Xu, Y., Liu, Q., Guan, C. & Ayrton, A. Hydrophilic anti-migraine triptans are substrates for OATP1A2, a transporter expressed at human blood-brain barrier. *Xenobiotica* **42,** 880–890 (2012).

5. Tsuji, A., Saheki, A., Tamai, I. & Terasaki, T. Transport mechanism of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors at the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **267,** 1085–1090 (1993).

6. Yamazaki, M., Terasaki, T., Yoshioka, K., Nagata, O., Kato, H., Ito, Y. & Tsuji, A. Carrier-mediated transport of H1-antagonist at the blood-brain barrier: A common transport system of H1-antagonists and lipophilic basic drugs. *Pharm. Res.* **11,** 1516–1518 (1994).

7.   Kang, Y. S., Terasaki, T. & Tsuji, A. Acidic drug transport in vivo through the blood-brain barrier. A role of the transport carrier for monocarboxylic acids. *J. Pharmacobiodyn.* **13,** 158–163 (1990).

8.   Geier, E. G., Chen, E. C., Webb, A., Papp, A. C., Yee, S. W., Sadee, W. & Giacomini, K. M. Profiling solute carrier transporters in the human blood-brain barrier. *Clin. Pharmacol. Ther.* **94,** 636–639 (2013).

9.   Henthorn, T. K., Liu, Y., Mahapatro, M. & Ng, K. Y. Active transport of fentanyl by the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **289,** 1084–1089 (1999).

10.   Moriki, Y., Suzuki, T., Fukami, T., Hanano, M., Tomono, K. & Watanabe, J. Involvement of P-glycoprotein in blood-brain barrier transport of pentazocine in rats using brain uptake index method. *Biol. Pharm. Bull.* **27,** 932–935 (2004).

11.   Motohashi, H. & Inui, K. I. Multidrug and toxin extrusion family SLC47: Physiological, pharmacokinetic and toxicokinetic importance of MATE1 and MATE2-K. *Mol. Aspects Med.* **34,** 661–668 (2013).

12.   Hediger, M. A., Clémençon, B., Burrier, R. E. & Bruford, E. A. The ABCs of membrane transporters in health and disease (SLC series): Introduction. *Mol. Aspects Med.* **34,** 95–107 (2013).

13.   Schlessinger, A., Matsson, P., Shima, J. E., Pieper, U., Yee, S. W., Kelly, L., Apeltsin, L., Stroud, R. M., Ferrin, T. E., Giacomini, K. M. & Sali, A. Comparison of human solute carriers. *Protein Sci.* **19,** 412–428 (2010).

14. Schlessinger, A., Geier, E., Fan, H., Irwin, J. J., Shoichet, B. K., Giacomini, K. M. & Sali, A. Structure-based discovery of prescription drugs that interact with the norepinephrine transporter, NET. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 15810–15815 (2011).

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