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Characterization of the membrane transporter OATP1A2 activity towards different classes of drugs

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Résumé

Les transporteurs membranaires sont des éléments importants dans le devenir, l'efficacité, et la toxicité du médicament. Ils influencent la pharmacocinétique et la pharmacodynamie de ces derniers. Plusieurs interactions médicamenteuses observées cliniquement sont attribuables à la fois aux enzymes responsables du métabolisme des médicaments et aux transporteurs membranaires. Il est connu qu'une variabilité existe entre différents individus dans la réponse à un médicament et les polymorphismes génétiques retrouvés dans les gènes codant pour les transporteurs membranaires peuvent partiellement expliquer cette variabilité.

OATP1A2 est un transporteur membranaire exprimé sur des organes importants, comme le cerveau et le rein. Plusieurs médicaments utilisés en clinique sont des substrats d'OATP1A2 et l'expression localisée de ce transporteur suggère un rôle important dans le devenir du médicament. Donc, mon projet de doctorat consistait à caractériser l'activité d'OATP1A2 en relation avec ses substrats et inhibiteurs, et de plus, à évaluer l'impact de différents variants génétiques d'OATP1A2 sur leur transport.

Dans le premier article, la rosuvastatine a été utilisée comme substrat-type pour étudier le transport d'OATP1A2. Les expériences ont été menées en introduisant la rosuvastatine en compétition avec différent β-bloqueurs, une classe de médicaments rapportée dans la littérature comme substrats d'OATP1A2. Parmi les β-bloqueurs évalués, le carvédilol était l'inhibiteur le plus puissant. Dans la deuxième partie de l'étude, des médicaments ayant une structure similaire au carvédilol, tels que les antidépresseurs tricycliques, ont été évalués quant à leur potentiel d'inhibition sur OATP1A2. Une relation structure-activité a été définie à l'aide de ces données. Nous avons démontré que des composés tricycliques avec une courte chaîne aliphatique pouvaient inhiber OATP1A2.

Dans le deuxième article, OATP1A2 a été étudié en considérant son expression et son rôle au sein de la barrière hémato-encéphalique (BHE). Des études précédentes ont démontré qu'OATP1A2 est exprimé sur la membrane luminale des cellules endothéliales formant la BHE. Nos données démontrent que les triptans, une classe de médicaments couramment utilisées pour traiter la crise migraineuse, sont des substrats d'OATP1A2 et que les composés tricycliques identifiés comme inhibiteurs d'OATP1A2 dans nos études précédentes peuvent inhiber le transport des triptans par OATP1A2. Ces résultats sont importants puisque: 1) il a été suggéré que les triptans peuvent agir au niveau du système nerveux central en se liant aux récepteurs trouvés sur les neurones centraux; 2) comme les triptans sont des molécules hydrophiles, un mécanisme de transport facilité est nécessaire pour qu'ils pénètrent la BHE et OATP1A2 pourrait être l'élément clé; 3) l'inhibition d'OATP1A2 par les composés tricycliques pourrait limiter l'accès des triptans à leur site d'action.

Le troisième article caractérise l'activité associée à deux variants génétiques d'OATP1A2 (OATP1A2*2 et *3). Leur capacité à transporter les triptans et leur potentiel d'inhibition par les médicaments tricycliques ont été évalués. Des résultats supplémentaires caractérisant OATP1A2, mais sans liens directs avec les trois articles, seront présentés en annexe.

Dans l'ensemble, les résultats présentés dans cette thèse servent à caractériser le transporteur membranaire OATP1A2 en relation avec ses substrats et inhibiteurs, et en fonction de ses variants génétiques.

Mots-clés : Transporteurs de médicaments, OATP1A2, interactions médicamenteuses, triptans, barrière hémato-encéphalique, rosuvastatine, antidépresseurs tricycliques, carvédilol, polymorphisme d'un seul nucléotide.

Abstract

Drug transporters are important determinants in drug disposition, efficacy, and toxicity. They influence the pharmacokinetics and pharmacodynamics of drugs. Several clinically-observed drug-drug interactions are mediated through drug metabolizing enzymes and drug transporters. It is well known that there is an interindividual variability in the response to medications and polymorphisms found in genes encoding for drug transporters partially account for it.

OATP1A2 is a membrane drug transporter expressed on important organs, such as the brain and the kidney. A wide spectrum of drugs used in the clinic are substrates of OATP1A2. Its localisation suggests an essential role in drug disposition. Thus, my PhD project consisted of characterizing the activity of OATP1A2 in regards to its substrates, inhibitors, and different protein variants due to genetic polymorphisms.

In the first article, rosuvastatin was used as the probe substrate to study OATP1A2 transport activity. Experiments were conducted by putting rosuvastatin in competition with different β-blockers, a class of drugs known in the literature to be transported by OATP1A2. One of the drugs evaluated, carvedilol, inhibited OATP1A2 with much more potency than the others. In the second part of the study, drugs with a structure similar to carvedilol, such as tricyclic antidepressants, were tested for their potential to inhibit OATP1A2. A structure-activity relationship was defined using the data. It was demonstrated that drugs composed of a tricyclic ring with a short aliphatic amine chain were potent OATP1A2 inhibitors.

In the second article presented, OATP1A2 was studied in the context of its localization at the blood-brain barrier (BBB). OATP1A2 expression at the luminal membrane of the endothelial cells making up the BBB was demonstrated in the literature. Our article showed that triptans, a class of commonly used anti-migraine drugs, were OATP1A2 substrates. The tricyclic drugs previously evaluated were shown to potently inhibit triptan transport through OATP1A2.

These findings are important for three reasons: 1) it has been postulated that triptans may act at the central nervous system by binding to receptors found on central neurons; 2) as triptans are hydrophilic molecules, a facilitated transport mechanism is required for them to penetrate the BBB and OATP1A2 may be the key player; and 3) the inhibition of OATP1A2 by the tricyclic drugs may limit the entrance of triptans to their site of action.

The third article characterized the transport activity of two OATP1A2 protein variants (OATP1A2*2 and *3). Their capacities to transport triptans and their potential of being inhibited by tricyclic drugs were evaluated. Additional data characterizing OATP1A2 but considered out of the scope of the three articles will be presented in appendices.

In overall, the central theme of this thesis looks into the characterization of the OATP1A2 membrane drug transporter in regards to its substrates, inhibitors, and proteins variants.

Keywords: Drug transporters, OATP1A2, drug-drug interactions, triptans, blood-brain barrier, rosuvastatin, tricyclic antidepressants, carvedilol, single-nucleotide polymorphisms.

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List of abbreviations

ABC: ATP-binding cassette

ADME: absorption, distribution, metabolism, and excretion

ATP: Adenosine triphosphate

BBB: blood-brain barrier

BCRP: breast cancer resistance protein

CLint: intrinsic clearance

CNS: central nervous system

CYP450: cytochromes P450

DDI: drug-drug interaction

DNA: deoxyribonucleic acid

DPDPE: Deltorphin II, [D-Pen^{2,5}]enkephalin

FDA: Food and Drug Administration

HEK293: human embryonic kidney cells 293

HGNC: HUGO Gene Nomenclature Committee

HMG-CoA reductase: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

HPLC: high pressure liquid chromatography

IC_{50}: half maximal inhibitor constant

ITC: International Transporter Consortium

K_i: inhibitory constant

K_m: Michaelis constant

LC-MS/MS: liquid chromatography-tandem mass spectrometry

logD: log10(coefficient of distribution)

logP: log10(coefficient of partition)

MCT: monocarboxylate transporter

MDR1: multidrug resistance protein 1

MRI: magnetic resonance imaging

mRNA: messenger ribonucleic acid

MRP: multidrug resistance-associated protein

NBD: nucleotide binding domains

OAT: organic anion transporter

OATP1A2: organic anion transporting polypeptide 1A2

OCT: organic cation transporter

PET: positron-emission tomography

P-gp: P-glycoprotein

PXR: pregnane X receptor

S.D.: standard deviation

SLC: solute carrier

SNP: single nucleotide polymorphism

SPECT: single-photon emission computed tomography

T_3: triiodothyronine

T_4: thyroxine

TMD: transmembrane domain

UV: ultraviolet

To my parents and my fiancé Vincent,
Nothing would be possible without them

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Although it was not always a smooth process getting there and getting through it, I am proud of what was accomplished. The knowledge, experience, and hard labor can never be taken away.

Preface

As we are coming to understand that a drug's pharmacokinetic and pharmacodynamic profile depends not only on drug metabolizing enzymes but also on its interaction with specific drug transporters in the body, we are moving forward into a new era in drug development where smarter and more specific strategies will be developed. Indeed, research in the drug transporter field will help us understand how to specifically target a drug to its active site, ameliorating its pharmacokinetic profile, increasing efficacy, reducing toxicity and adverse events.

Characterizing drug transporters will help in understanding the interindividual variability in the response to drugs. Single nucleotide polymorphisms and drug-drug interactions mediated via transporters may modify the pharmacokinetic profile of drugs; thus, modifying the drug response. More and more examples of variability in drug disposition due to the activity of drug transporters are being demonstrated in the literature. To move forward in understanding drug response variability mediated by drug transporters, it is crucial to investigate the fundamental characteristics of known drug transporters. The work presented in this thesis aimed at characterizing the OATP1A2 drug transporter.

My thesis will first introduce the broad family of transporters, and then it will focus on the OATP1A2 drug transporter. Its potential role at the blood-brain barrier will be presented. The concept of how drug-drug interactions and single-nucleotide polymorphisms found in drug transporters influence the intervindividual variability in the response to drugs will be presented. The second section contains three scientific articles representing the work done during my doctorate. For each article, the study objectives will be listed, followed by a short introduction, then the article will be presented and discussed. Additional data generated following the theme of this thesis but considered out of the scope of the three articles will be presented in appendices.

The first two articles characterize the OATP1A2 transporter in regards to its various substrates and inhibitors. The second article places OATP1A2 in the context of the blood-brain barrier and CNS-active drugs were evaluated. The articles are: 1) Effects of β-blockers and tricyclic antidepressants on the activity of human organic anion transporting polypeptide 1A2 (OATP1A2) and 2) Effects of tricyclic compounds on the transport of anti-migraine triptans through human organic anion transporting polypeptide 1A2 (OATP1A2). The third article investigates two common protein variants of OATP1A2 for their transport activity: Impact of single nucleotide polymorphisms found in human organic anion transporting polypeptide 1A2 (OATP1A2) on the transport of triptans.

Finally, my thesis ends with an overall discussion on the future directions to take in understanding the role of OATP1A2 at the blood-brain barrier and wraps up with a short conclusion.

SECTION 1: INTRODUCTION

1.1 TRANSPORTERS

1.1.1 GENERAL INTRODUCTION

Transporters are transmembrane proteins that span biological membranes and mediate the translocation of substrates across membranes. At the plasma membrane, they control the uptake or efflux of endogenous compounds (sugars, amino acids, nucleotides, and ions) and xenobiotics in and out of the cells. On membranes forming subcellular organelles, they regulate entrance of substrates into organelles. They are classified into two superfamilies: 1) solute carrier (SLC) and 2) ATP-binding cassette (ABC). In humans, the SLC superfamily is composed of 395 members divided into 52 families (SLC1 – SLC52) based on the number of α-helices and sequence homology (Table 1) [1]. SLCs that function by moving solutes down their concentration gradient across membranes using the electrochemical potential difference as the driving force are classified as facilitated transporters. SLCs transporting substrates against their concentration gradient using the ion gradient, such as sodium or proton, generated by ATP-dependent pumps are classified as secondary-active transporters. SLCs using the gradient generated by secondary-active transporters to function are classified as tertiary-active transporters. The ABC superfamily is composed of 49 members divided into seven families (ABCA – ABCG) (Table 2). ABC transporters use the energy of adenosine triphosphate (ATP) hydrolysis to move molecules against their concentration gradient and they are also classified as active transporters.

Table 1: List of the 52 SLC families.

The total numbers of members in each family classified in 2004 and in 2013 are shown on the right. The families known to transport drugs are shown in boxes. *Table adapted from Hediger et al. (Mol Aspects Med 2013).*

The HGNC Solute Carrier Family Series	Total 2004	Total 2013
SLC1: The high affinity glutamate and neutral amino acid transporter family	7	7
SLC2: The facilitative GLUT transporter family	14	14
SLC3: The heavy subunits of the heteromeric amino acid transporters	2	2
SLC4: The bicarbonate transporter family	10	10
SLC5: The sodium glucose cotransporter family	8	12
SLC6: The sodium- and chloride-dependent neurotransmitter transporter family	16	21
SLC7: The cationic amino acid transporter/glycoprotein-associated amino-acid transporter family	14	14
SLC8: The Na+/Ca2+ exchanger family	3	3
SLC9: The Na+/ H+ exchanger family	8	13
SLC10: The sodium bile salt cotransport family	6	7
SLC11: The proton coupled metal ion transporter family	2	2
SLC12: The electroneutral cation-Cl cotransporter family	9	9
SLC13: The human Na+-sulfate/carboxylate cotransporter family	5	5
SLC14: The urea transporter family	2	2
SLC15: The proton oligopeptide cotransporter family	4	5
SLC16: The monocarboxylate transporter family	14	14
SLC17: The vesicular glutamate transporter family	8	9
SLC18: The vesicular amine transporter family	3	4
SLC19: The folate/thiamine transporter family	3	3
SLC20: The type III Na+-phosphate cotransporter family	2	2
SLC21/SLCO: The organic anion transporting family	11	12
SLC22: The organic cation/anion/zwitterion transporter family	18	23
SLC23: The Na+-dependent ascorbic acid transporter family	4	4
SLC24: The Na+/(Ca2+–K+) exchanger family	5	6
SLC25: The mitochondrial carrier family	27	53
SLC26: The multifunctional anion exchanger family	10	11
SLC27: The fatty acid transport protein family	6	6
SLC28: The Na+-coupled nucleoside transport family	3	3
SLC29: The facilitative nucleoside transporter family	4	4
SLC30: The zinc efflux family	9	10
SLC31: The copper transporter family	2	2
SLC32: The vesicular inhibitory amino acid transporter family	1	1
SLC33: The Acetyl-CoA transporter family	1	1
SLC34: The type II Na+-phosphate cotransporter family	3	3
SLC35: The nucleoside-sugar transporter family	17	30

SLC36: The proton-coupled amino acid transporter family	4	4
SLC37: The sugar-phosphate/phosphate exchanger family	4	4
SLC38: The System A & N, sodium-coupled neutral amino acid transporter family	6	11
SLC39: The metal ion transporter family	14	14
SLC40: The basolateral iron transporter family	1	1
SLC41: The MgtE-like magnesium transporter family	3	3
SLC42: The Rh ammonium transporter family (pending)	3	3
SLC43: Na+-independent, system-L like amino acid transporter family	2	3
SLC44: Choline-like transporter family		5
SLC45: Putative sugar transporter family		4
SLC46: Folate transporter family		3
SLC47: Multidrug and Toxin Extrusion (MATE) family		2
SLC48: Heme transporter family		1
SLC49: FLVCR-related transporter family		4
SLC50: Sugar efflux transporters		1
SLC51: Transporters of steroid-derived molecules		2
SLC52: Riboflavin transporter family		3
Total	**298**	**395**

Table 2: List of ABC families.

The total numbers of members in each family are shown on the right. The families known to transport drugs are shown in boxes. *Table adapted from Vasiliou et al. (Hum. Genomics 2009).*

Subfamily name	**Aliases**	**Number of genes**
ABCA	ABC1	12
ABCB	MDR	11
ABCC	MRP	13
ABCD	ALD	4
ABCE	OABP	1
ABCF	GGN20	3
ABCG	White	5
Total		**49**

As the transporter field is continuously growing, the gene nomenclature system was developed and approved by the HUGO Gene Nomenclature Committee to facilitate gene classification [2]. The SLCs genes are named using the root symbol SLC, followed by a numeral to indicate the family (e.g., *SLC1*, solute carrier family 1), followed by a letter to indicate the subfamily (e.g. *SLC1A*), and ending with a number to indicate the individual transporter gene (e.g. *SLC1A1*). SLC transporters are classified in the same family based on their biochemical function and when their amino acid sequence has at least 20% identity [3]. The SLC21 family is an exception to this classification method because this research field has evolved rapidly. Hagenbuch and Meier have developed an updated evolutionary-based nomenclature system in order to accommodate a species-independent classification system with less ambiguity [4]. *SLC21* is replaced by the root symbol *SLCO (Slco* for other animal species) followed by a number, a letter (capital for human genes and small letter for other animal species), and another number to designate the family, subfamily, and individual transporter gene, respectively (e.g. *SLCO1A2* for humans and *Slco1a2* for animals). Members with more than 40% amino acid sequence identity are grouped into the same family and those with more than 60% amino acid sequence identity are grouped into the same subfamily.

Whereas for *ABC* genes, the nomenclature is based on divergent evolution from a common ancestor and sequence similarity [5]. Family members share 30-50% sequence homologies [6]. They are named by the root symbol *ABC*, followed by a letter (A to G) to designate the family, and a number to designate individual members [2]. In general, the ABC transporter consists of two nucleotide binding domains (NBDs), also known as ATP binding cassettes, and two transmembrane domains (TMDs) [5]. In order to power translocation of substrates against their gradient, the two NBDs have to work together to bind and hydrolyse ATP molecules. Several highly conserved motifs, crucial for the ATPase activity, are found in the NBD: Walker A and Walker B sequences, the ABC signature motif, the H loop and the Q loop. The TMD, made up of several hydrophobic α-helices, is responsible for substrate recognition and translocation. Some ABC proteins (e.g. BCRP) are considered as “half-transporters” as their gene encode a single NBD and a single TMD. To gain functionality, the protein subunits need to homodimerize, heterodimerize or oligomerize.

1.1.2 DRUG TRANSPORTERS

Drug transporters are important determinants in drug absorption, distribution, organ-specific targeting, metabolism, excretion, efficacy, and toxicity. It is evident that drug transporters influence the pharmacokinetics and pharmacodynamics of a drug. Among the growing numbers of transporters, only a few members, organized in 13 families (Table 3), have been identified as essential in drug disposition [7].

Table 3: List of transporters involved in the disposition of drugs.

Table adapted from You & Morris (2014).

Gene family	Gene name	Protein name
SLC22	*SLC22A1*	OCT1
	SLC22A2	OCT2
	SLC22A3	OCT3
	SLC22A4	OCTN1
	SLC22A5	OCTN2
	SLC22A21	OCTN3
	SLC22A16	CT2
	SLC22A6	OAT1
	SLC22A7	OAT2
	SLC22A8	OAT3
	SLC22A11	OAT4
	SLC22A10	OAT5
	SLC22A20	OAT6
	SLC22A12	URAT1
SLC21/SLCO	***SLCO1A2***	**OATP1A2**
	SLCO1B1	OATP1B1
	SLCO1B3	OATP1B3
	SLCO1C1	OATP1C1
	SLCO2A1	OATP2A1
	SLCO2B1	OATP2B1
	SLCO3A1	OATP3A1
	SLCO4A1	OATP4A1
	SLCO4C1	OATP4C1
	SLCO5A1	OATP5A1
	SLCO6A1	OATP6A1
SLC15	*SLC15A1*	PEPT1
	SLC15A2	PEPT2
	SLC15A4	PHT1

	SLC15A3	PHT2
SLC16	*SLC16A1*	MCT1
	SLC16A7	MCT2
	SLC16A8	MCT3
	SLC16A3	MCT4
SLC5	*SLC5A8*	SMCT1
	SLC5A12	SMCT2
SLC28	*SLC28A1*	CNT1
	SLC28A2	CNT2
	SLC28A3	CNT3
SLC29	*SLC29A1*	ENT1
	SLC29A2	ENT2
	SLC29A3	ENT3
	SLC29A4	ENT4
SLC47	*SLC47A1*	MATE1
	SLC47A2	MATE2
SLC51	*SLC51A*	OST-α
	SLC51B	OST-β
SLC10	*SLC10A1*	NTCP
	SLC10A2	ASBT
ABCB	*ABCB1*	MDR1/P-gp
	ABCB11	BSEP
ABCC	*ABCC1*	MRP1
	ABCC2	MRP2
	ABCC3	MRP3
	ABCC4	MRP4
	ABCC5	MRP5
	ABCC6	MRP6
	ABCC10	MRP7
	ABCC11	MRP8
	ABCC12	MRP9
ABCG	*ABCG2*	BCRP/ABCG2

While drug transporters are found on every tissue of the human body, they are particularly highly expressed on the epithelia of tissues functioning as barriers for drug entry such as the intestine, liver, kidney, blood-brain barrier (BBB), and placenta. This expression pattern corresponds well to their function as cells gatekeepers. Epithelial cells are typically polarized into apical and basolateral membranes. For example, the brain capillary endothelial cells that make up the BBB are polarized into apical/luminal and basolateral membranes which correspond to the side facing the blood and the central-nervous system (CNS), respectively. The expression pattern of drug transporters usually differs on the two membranes. The polarization is essential for directing substrates towards the same direction. In addition, their

presence on plasma membranes of epithelial cells can cause variability in drug concentrations between the plasma and the target organ.

Drug transporters represent one of the rate-limiting steps in drug disposition. In drug development, studies have predominantly focused on transporters expressed on the intestine, liver, kidney, and BBB as they are the most common sites of drug-drug interactions [8].

Irregularity in transporter expression and activity may lead to inter-individual variability in the response to drugs. Drug-drug interactions (DDIs) and genetic polymorphisms on drug transporters can contribute to this variability. Due to the emerging role that transporters play in DDIs, experts from academia, industry, and the US Food and Drug Administration (FDA) were united to form the International Transporter Consortium (ITC) in 2007. Their mission is to determine which transporter is clinically important in DDIs, establish standardized protocols for the *in vitro* and *in vivo* study of DDIs, and establish a consensus on current knowledge of clinically relevant drug transporters [9]. They generate decision trees that guide industry into when to perform clinical studies for DDI with new molecular entities. Up to now, the ITC suggested that substrates of multidrug resistance protein 1 (MDR1; also known as P-glycoprotein (P-gp) or ABCB1), organic anion-transporting polypeptide 1B1 (OATP1B1), organic anion-transporting polypeptide 1B3 (OATP1B3), organic anion transporter 1 (OAT1), organic anion transporter 3 (OAT3), organic cation transporter 2 (OCT2), breast cancer resistance protein (BCRP; also known as ABCG2) should be evaluated further. These recommendations are based on the observation that clinically significant interactions were observed with drugs that are substrates of these transporters. This list will most likely expand as more studies are being done in the field of transporter related DDI.

1.1.3 ENDOGENOUS ROLES OF DRUG TRANSPORTERS

Transporters perform many physiological roles as they have endogenous substrates, such as metabolites, nutrients, antioxidants, gastrointestinal microbiome products, bile salts, neuro-active molecules, hormones and signalling molecules [10]. Some transporters are key players

in certain metabolic processes, such that sequence variants in the genes encoding these transporters can lead to diseases. For example, ABCC2 (MRP2) located on the hepatocyte canalicular membrane is the main efflux transporter for the elimination of bilirubin conjugates into the bile [7]. Mutations that abolish the cell surface expression or activity of ABCC2 lead to Dubin-Johnson syndrome which is characterized by a dark liver and an increase in conjugated bilirubin in the blood [11, 12]. OATP1B1 and OATP1B3 transporters, located on the sinusoidal membrane of hepatocytes, are responsible for the uptake of conjugated bilirubin from the blood. Mutations causing complete loss of both transporters result in Rotor syndrome, a rare benign autosomal recessive disease, characterized by conjugated hyperbilirubinaemia and jaundice [13]. In contrast to patients with Dubin-Johnson syndrome, patients with Rotor syndrome do not have a pigmented liver because the conjugated bilirubin cannot re-enter the hepatocyte through OATP1B1 nor OATP1B3 [14].

Drug transporters are also implicated in other diseases and their expression is altered in various diseased states. For example, several members of the OATPs are up- or downregulated in different cancers and it has been suggested that their expression state might affect cancer development [15]. *SLCO1A2* mRNA expression is upregulated 8 times in breast cancerous tissue compared to adjacent normal tissue [16]. OATP1A2 protein expression has been confirmed in breast carcinoma cells from patients but not in nonneoplastic epithelial cells, stroma, or adipocytes adjacent to the tumour [17]. It has been suggested that OATP1A2 on breast cancer cells contribute to the hormone-induced progression of breast cancer since steroid hormones are substrates of this transporter. Some ABC transporters are also overexpressed in tumors, and they may contribute to resistance to chemotherapy. In fact, repeated administration of anti-cancer drugs is associated with chemotherapy resistance and treatment failure [18]. Once drug resistance has developed, the tumor is typically cross-resistant to multiple drugs even if their structures are unrelated. Overexpression of ABC transporters, such as P-gp/MDR1, BCRP/ABCG2, and members of the MRP, contributing to pumping drugs out of the cancerous cells is the main reason for multi-drug resistance [19].

1.2 OATP1A2

1.2.1 OATP1A2 CHARACTERISTICS

OATP1A2 (previously known as OATP-A, OATP1, and OATP) is a member of the SLC21/SCLO family. It is made up of 12 transmembrane domains with a large fifth extracellular loop and both the C- and N-termini oriented towards the cytoplasmic space. N-glycosylation sites, important for targeting the protein to the plasma membrane, are found in extracellular loops 2 and 5 [20]. OATP1A2 exists in unglycosylated and several glycosylated forms and molecular weights between 60 and 150 kDa have been found [21-23]. The OATP proteins are distinguished by their signature amino acid sequence (D-X-RW-(I,V)-GAWW-X-G-(F,L)-L) which is found between extracellular loop 3 and the transmembrane domain 6 for OATP1A2 [24]. Transport through OATP1A2 is considered to be bidirectional as it moves substrates down its gradient across the membrane regardless of the orientation. It has been suggested that the OATPs function like a rocker-switch type of mechanism to translocate substrates through a central pore [25]. The mechanism of transport is recognized to be sodium-independent but the driving force is still unknown.

OATP1A2 mRNA expression is nearly ubiquitous. It has been detected at various expression levels in the human lung, brain, intracranial artery, optic nerve, retina, spinal cord, prostate, testis, lymph node, pituitary gland, duodenum, esophagus, kidney, liver, spleen, tonsil, [26-28]. However, mRNA is not necessarily representative of protein expression. OATP1A2 protein expression, confirmed by either immunofluorescence or Western blot, is found on the luminal membrane of the brain capillary endothelial cells which make up the blood-brain barrier, apical membranes of cholangiocytes in the liver, apical membrane of the distal nephrons in the kidney, the apical membrane of enterocytes in the duodenum, apical cell layers of the urothelium, placenta, red blood cells, brain neurons, and retina [2, 21-23, 27, 29-31].

A lot of attention has been drawn to OATP1A2 due to its postulated location on the intestine and its possible role in mediating drug absorption. Many food-drug interactions were attributed to the inhibition of intestinal OATP1A2 by flavonoids found in fruits and vegetables. It has been demonstrated that fexofenadine bioavailability is decreased when co-administered with fruit juices (grapefruit, apple, orange) in healthy volunteers and *in vitro* data supports the role of OATP1A2 in mediating this interaction [29, 32, 33]. Misaka *et al.* demonstrated that green tea reduced plasma concentrations of the β-blocker nadolol and their findings are also supported by *in vitro* results [34, 35]. However, all this excitement has declined lately because recent studies could not detect OATP1A2 in the intestine by liquid chromatography-tandem mass spectrometry (LC-MS/MS) nor the mRNA transcript [36, 37]. Thus, OATP1A2 expression in the small intestine remains controversial. The previously observed food-drug interaction may be attributed to the inhibition of another intestinal transporter, such as OATP2B1. Indeed, grapefruit juice, orange juice, and their constituents can also inhibit OATP2B1 [38].

OATP1A2 transports a wide spectrum of substrates including endogenous molecules, xenobiotics, and clinically relevant drugs. A list of currently known OATP1A2 substrates and inhibitors classified into categories is presented in Table 4.

Table 4: List of OATP1A2 substrates and inhibitors.

Adapted from Franke *et al. (Pharmacogenomics 2009)* [39].

Category	Substrates	Inhibitors	Reference
Bile salts	Taurocholate, cholate, glycocholate, taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid		[28, 40]

	(TUDCA)		
Hormones	Estrone-3-sulfate (E3S), estradiol 17β-glucuronide ($E_2$17βG), dehydroepiandrosterone (DHEAS), triiodothyronine (T_3), thyroxine (T_4),	Steroid hormones	[23, 40, 41]
Peptides	Deltorphin II, [D-Pen^{2,5}]enkephalin (DPDPE), BQ-123, substance P, vasoactive intestinal peptide		[21, 30, 40]
Organic anions	Bromosulfophthalein (BSP), sodium fluorescein, all-trans-retinol		[2, 28, 40, 42]
Organic cations	*N*-methyl-quinine, *N*-methyl-quinidine, APD-ajmalinium,		[43]
Drugs	Imatinib, quinine, fexofenadine, methotrexate, atorvastatin, pitavastatin, pravastatin, rosuvastatin, rocuronium, celiprolol, acebutolol, atenolol, nadolol, sotalol, labetalol, EDDP, docetaxel, mirabegron, glibenclamide, triptans (almotriptan, eletriptan, frovatriptan, rizatriptan, sumatriptan, zolmitriptan), aliskiren,	Chloroquine, hydroxychloroquine, multikinase inhibitors (lapatinib, bosutinib, cediranib, afatinib, erlotinib, foretanib, gefitinib, nilotinib, pelitinib, sunitinib, vandetanib), rifampicin, clarithromycin, everolimus, sirolimus, tacrolimus, cyclosporine	[26, 27, 31, 41, 43-63]

	tebipenem pivoxil, levofloxacin, trospium chloride, doxorubicin		
Toxins	Ouabain, microcystin		[40, 64]
Eicosanoids	Prostaglandin E2 (PGE2)		[40]
Flavonoids		Naringin, hesperidin, apigenin, kaempferol, quercetin, epicatechin gallate, epigallocatechin gallate	[29, 34, 35, 65]

OATP1A2 activity is modulated by different proteins. The chaperon proteins PDZK1 and NHERF1 enhance OATP1A2 stability at the plasma membrane and decrease the transporter internalization [66]. Post-translational modifications of OATP1A2 regulate its activity. Phosphorylation by protein kinase C or casein kinase 2 increases OATP1A2 internalization through clathrin mediated endocytosis [67, 68]. Five putative N-glycosylation sites were identified in OATP1A2 and such modification targets the transporter to the plasma membrane [23]. The transporter is also regulated at the transcriptional level. It has been shown that the vitamin D receptor and the xenobiotic sensor pregnane X receptor (PXR) upregulate the transcription of *SLCO1A2* gene [16, 69].

Several animal transporters share a certain degree of homology with the human OATP1A2. Bovine Oatp1a2 share 83% homology with its human counterpart but the bovine protein differs with its 11 predicted transmembrane domains and multiple binding sites [70-72]. Five rat proteins (Oatp1a1, Oatp1a3, Oatp1a4, Oatp1a5, and Oatp1a6) share between 66-72% homology with OATP1A2. The pattern of expression differs among the five members. Four mouse proteins (Oatp1a1, Oatp1a4, Oatp1a5 and Oatp1a6) share between 66-73% homology with OATP1A2 and their pattern of expression is also variable. Multiple members are found in the rodent due to gene duplication. Pig Oatp1a2 (84% homology) contains 12 transmembrane

domains and its mRNA has been found in the liver, brain, and intestine [73, 74]. Dog Oatp1a2 share 87% homology with OATP1A2 and its mRNA has been found in the liver and kidney [73]. Rodents are not considered good animal models for the study of the OATP1A2 transporter due to the multiplicity in protein members and their low sequence homology. Further studies are needed to determine if other animals are more representative.

1.2.2 OATP1A2 AT THE BLOOD-BRAIN BARRIER

The brain is separated from the circulating blood by the BBB and from the cerebrospinal fluid by the choroid plexus. This delimitation is important to protect the brain from potentially harmful agents, regulate ions level, confine central neurotransmitters, prevent leakage of plasma proteins, and tightly control nutrients and metabolites essential for the brain [75]. The BBB is formed by the endothelial cells lining the brain microvessels. The surface area formed by the BBB represents a vast interface for exchange and is in average between 12 and 18 m^2 in adults [76]. The structure of the endothelial cells is maintained by astrocytes feet and pericytes. The particularity with these endothelial cells is the organization of the proteins in the tight junctional complexes found between the cells that maintains the BBB "tightness" and integrity. The tight junctions prevent paracellular diffusion of ions and macromolecules between endothelial cells. The effectiveness of the tight junctions results in a high transendothelial electrical resistance of the BBB (1500 to 2000 $\Omega \cdot cm^2$) [77]. Under physiological conditions, the BBB is almost impermeable to endogenous and exogenous substances.

The BBB tightly controls the access of substances to the brain in order to maintain a stable environment for the CNS. Specific receptors, ions channels, and transporters are expressed to regulate the entrance of nutrients. Several mechanisms of transport exist for molecules to cross the BBB: cell migration, passive diffusion, carrier-mediated efflux, carrier-mediated influx, receptor-mediated transcytosis, and adsorptive-mediated transcytosis (Figure 1). Lipid-soluble molecules and certain gases (oxygen and carbon dioxide) can diffuse passively through the

BBB. Substrates attempting to cross the BBB can be pumped out of the endothelial cells by the ABC transporters located on the luminal membrane. In contrast, SLC transporters may move substrates from the circulating blood into the CNS. Many vital molecules, such as glucose, amino acids and nucleosides, are directed to the brain through the SLC transporters. Several macromolecules, such as transferrin and insulin, bind to cell surface receptors and are taken up in the cells by endocytosis [78, 79]. Positively charge proteins, such as albumin and the SynB5 peptide, can also be transported by vesicles but in a non-specific and non-receptor mediated manner. The cationic macromolecules adsorb to the endothelial cell surface and induce endocytosis. The endocytosed macromolecules then move through the cell before being release at the abluminal membrane [80, 81]. Mononuclear leukocytes, monocytes and macrophages are able to cross the BBB by a process of diapedesis directly through the endothelial cells [82].

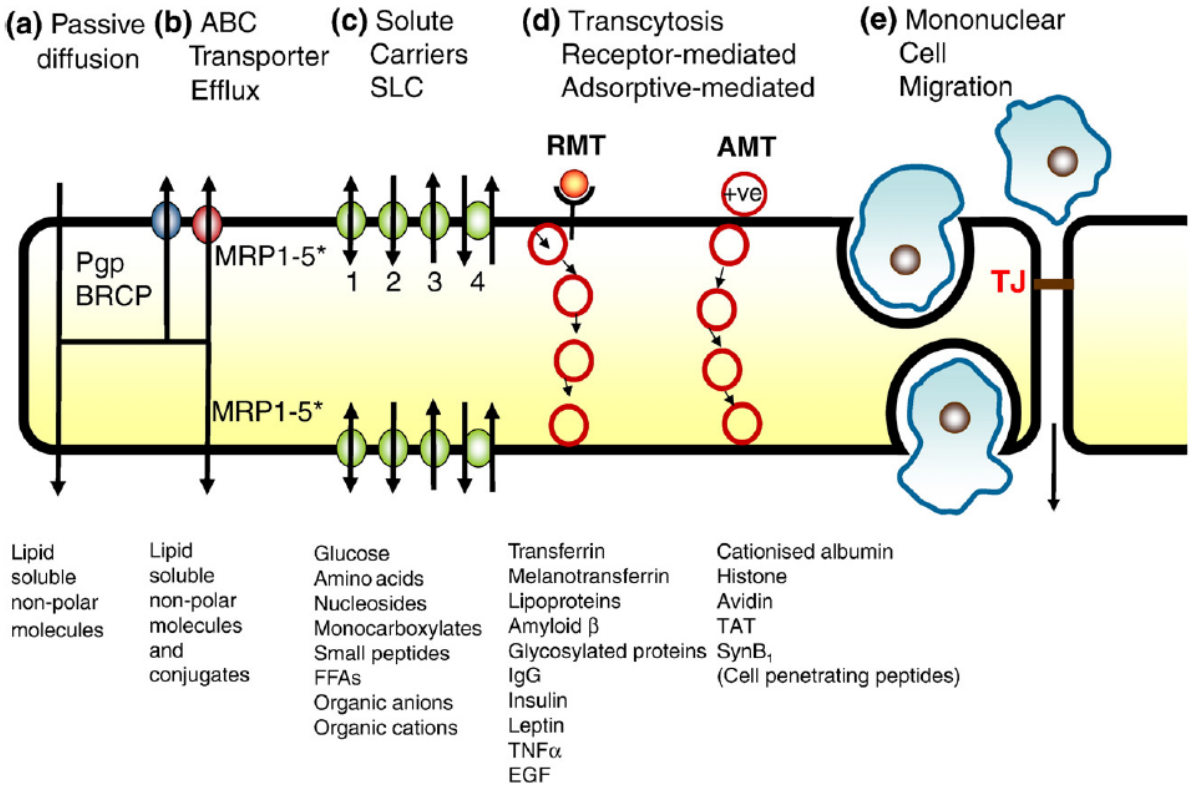

Figure 1: Different routes of transport across the BBB.

Adapted from Abbott *et al. (Neurobiology of Disease 2010)*[75].

Several CNS diseases, such as multiple sclerosis and Alzheimer's disease, may involve the disruption of the BBB [83, 84]. The tight junctions between endothelial cells are affected and the expression of transporters and enzymes may be modified. Consequently, the entry of immune cells, endogenous molecules, and xenobiotics is facilitated.

Targeting the CNS in drug therapy is challenging due to the limited permeation of the BBB and the blood-cerebrospinal fluid barrier. The level of difficulty is increased when the drug target is within cellular compartments of the brain parenchyma (i.e., astrocytes, microglia, oligodendrocytes, and neurons). Accessibility of pharmaceuticals to the brain is therefore highly dependent on influx and efflux transporters.

Drug transporters found on the luminal membrane of the BBB are potential entry sites for drugs or toxins. Those found on both the luminal and abluminal membranes may allow a direct flow of their substances from the blood to the CNS. Drug transporters detected at the human BBB are listed in Table 5.

Table 5: Expression of drug transporters at the human blood-brain barrier.

Adapted from Stieger *et al*. (*Clin Pharmacokinet* 2015) [85].

Transporter	Gene	Protein expression confirmed
OATP1A2	*SLCO1A2*	[21, 23, 30, 86]
OATP1C1	*SLCO1C1*	[87]
OATP2B1	*SLCO2B1*	[30]
OCT1	*SLC22A1*	[88]
OCT2	*SLC22A2*	[88]
OCT3	*SLC22A3*	[89]
OCTN2	*SLC22A5*	[90]
ENT1	*SLC29A1*	[91]

MATE1	*SLC47A1*	[89]
MCT1	*SLC16A1*	[92]
BCRP/ABCG2	*ABCG2*	[91]
MDR1/P-gp	*MDR1*	[91]
MRP1	*ABCC1*	[93]
MRP4	*ABCC4*	[91, 93]
MRP5	*ABCC5*	[93]

Toxins may also gain access to the brain through drug transporters. For example, 126 patients undergoing haemodialysis in Brazil developed symptoms of acute neurotoxicity and subacute hepatotoxicity following the use of water from a lake with cyanobacteria overgrowth [94]. Among these patients, 60 subsequently died. Microcystins, a class of toxins produced by cyanobacteria, were detected in the patients' serum, dialysis filters, and water-treatment column. It was later demonstrated that microcystins are transported in oocytes by OATP1B1, OATP1B3, and OATP1A2 [64]. OATP1B1 and OATP1B3, found on the sinusoidal membrane of hepatocytes, may be responsible for the hepatotoxicity; whereas, OATP1A2 may play a role in neurotoxicity. Microcystins caused damage to oocytes but only in the presence of OATP1A2. In addition, neurons in the brain express OATP1A2 [30]. As a result, microcystins are able to exert their toxicity in neurons after crossing the BBB.

In terms of normal physiology, drug transporters are also implicated in the passage of endogenous compounds and removal of neurotoxins to and from the brain, respectively. Thyroid hormones play a pivotal role in the development and differentiation of the brain as well as the maintenance and metabolic regulation of the adult CNS [95]. Triiodothyronine (T_3) and thyroxine (T_4) are substrates of OATP1C1 and OATP1A2 and they might access the brain through one of these routes [40]. Monocarboxylate transporter 1 (MCT1) may be involved in bringing lactate and ketone bodies (β-hydroxybutyrate and acetoacetate) to the brain to use as energy substrates when the level of glucose is reduced, such as conditions of prolonged starvation, diabetes, or under hypoglycaemia [96].

The numerous ABC transporters found at the luminal side of the BBB play a neuro-protective role. This is exemplified in a phase I clinical trial where PSC 833, a second-generation MDR1 inhibitor, was given concomitantly with etoposide, an anticancer drug. Severe ataxia was observed in patients who received the highest doses of PSC 833 [97]. This adverse event was attributed to a higher permeation of etoposide to the brain caused by the inhibition of MDR1 efflux by PSC 833. While ABC transporters protect the brain from potential neurotoxins, they also represent a considerable challenge for the delivery of CNS-active drugs to their target site. Consequently, the development of drugs treating brain diseases such as brain tumors and bacterial or viral infections has been severely hindered.

On the other hand, SLC transporters at the BBB may have an opposite role. OATP1A2 expression on the luminal membrane of the endothelial cells suggests an important function of this transporter on brain penetration of drugs. Deltorphin II and [D-penicillamine^{2,5}]enkephalin (DPDPE) are δ-opioid receptor agonists that have previously been considered as potential central analgesics in humans [98]. To cause analgesia, they need to reach the δ-opioid receptor located within the CNS. Since they are peptides, they don't easily penetrate the BBB. Nonetheless, they have been found to enter the animal brain in a saturable manner, suggesting the involvement of a transporter [99-101]. They have been found to be OATP1A2 substrates and it has been suggested that deltorphin II and DPDPE can cross the human BBB using OATP1A2 [21].

Triptans (e.g. almotriptan, eletriptan, frovatriptan, naratriptan, rizatriptan, sumatriptan, and zolmitriptan) are commonly used anti-migraine medications that are believe to act at the CNS. All triptans, with the exception of eletriptan, are hydrophilic; thus, limiting their penetration to the brain by passive diffusion. Studies have found that zolmitriptan is not only capable of crossing the human BBB but it can also bind to its target receptor at the CNS [102, 103]. OATP1A2 has been suggested as a key player in triptans distribution to the brain as they are substrates [55].

In conclusion, the expression of different drug transporters at the brain suggests a highly coordinated and regulated environment for the permeation of xenobiotics to the brain. It is becoming more and more recognized that drug transporters at the BBB play a crucial role in the pharmacokinetic and pharmacodynamic profiles of a drug. Therefore, it is important to understand the molecular properties of drug transporters expressed at the BBB in the development of CNS-active drugs in order to optimize their concentrations and effects at the target site.

1.3 INTERINDIVIDUAL VARIABILITY IN RESPONSE TO DRUGS

A major setback in treating patients suitably in the clinic is the impact of interindividual variability in the response to medications. This heterogeneity in drug response influences the efficacy, safety and toxicity of the medication. The origin of the variability is multifactorial and comprises of genetic, environmental (dietary constituents), physiological (age and gender), epigenetic, and pathological factors.

Adverse reactions to drugs is an important public health concern. Conclusions from a meta-analysis revealed that the incidence of suffering from an adverse reaction (including non-serious, serious, and fatal reactions) related to a medication is around 15.1% in hospitalized patients and that adverse drug reactions is the 4th cause of mortality in the United States [104, 105]. In addition, an estimate of 6.5% of hospital admission is related to adverse drug reactions [106]. At the other end of the spectrum, there are patients who do not benefit from a medication due to a lack of efficacy. In 1969, the FDA initiated a post-marketing surveillance system on drug safety. They revealed that the most common undesirable side effect encountered until 2002 is the lack of drug efficacy [107].

For those reasons, it is crucial to understand the underlying influences on the response to drugs in order to improve their safety and efficacy profile. The concept of personalized medicine is born from these efforts. Personalized medicine is an emerging approach for treating diseases that takes into accounts the patient's genetic background, environmental influences, and lifestyle. More broadly, it can be defined as the tailoring of medical treatment to the individual characteristics, needs and preference during all stages of care, including prevention, diagnosis, treatment and follow up [108].

The next two sections introduce how drug transporters are involved in the interindividual variability in the response to drugs.

1.3.1 DRUG-DRUG INTERACTIONS

Drug-drug interactions (DDIs) can result when one drug alters the pharmacokinetic of another drug or its metabolites. The additive pharmacodynamic effect of multiple drugs can also lead to a DDI. The underlying mechanisms of a DDI affecting the pharmacokinetic of a drug are caused by either inhibition or induction of drug metabolizing enzymes [e.g. cytochromes P450 (CYP450)] or drug transporters. Dietary supplements and some foods may alter drug metabolism and/or transport. The result of those interactions is a sudden alteration in metabolism or transport in individuals who otherwise would have tolerated a particular dose of that drug. The known safety and efficacy of that drug is changed.

The desirable and undesirable effects of a drug are related to its concentration at various sites of action, which is usually related to the blood or tissue concentration of the drug. The blood and tissue concentrations are determined by the drug's absorption, distribution, metabolism, and excretion (ADME). DDIs related to metabolism are well-recognized but effects related to transporters are being documented with increasing frequency and are, therefore, important to consider in drug development. Transporters can affect the safety and efficacy profile of a drug by affecting the concentration of a drug or its metabolites in various tissues.

Several DDIs of clinical significance with mechanisms attributed to the inhibition or induction of transporters have been reported in the literature over the years. A few examples are listed in Table 6 [7, 109].

Table 6: Transporter mediated drug-drug interactions observed in clinical studies

Transporter	Perpetrator drug / compound	Victim drug	Pharmacokinetic effect of victim drug	Reference
OATP1A2/ OATP2B1 (intestinal)	Grapefruit juice (6',7'-Dihydroxybergamottin, bergamottin)	Fexofenadine	p.o. ↓AUC, Cmax	[29, 32, 33, 38]
	Orange (hesperidin, tangeritin, nobiletin) and apple juice (quercetin, kaempferol)	Aliskiren	p.o. ↓AUC, Cmax	[110]
OATP1B1 (hepatic)	Gemfibrozil	Pravastatin	p.o. ↑AUC, Cmax; ↓CLr	[111]
	Cyclosporine	Pravastatin	p.o. ↑AUC, Cmax	[112, 113]
	Cyclosporine	Rosuvastatin	p.o. ↑AUC, Cmax	[114]
OAT1/ OAT3 (renal)	Probenecid	Furosemide	p.o. ↑AUC, Cmax, T½, Tmax; ↓CLt, CLr, CLnr iv. ↑AUC, T½; ↓CLt, CLr	[115-117]
	Probenecid	Methotrexate	iv. ↑Cserum, T½	[118]
OCT2	Cimetidine	Metformin	p.o. ↑AUC, Cmax; ↓CLr	[119]

(renal)	Cimetidine	Ranitidine	p.o. ↑AUC, T½; ↓CLr	[120]
	Cimetidine	Dofetilide	p.o. ↑AUC, Cmax, T½; ↓CLt, CLr, CLnr	[121]
	Cimetidine	Pindolol	p.o. ↑AUC, Cmax; ↓CLr	[122]
	Trimethoprim	Procainamide	p.o. ↑AUC; ↓CLt, CLr	[123]
ABCB1 (P-gp) (intestinal)	St-John's wort (hypericin, pseudohypericin) [inducer]	Digoxin	p.o. ↓ AUC, Cmax, Ctrough	[124]
	Quinidine	Digoxin	iv. ↑ T½, Cserum; ↓CLt, CLr, CLnr	[125, 126]
	Clarithromycin	Digoxin	p.o. ↑AUC, Cmax, T½; ↓CLrng	[127]
	Ritonavir	Digoxin	iv. ↑AUC, Vd, T½; ↓CLt, CLr, CLnr	[128]
ABCG2 (BCRP) (intestinal)	Omeprazole/ pantoprazole	Methotrexate	p.o. ↑AUC, Cmax; ↓CLr	[129, 130]
	Elacridar	Topotecan	p.o. ↑AUC, Cmax, F	[131]

↑: Increased; ↓: Decreased; AUC: area under the plasma/serum concentration time curve; CLnr: Nonrenal clearance; CLr: Renal clearance; CLrng: Renal nonglomerular clearance; CLt: Total clearance; Cmax: Maximal plasma/serum concentration; Cserum: Serum concentration; Ctrough: Minimum plasma/serum concentration during steady state; F: Bioavailability; iv.: Intravenous administration of drug; p.o.: Oral administration of drug; T½: Plasma/serum concentration half-life; Vd: Volume of distribution.

Although an interaction is observable when fexofenadine is administered concomitantly with fruit juices, it is not likely to be mediated by OATP1A2 as previously considered. As mentioned earlier, the interaction may be mediated by OATP2B1 instead. OATP2B1 is found in the intestine and is also capable of transporting fexofenadine [36, 132].

DDIs are not always as straightforward as the examples listed above. Sometimes, multiple mechanisms are involved, such that multiple enzymes and/or transporters are implicated. Other times, when a cocktail of medication is given, more than one drug may inhibit the same enzyme or transporter. As the transporter field is a relatively new but rapidly evolving field, other less characterized transporters may also be involved in mediating DDIs.

Transporter-mediated DDIs have the potential to seriously influence drug efficacy and toxicity. Understanding DDIs will help us better prescribe medications and make a better use of our resources available. For instance, it can help in adjusting the dosage, determine if additional therapeutic monitoring if required, and establishing contraindication to concomitant use. Extensive research has led to contraindicating the consumption of grapefruit juice and orange juice with certain medications as described on the drug's label (e.g. fexofenadine). Dosage adjustments were done following the description of a clinically significant DDI between the two HIV protease inhibitors, ritonavir and saquinavir. Coadministration of ritonavir with saquinavir increased saquinavir bioavailability dramatically without affecting the pharmacokinetics of ritonavir [133]. As a result, ritonavir is now used as a booster to increase the bioavailability of other protease inhibitors instead of being used alone.

1.3.2 SINGLE-NUCLEOTIDE POLYMORPHISMS

Another factor leading to interindividual differences in the response to drugs is found in the genes encoding drug transporters. These genes are polymorphic; as a result, their protein expression level and transport efficiency is variable.

A single-nucleotide polymorphism (SNP) is a genetic variation where a single nucleotide is altered. SNPs are the most common type of genetic variations [134]. For a variation to be defined as a polymorphism, it has to occur at a frequency higher than 1% in the population. Most SNPs do not affect an individual health and normal development. Yet, some SNPs have been found to play a role in an individual's response to certain drugs, response to environmental factors, and susceptibility of developing particular diseases [134].

Polymorphisms found in the genes implicated in the pharmacodynamics and especially the pharmacokinetics of a drug are responsible for 50% of the variability observed in drug responses [135].

Pharmacogenomics, also interchangeably referred to pharmacogenetics, is the field of study that concentrate on understanding how genes affect an individual's response to medications. By understanding how genetic variations influence the drug's pharmacokinetics, the drug's dosage can be tailored in advance to each patient in order to improve efficacy and safety. However, not all drugs will benefit from such optimisation process. The cost-efficiency of genotyping is not recognized for drugs with a large therapeutic window, good safety profile, and those where multiples genes are implicated in their pharmacokinetics. Drugs with a narrow therapeutic index are most likely to profit from this approach. In recent years, pharmacogenomic tests have become available but their usage remained limited. The main reason is the lack of scientific proof in the improvement of patients' care with genotyping [136]. In spite of this setback, this field is still in its early stages and additional unknown cofactors may have influenced the output. Further studies are necessary to determine which drug can profit from genotyping.

Two approaches are used to investigate the importance of genetic variations in drug response. The phenotype-to-genotype approach associates an observable drug response to a gene and polymorphisms in that gene. The genotype-to-phenotype approach examines all naturally occurring polymorphisms of that gene before conducting functional *in vitro* experiments and/or clinical assessments. The second method is most frequently used for drug transporters. For example, the *ABCB1* variants, 267G>T/A and 3435C>T, were first characterized *in vitro* before being associated with drug pharmacokinetics, response, and toxicity [7]. Unfortunately, genotype-to-phenotype studies have been less successful at identifying polymorphisms that result in substantial clinical effects. This could be explained by the overlap in drug transporters substrates and the complexity of a drug response which is likely to involve multiples genes.

Many SNPs found in drug transporter genes have been reported in the public database Pharmacogenetics Knowledge Base (PharmGKB), which documents genotypic and phenotypic pharmacogenetic data (www.pharmgkb.org). Nonsynonymous SNPs found in the coding region can influence transporter function. SNPs found in untranslated regions may influence mRNA stability and translation, while SNPs found in the promoter may influence transcription and gene/protein expression.

In 2003, a study screened 24 drug transporters genes for polymorphisms in exonic and flanking intronic regions in 247 DNA samples from an ethnically diverse population (100 European Americans, 100 African Americans, 30 Asians, 10 Mexicans, and 7 Pacific Islanders) and made several interesting observations [137]. The number of synonymous (silent) and nonsynonymous (resulting in an amino acid change) SNPs identified was similar (175 and 155, respectively). However, genetic variation was three- to fourfold more frequent at synonymous positions than at nonsynonymous position. It is suggestive of a selective pressure to suppress major changes in transporter function. Also, more SNPs were found in the loop domains than in the transmembrane regions. Similar to other studies, genetic variations differ across ethnic groups. Among the 680 SNPs identified, 421 were population-specific and were mostly found at low frequency.

Functional analysis of nonsynonymous variants has been investigated mostly using *in vitro* heterologous expression systems. One very peculiar observation is that the polymorphism affecting the transporter activity is substrate dependent. In other words, the same variant may have enhanced activity with certain substrates but reduced transport for others. Furthermore, *in vitro* findings are not always correlated with clinical data. Consequently, providing *in vitro* as well as clinical evidences should be stressed when establishing an association between a genotype and its effects on drug disposition.

There are cases where *in vitro* findings have been validated with clinical data. ABCB1 and ABCG2 are efflux transporters expressed in the intestine and they are implicated in limiting drug absorption. When their activity is modified by a SNP, it is reasonable to suppose that the bioavailability of drugs transported by ABCB1 or ABCG2 is also affected. ABCB1 A893S and A893T variants showed increased transport of fexofenadine in membrane vesicles preparation from cells expressing those variants [138]. These two variants are thus hyperfunctional compared to the wild-type. Subjects with the *ABCB1* 2677G>T and 2677G>A genotypes, leading to the A893S and A893T variants, have a decreased plasma exposure after a single dose administration of fexofenadine [139, 140]. In addition, *in vitro* experiments demonstrated that cells expressing the ABCG2 Q141K variant have increased intracellular levels of topotecan compared to ABCG2 wild-type [141]. The Q141K variant is thus hypofunctional. In the same study, patients who are heterozyogous for *ABCG2* 421C>A, the SNP that gives rise to the Q141K variant, have an increased bioavailability compared to patients with the *ABCG2* 421CC genotype when topotecan is administered orally. Furthermore, the OATP1B1 V174A variant membrane expression is reduced compared to the wild-type [142]. OATP1B1 is expressed exclusively on the sinusoidal membrane of hepatocytes and it is implicated in the uptake of drugs from the blood into the liver. In the clinic, subjects heterozygous for *SLCO1B1* 521T>C have an increased exposure to pravastatin than subjects with the *SLCO1B1* 521TT genotype and homozygous subjects have an even higher exposure to pravastatin than heterozygous subjects [143].

Several nonsynonymous SNPs have been identified in the *SLCO1A2* gene from a collection of ethnically diverse genomic DNA samples and a few variant proteins have been characterized in vitro using overexpression systems (Table 7).

Table 7: List of nonsynonymous *SLCO1A2* genetic polymorphisms.

Nucleotide position	BP change	AA position	AA change	Effects of the transporter activity
38	T>C	13	I>T	↔ for E3S, Deltorphin II, DPDPE [23] ↑ for E3S and methotrexate [44]
382	A>T	128	N>Y	↔ for E3S, Deltorphin II, DPDPE [23] ↔ for E3S and methotrexate [44]
404	A>T	135	N>I	↓ for E3S, Deltorphin II, DPDPE [23] ↔ for E3S and methotrexate [44]
502	C>T	168	R>C	↓ for E3S and methotrexate [44]
516	A>C	172	E>D	↓ for E3S, Deltorphin II, DPDPE [23] ↓ for E3S and methotrexate [44]
550	G>A	184	E>K	↓ for E3S, imatinib, methotrexate [144]
553	G>A	185	D>N	↓ for E3S, imatinib, methotrexate [144]
559	G>A	187	A>Y	↓ for Deltorphin II; ↔ for E3S and DPDPE [23] ↔ for E3S and methotrexate [44]
763	G>A	255	V>I	↔ for E3S, imatinib, methotrexate [144]
775	A>C	259	T>P	↓ for E3S, imatinib, methotrexate [144]
830	C>A	277	T>N	↔ for E3S and methotrexate [44]
833	A>−	278	N>del	↓ for E3S and methotrexate [44]
841	A>G	281	I>V	↔ for E3S and methotrexate [44]
862	G>A	288	D>N	↓ for E3S, imatinib, methotrexate [144]
968	T>C	323	L>P	↔ for E3S and methotrexate [44]
1063	A>G	355	I>V	↔ for E3S and methotrexate [44]
2003	C>G	668	T>S	↔ for E3S, Deltorphin II, DPDPE [23] ↔ for E3S and methotrexate [44]

BP: base pair; AA: amino acid; DPDPE: Deltorphin II, [D-Pen^{2,5}]enkephalin; E3S: estrone-3-sulfate; ↔: unchanged function; ↑: increased function; ↓: decreased function; −: deletion

Since OATP1A2 is found on organs important in drug disposition, such as the BBB and the kidney, it is of interest to investigate this transporter further. In addition, OATP1A2 is expressed on different cell types and transports a broad spectrum of substrates, rendering it a good candidate for the distribution of drugs in organs. It must be remembered that the efficacy of drugs with intracellular target sites is related to their ability to penetrate their target organ.

1.4 Rationale, Hypothesis, and Objectives

PK and PD concepts were initially developed assuming that the free concentration of a drug freely distributes across cell membranes and that equilibrium was reached across all tissues. The discovery of influx and efflux drug transporters with substrate specificity and selectivity expressed on all tissues is modifying these PK/PD concepts. Drug transporters are becoming increasingly recognized as important determinants in drug disposition, efficacy, and toxicity. Previous sections illustrate the role they play in interindividual variability in the response to drugs by demonstrating variable activity when carrying a SNP and by mediating DDIs.

OATP1A2 is a membrane drug transporter expressed on important organs, such as the brain and the kidney. Its localisation suggests an essential role in drug disposition such that a DDI or genetic variability may affect the local concentrations of the drug and ultimately the PD effects may be modified. Previous studies, assuming the localisation of OATP1A2 in the small intestine, demonstrated that OATP1A2's activity in transporting drugs used in the clinic is modifiable by flavonoids found in fruit juices and green tea. These studies put in evidence OATP1A2's potential for being inhibited. Although OATP1A2's intestinal expression is controversial, its expression on the luminal membrane of the endothelial cells forming the BBB is well established. A wide spectrum of drugs used in the clinic are substrates of OATP1A2 and some of these drugs may depend on OATP1A2 to reach their site of action (e.g. hydrophilic anti-migraine triptan drugs crossing the BBB to reach their receptors in the brain).

This leads us to propose the following central hypothesis: OATP1A2 is a key determinant in drug concentrations for organs in which they are expressed and thus, may be essential for drug PD effects.

The primary objectives of my PhD project consist of:

1. Characterizing the transport activity of OATP1A2 in regards to its substrates and inhibitors.
    a. Characterizing the HEK293-OATP1A2 and the HEK293-VC cells using rosuvastatin as the probe substrate.
    b. Identifying subsequent OATP1A2 substrates and inhibitors using rosuvastatin as the probe substrate in competition experiments.
2. Characterizing the activity of different OATP1A2 protein variants due to genetic polymorphisms.

Data obtained from the first objective of the project defined a structure-activity relationship where drugs composed of a tricyclic ring with a short aliphatic amine chain are potent OATP1A2 inhibitors. This leads to a second more specific hypothesis: tricyclic compounds, such as tricyclic antidepressant drugs, may block OATP1A2 located at the BBB; thus preventing the passage of CNS-active hydrophilic drugs depending on OATP1A2 to reach their site of action.

Triptans are commonly used in the treatment of acute migraine attacks. It has been postulated that triptans may act at the central nervous system by binding to receptors found on central neurons. As these molecules are hydrophilic, facilitated transport is required for their passage across the BBB and OATP1A2 may be the key player. Although triptans are successful in treating migraine attacks, a proportion of patients fail to respond to their action. We believe that certain cases of non-responder may be explained by the failure of the drug in penetrating

the brain. The two factors potentially involved in restricting triptans access to the brain are DDIs through OATP1A2 or genetic variability affecting the activity of the transporter.

The objectives of the second hypothesis consist of:

1. Characterizing the transport of hydrophilic anti-migraine triptan drugs through OATP1A2.
2. Characterizing the potential for tricyclic compounds to inhibit triptans transport via OATP1A2.
3. Characterizing the potential for tricyclic compounds to inhibit triptans transport via OATP1A2 at clinically significant concentrations.
4. Characterizing the transport of triptans through OATP1A2 genetic variants.
5. Characterizing the potential for tricyclic compounds to inhibit triptans transport through OATP1A2 genetic variants.

Compounds with a tricyclic chain and aliphatic amine chain, such as tricyclic antidepressants and the β-blocker carvedilol, are expected to inhibit the transport of triptans through OATP1A2 at high potency. The inhibition is still expected with the most potent inhibitors at concentrations observed in the clinic. The relevance of such an interaction is that the concentration of the victim drug might fall below its therapeutic window in the brain. Consequently, the antimigraine activity may be abolished.

There are several lines of evidence supporting a mechanism of action of triptans in the CNS: 1) 5-HT_{1B} and 5-HT_{1D} receptors proteins, the receptors to which triptans bind to, are found on trigeminal sensory neurons; 2) activation of the trigeminal nucleus neurons by electrical stimulation is inhibited after administration of a triptan in animal models; 3) some patients experience CNS adverse events related to triptans; 4) studies using positron emission tomography (PET) show that zolmitriptan can penetrate the brain at therapeutic doses and can

bind to their receptors located in the CNS [102, 103, 145-150]. As the triptans mechanism of action may reside centrally, the characterization of OATP1A2 transport activity and its potential for being inhibited should be investigated.

The co-prescription of a triptan with a tricyclic antidepressant is not unusual. Migraine is often diagnosed in patients with mood disorders, such as depression, anxiety, panic disorder, and bipolar disorder [151, 152]. As a result, treatments for both conditions are commonly prescribed. Tricyclic antidepressants are not only prescribed for depression but also for other off-label uses such as obsessive-compulsive disorder, panic disorder, chronic pain, insomnia, premenstrual symptoms and bulimia. In addition, β-blockers and antidepressants, especially amitriptyline, are occasionally prescribed for the prevention of migraine attacks [153].

The next two sections present the work accomplished throughout my doctoral studies. The work introduced is to characterize the OATP1A2 transporter in relation to its substrates and inhibitors. In addition, pharmacogenomic studies with its two most common variant proteins will be presented. First, three manuscripts will be presented. Then, experiments not included in the manuscripts but still following the theme of the thesis will be presented.

SECTION 2: MANUSCRIPTS

SECTION 2.1

ARTICLE #1

Effects of β-blockers and tricyclic antidepressants on the activity of human organic anion transporting polypeptide 1A2 (OATP1A2)

2.1.1 OBJECTIVES

The primary objective of the first article was to characterize OATP1A2 for its substrates and inhibitors. The secondary objective was to establish a correlation between the structure of the drugs evaluated and their potential to inhibit OATP1A2.

2.1.2 INTRODUCTION

Choosing an appropriate *in vitro* method to study drug transporters is important for reproducibility purposes. Different *in vitro* models have been developed to study OATP1A2 transport. Transient models have been used such as *Xenopus Laevis* oocytes injected with the complementary RNA, transiently transfected HeLa cells, and HEK293 cells transduced with baculoviruses genetically modified to contain the OATP1A2 gene [46, 49, 55]. *Xenopus Laevis* oocytes can efficiently translate exogenous mRNA into proteins, very few endogenous membrane transporters are expressed on their surface, and since they are very large in size they can be used as a single cell model. However, the protein expression is transient, the oocytes have a limited lifespan, and the system is low-throughput. Transiently transfected cells are easy to work with and very high levels of expression can be achieved dependent on the type of transfection reagent. However, transfection efficiency is variable from one experiment to another, the system stability is limited to a few days, and transfection reagents are expensive at large scale. The baculovirus system is high-throughput, it allows the recombinant protein expression level to be modulated, it is relatively simple to use, and a broad range of cell lines and primary cells can be transduced. Although the baculovirus system offers many advantages, large quantities of virus are needed for scaling up and transduction of the cells is required before every assay, adding an extra step which results in additional days to the experiment. A HEK293 cell line stably overexpressing OATP1A2 was developed and used for the study of food-drug interactions [65]. The advantages of a stable model include reproducibility, possibility for high-throughput screening, and ease of use once the cell line has been established. For these reasons, a HEK293 cell line overexpressing OATP1A2 was used as *in vitro* model in all experiments presented in this thesis. The HEK293-OATP1A2 and

HEK293-VC cells were kindly provided by Dr. Markus Keiser and Dr. Werner Siegmund (Department of Clinical Pharmacology, Center of Drug Absorption and Transport, University Medicine Greifswald, Felix-Hausdorff-Str. 3, D-17487 Greifswald, Germany). The cell lines were previously characterized for their expression, localization, and function of OATP1A2 [65].

β-blockers have been shown to be transported by OATP1A2 in a *Xenopus Laevis* oocyte model. We wanted to first confirm these substrates in our HEK293 cell model. The most straightforward method to determine if a compound interacts with the transporter was to perform competition experiments. The test compound was placed in competition with a probe substrate, which was monitored for uptake in the HEK293 cells. When the transport of the probe substrate was diminished, it was an indication that the test compound was either a substrate or inhibitor of OATP1A2. The HMG-CoA reductase inhibitor, rosuvastatin, was chosen as a probe substrate for OATP1A2. Therefore, an analytical method sensitive enough to quantify intracellular rosuvastatin concentrations was also developed in this article.

2.1.3 ARTICLE

The authors' specific contributions in this article were as follow:

- Participated in research design: Jennifer Lu, Veronique Michaud, Jacques Turgeon
- Conducted experiments: Jennifer Lu, Liliam Gabriela Guilarte Moya, Henry Leung
- Contributed new reagents or analytic tools: Fleur Gaudette
- Performed data analysis: Jennifer Lu, Fleur Gaudette
- Wrote or contributed to the writing of the manuscript: Jennifer Lu, Veronique Michaud, Jacques Turgeon

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Effects of β-Blockers and Tricyclic Antidepressants on the Activity of Human Organic Anion Transporting Polypeptide 1A2 (OATP1A2)[S]

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Running Title: OATP1A2 block by β-blockers, tricyclic antidepressants

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Abbreviations: K_i, inhibitory constant; K_m, Michaelis constant; OATP, organic anion transporting polypeptide; SNP, single nucleotide polymorphism; V_{max}, maximal velocity

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Abstract

OATP1A2, a membrane drug-transporter expressed on important organs (such as the brain, kidney and intestine) may be a key element in the disposition of drugs. Previous studies demonstrated that it could transport a broad spectrum of substrates including endogenous molecules and clinically relevant drugs such as several β-blockers and HMG-CoA reductase inhibitors. The primary objective of this study was to investigate OATP1A2 transport activity using rosuvastatin as a probe-substrate and to evaluate competitive inhibition of its transport by β-blockers. Rosuvastatin transport was saturable with a K_m of 60.2 μM. With the exception of carvedilol (IC_{50} of 3.2 μM), all other β-blockers evaluated had a small or insignificant effect on OATP1A2-mediated uptake of rosuvastatin. Carvedilol differs from the other β-blockers by its tricyclic moiety in its chemical structure. As a secondary objective, the transport of a series of tricyclic compounds by OATP1A2 and their potential for rosuvastatin transport inhibition were evaluated. Tricyclic compounds were not OATP1A2 substrates. On the other hand, tricyclic compounds with a short aliphatic amine chain inhibited OATP1A2-mediated rosuvastatin transport. Our data suggest that these drugs may modulate the transport of OATP1A2 substrates and may affect drug actions.

Introduction

OATP1A2 is a membrane drug transporter expressed on organs important for drug disposition. The mRNA expression is found the highest in the brain, followed by the kidney, liver, lung, and the testes (Kullak-Ublick et al., 1995). OATP1A2 protein is found on the luminal membrane of the brain capillary endothelial cells which make up the blood-brain barrier, apical membranes of cholangiocytes in the liver, apical membrane of the distal nephrons in the kidney, and the apical membrane of enterocytes in the duodenum (Gao et al., 2000; Lee et al., 2005; Glaeser et al., 2007). OATP1A2 expression in the small intestine is controversial as recent studies could not detect its presence in the entire intestine (Groer et al., 2013; Drozdzik et al., 2014). Due to its location, the following roles have been attributed to OATP1A2: distribution of substrates to the brain, reabsorption of substrates excreted in the bile, reabsorption or secretion of xenobiotics into urine, and oral absorption of xenobiotics.

OATP1A2 transports various endogenous molecules such as bile salts and hormones (triiodothyronine, thyroxine, and steroid conjugates) (Kullak-Ublick et al., 1995; Kullak-Ublick et al., 1998; Fujiwara et al., 2001; Lee et al., 2005). Based on the endogenous substrates it transports and its localization, it has been proposed that OATP1A2 may be involved in the regulation of several physiological processes. For instance, it may be implicated in the delivery of thyroid hormones to the brain and the kidney as well as removal of thyroid hormones from the periphery (Hagenbuch, 2007). In addition, it may play a role in bile acids transport as a study has shown that OATP1A2 mRNA is upregulated in patients with cholestatic liver disease (Kullak-Ublick et al., 1997).

OATP1A2 can also transport several exogenous substances including peptide agonists of the σ-opioid receptor and bromosulfophthalein (Kullak-Ublick et al., 1995; Gao et al., 2000). Several clinically important drugs such as fexofenadine, imatinib, methotrexate, pravastatin, and rosuvastatin are also transported by OATP1A2 (Cvetkovic et al., 1999; Badagnani et al., 2006; Ho et al., 2006; Hu et al., 2008; Shirasaka et al., 2010). Rosuvastatin is a hydrophilic molecule and therefore, depends on transporters to move across the plasma membrane. Rosuvastatin has high affinity for OATP transporters as its K_m was determined to be 2.6 μM, 4.0 μM, 9.8 μM, 2.4 μM for OATP1A2, OATP1B1, OATP1B3, and OATP2B1, respectively (Ho et al., 2006). Finally, OATP1A2 is inhibited by various flavonoids, such as naringin, apigenin, kaempferol, quercetin, and several flavonoids found in green tea (Bailey et al., 2007; Mandery et al., 2010; Roth et al., 2011; Misaka et al., 2014).

Flavonoids are found in vegetables, fruits, and plants; thus, pharmacokinetic studies using flavonoids focused on intestinal interactions. Bailey et al. have demonstrated that ingestion of fexofenadine and a solution of naringin decreased fexofenadine maximum plasma concentration (C_{max}) and the area under the plasma concentration-time curve (AUC) (Bailey et al., 2007). Misaka et al. have shown that green tea decreased nadolol C_{max} and AUC (Misaka et al., 2014). However, these interactions assume that OATP1A2 is an intestinal uptake transporter in the human intestine.

Single nucleotide polymorphisms (SNPs) in the gene encoding OATP1A2, SLCO1A2, resulting in impaired cell surface expression and reduced OATP1A2 activity have been discovered in healthy individuals (Lee et al., 2005; Badagnani et al., 2006; Laitinen and Niemi, 2011). This suggests that OATP1A2 may not play a fundamental role in physiological functions but it may act as a secondary transporter for endogenous molecules. However, there is evidence that OATP1A2 may be important in drug disposition as Yamakawa et al. demonstrated that imatinib clearance is affected in chronic myeloid leukemia patients with the *SLCO1A2* –361G>A genotype (Yamakawa et al., 2011).

A previous study has proposed that several β-blockers are OATP1A2 substrates (Kato et al., 2009). Initially, their study aimed at determining the transporters involved in the gastrointestinal absorption of celiprolol as to understand the food-drug interaction induced by citrus juices using an animal model. They demonstrated an increase in plasma concentrations of celiprolol in *mdr1a/b*$^{-/-}$ mice compared to wild-type mice. Using isolated tissues of the mouse small intestine, they demonstrated competitive inhibition between celiprolol and bromosulfophthalein for transport from the apical to basal side. Their results suggested the involvement of P-glycoprotein and an influx transporter in the absorption of celiprolol. Subsequently, using *Xenopus Laevis* oocytes the uptake transporter involved was shown to be OATP1A2. In addition, they tested several other β-blockers and showed that acebutolol, atenolol, nadolol, sotalol, and labetalol are OATP1A2 substrates. More recently, a study has also shown that nadolol is transported by OATP1A2 in HEK293 cells stably expressing the transporter (Misaka et al., 2014).

Considering OATP1A2 location on important organs involved in drug disposition (such as the brain), it is of interest to investigate this transporter further. Based on current knowledge, the primary objectives of this study were 1) to assess rosuvastatin as a probe-substrate for OATP1A2; and 2) to determine whether there is competition between rosuvastatin and β-blockers for transport through OATP1A2. Considering the results obtained throughout the course of our studies, secondary objectives were 1) to evaluate the transport of different tricyclic compounds through OATP1A2; and 2) to determine whether there is competition between rosuvastatin and tricyclic compounds for transport through OATP1A2. Experiments were conducted using a HEK293 cell line stably overexpressing OATP1A2. This *in vitro* model was used since it offers many advantages over transient models such as stable expression, possibility for high-throughput screening, and ease of use once the cell line has been established.

Materials and Methods

Materials

Acebutolol hydrochloride, alprenolol tartrate salt, amitriptyline hydrochloride, atenolol, carbamazepine, carbazole, chlorpromazine hydrochloride, clomipramine hydrochloride, desipramine hydrochloride, imipramine hydrochloride, metoprolol tartrate salt, nadolol, naproxen, nortriptyline hydrochloride, phenothiazine, propranolol hydrochloride, timolol maleate salt, trimipramine maleate salt were purchased from Sigma-Aldrich (St-Louis, MO, USA). Carazolol hydrochloride, carvedilol, celiprolol hydrochloride, doxepin hydrochloride, rosuvastatin calcium salt were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Sotalol hydrochloride was a gift from Bristol-Myers Squibb (Montreal, Canada). All chemicals and solvent were obtained from Sigma-Aldrich, Fisher Scientific (Fair Lawn, NJ, USA) or J.T. Baker (Center Valley, PA, USA).

Cell culture

HEK293-OATP1A2 and HEK293-VC cells were kindly provided by Dr. Markus Keiser and Dr. Werner Siegmund (Department of Clinical Pharmacology, Center of Drug Absorption and Transport, University Medicine Greifswald, Felix-Hausdorff-Str. 3, D-17487 Greifswald, Germany). The cells were cultured in minimum essential medium eagle's (EMEM) containing 10% fetal bovine serum, 1X nonessential amino acids and 1X sodium pyruvate at 37°C and 5% CO_2. Cell culture media and supplements were purchased from Multicell Wisent Inc (St-Jean-Baptiste, QC, Canada); whereas, fetal bovine serum was obtained from HyClone Thermo Scientific (Logan, UT, USA).

Uptake assays and competition assays

HEK293-OATP1A2 and HEK293-VC cells were seeded in tissue culture plates (6-well or 12-well) previously treated with poly-L-lysine (Sigma-Aldrich). The number of cells seeded in 6-well plates was 1.5 x 10^6 cells/well and 7.5 x 10^5 cells/well in 12-well plates. After 24 h, the culture media was removed and the cells were pre-incubated with warm transport buffer (142 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3) at 37°C for 5 min. Following the pre-incubation period, the cells were incubated with transport buffer containing rosuvastatin in the presence or absence of an inhibitor at 37°C for 2 min. After incubation, the cells were washed twice with phosphate-buffer saline (PBS) containing 10% acetonitrile followed by a final wash with PBS. Rosuvastatin transport (60 μM) at different time points was done in 6-well plates by incubating HEK293-OATP1A2 and HEK293-VC cells. The K_m and V_{max} of rosuvastatin transport through OATP1A2 was determined by incubating HEK293-OATP1A2 and HEK293-VC cells in 6-well plates with rosuvastatin at concentrations ranging from 10 μM to 250 μM. To determine whether a compound can block OATP1A2, HEK293-OATP1A2 and HEK293-VC cells were co-incubated in 12-well plates with rosuvastatin (150 μM), which was used as the probe-substrate, in the absence or presence of different β-blockers (1.5 – 100 μM) or different tricyclic compounds (12.5 nM – 250 μM). A concentration of 150 μM rosuvastatin (3-times K_m value) was selected in order to saturate the OATP1A2 transporter with the probe-substrate. The inhibitory constant (K_i) of the tricyclic drugs for OATP1A2 was determined by incubating HEK293-OATP1A2 and HEK293-VC cells in 6-well plates with various concentrations of rosuvastatin (25 – 250 μM) in the absence or presence of the tricyclic drugs

(0.5 - 50 µM). Uptake of carvedilol was assessed in 12-well plates at a concentration of 2 µM for 2 min at 37°C. The pre-incubation and washing steps are the same as for rosuvastatin.

The protein concentration was measured in three wells of cells lyzed with 1% SDS + 0.2 N NaOH using the Pierce BCA Protein Assay Kit from Thermo Scientific (Rockford, IL, USA).

Quantification of rosuvastatin by HPLC-UV

The quantity of rosuvastatin transported in the cells was measured by HPLC with UV detection. The instrumentation consisted of a SpectraSystem P4000 pump, a SpectraSystem AS3000 autosampler, a Finnigan SpectraSystem UV6000 ultraviolet detector and a SpectraSystem SN4000 System Controller from Thermo Electron Corporation (San Jose, CA, USA). The ChromQuest Version 4.2.34 software was used for data acquisition. The samples were separated on a Phenomenex Luna 5u C8 column (150 mm x 4.6 mm 5 µM; Phenomenex, CA, USA). The mobile phase consisted of a mixture containing 10 mM ammonium formate pH 3 and acetonitrile (57:43 v/v). The flow rate was set at 1.2 ml/min and the column was heated at 40°C. Naproxen was used as the internal standard. The retention times of rosuvastatin and naproxen were 4.8 and 6.1 min., respectively. The peaks were monitored at a wavelength of 243 nm. The lowest limit of quantification was 25 ng/ml. The calibration curve was linear between 25 ng/ml to 25 000 ng/ml (r^2 of 0.996). The interday coefficient of variation (CV) for the calibration curve using four levels of quality controls (25, 100, 2 500, and 25 000ng/ml) ranged between 3.9 and 7.8%. The interday accuracy ranged between 94.4 and 98.4%. The intraday CV for the four levels of quality control ranged between 1.1 and 13.6%. The intraday accuracy ranged between 84.5 and 101.4 %.

After the final wash with PBS, the samples were processed as follow; the cells were lyzed with methanol containing naproxen (100 ng/ml). The cell lysate was transferred to a 1.7 mL

microtube and the samples were spun down at max speed for 10 min at room temperature. The supernatant was transferred to a culture borosilicate glass tube, evaporated to dryness, and reconstituted in 100 µl of 10 mM ammonium formate pH 3 and acetonitrile (57:43 v/v). A volume of 20 µl per sample was injected.

Quantification of carvedilol by HPLC-fluorescence

The same system was used for the quantification of carvedilol than for rosuvastatin except for the detector which was a SpectraSystem FL3000 ultraviolet detector from Thermo Electron Corporation (San Jose, CA, USA). The samples were separated on an Eclipse XDB-C8 column (150 mm x 4.6 mm 5 µM; Agilent, USA). The mobile phase consisted of a mixture of 50 mM potassium phosphate monobasic pH 3.5 and acetonitrile (60:40 v/v). The flow rate was set at 1.0 ml/min and the column was heated at 50°C. Propranolol was used as the internal standard. The retention times of carvedilol and propranolol were 2.9 and 2.2 min., respectively. The excitation and emission wavelength were 242 nm and 344 nm, respectively. After the final wash in PBS, the samples were processed similarly to the rosuvastatin samples with the following exceptions: the cells were lyzed with methanol containing propranolol (200 ng/ml) and the samples were reconstituted in 500 µl of 50 mM potassium phosphate monobasic pH 3.5. The lowest limit of quantification was 50 ng/ml. The calibration curve was linear over a concentration range of 50 ng/ml to 2000 ng/ml (r^2 of 0.998). The interday coefficient of variation (CV) for the calibration curve using four levels of quality controls (50, 80, 200, 2000 ng/ml) ranged between 3.9% and 8.2%. The interday accuracy ranged between 95.3 and 99.3%. The intraday CV for the four levels of quality control ranged between 3.0 and 10.3%. The intraday accuracy ranged between 91.4 and 103.3%.

Data analysis

The net transport of rosuvastatin through OATP1A2 was calculated by subtracting the value in VC cells from the value in OATP1A2 cells. Data were analyzed using GraphPad Prism5 (La Jolla, CA). Each data point is expressed as the mean ± S.D. K_m and V_{max} were calculated by fitting the data to the Michaelis-Menten equation. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation and the range given represents the 95% confidence interval. Transport inhibitions of different concentrations of rosuvastatin by increasing concentrations of inhibitors were plotted in a Dixon plot. Linear regression was used to fit each set of data and the intercept of all lines represents the - K_i. The K_i was accurately calculated by the equations:

$$y = V_{max} \times \left(\frac{x}{K_{mapp} + x} \right)$$

$$K_{mapp} = K_m \times \left(1 + \frac{l}{K_i} \right)$$

An initial value of 1.0 was set for all parameters. The rule for initial value for K_m was set to *XMID and V_{max} was set to *YMAX. A shared value for all data sets was set as default constraint for K_m, K_i, and V_{max} and a constant data set (=column title) was set for the parameter *l*.

Results

Transport of rosuvastatin through OATP1A2

Rosuvastatin transport via OATP1A2 was characterized using a HEK293 cell model stably expressing this transporter. Transport during different time-points demonstrated that rosuvastatin uptake was linear between 1 and 10 min (Figure 1A). An incubation time of 2 min was chosen for all experiments as it remains in the linear range. Overall, the transport activity varied from 1395 pmol/mg protein/min to 8056 pmol/mg protein/min over a range of concentrations of 10 µM to 250 µM of rosuvastatin. A saturable transport was observed with a K_m of 60.2 ± 6.0 µM, the V_{max} was 9741 ± 543 pmol/mg protein/min, and the intrinsic clearance was 161.8 µl/mg protein/min (Figure 1B).

Effect of β-blockers on rosuvastatin uptake through OATP1A2

To determine whether β-blockers are OATP1A2 inhibitors, competition studies were performed using rosuvastatin as probe-substrate (Figure 2). Carvedilol was the only β-blocker able to fully inhibit OATP1A2-mediated uptake of rosuvastatin and it was the most potent inhibitor with an IC_{50} of 3.2 µM. Metoprolol, propranolol, acebutolol, alprenolol, celiprolol, nadolol, and timolol had a small effect on OATP1A2-mediated uptake of rosuvastatin. Given that complete inhibition could not be achieved by these β-blockers at the concentrations tested, the IC_{50} could not be calculated appropriately. Sotalol and atenolol demonstrated no significant effect on rosuvastatin uptake.

Effect of tricyclic compounds on rosuvastatin uptake through OATP1A2

By observing the structures of all β-blockers tested, it can be noticed that carvedilol differs from the others by the presence of a tricyclic moiety in its structure (Figure 3 and Supplemental Figure 1A). Inhibition studies were performed with compounds with a similar

structure using rosuvastatin as the probe-substrate to determine whether the tricyclic ring is responsible for carvedilol strong inhibitory effect on OATP1A2 (Figure 3, Table 1, Supplemental Figures 1B and 2). Carazolol exerted the strongest inhibition with an IC_{50} of 3.7 µM. The inhibition potencies were followed by clomipramine (IC_{50} = 8.2 µM), amitriptyline (IC_{50} = 11.7 µM), chlorpromazine (IC_{50} = 12.0 µM), doxepin (IC_{50} = 12.1 µM), trimipramine (IC_{50} = 15.0 µM), imipramine (IC_{50} = 16.9 µM), nortriptyline (IC_{50} = 25.0 µM), and desipramine (IC_{50} = 37.0 µM). Carbamazepine, carbazole, and phenothiazine exerted no significant effect on OATP1A2-mediated uptake of rosuvastatin.

Transport of tricyclic compounds through OATP1A2

Comparison between structures of the compounds that inhibited OATP1A2 and those that exerted no effect reveals that a molecule composed of a tricyclic ring with a short aliphatic amine chain is able to inhibit OATP1A2 activity. As several tricyclic drugs inhibited rosuvastatin uptake, it was relevant to determine if they are also substrates of OATP1A2. Analytical methods were developed for each drug and transport assays were conducted. Given carvedilol showed the strongest inhibitory effect on rosuvastatin transport, it was the first tricyclic drug assessed. Incubation of carvedilol with HEK293-OATP1A2 and HEK293-VC showed no difference in intracellular concentrations of carvedilol between the two cell lines (Figure 4). Thus, carvedilol is not a substrate of OATP1A2 but an inhibitor. Other tricyclic drugs such as amitriptyline, doxepin, trimipramine, and imipramine were also evaluated but it was found that none of the drugs were OATP1A2 substrates. Only the results for carvedilol are presented as an example since all graphs were similar.

Determination of inhibition constant of tricyclic compounds

The inhibitory constant (K_i) of previously identified inhibitors for OATP1A2 transport were determined using rosuvastatin as a probe-substrate. A Dixon plot was drawn for each inhibitor and the K_i was calculated (Table 2, Figure 5, Supplemental Figure 3). Carvedilol showed the lowest K_i value (1.1 μM) implying it as the strongest inhibitor evaluated. The inhibition potencies were followed by trimipramine (2.8 μM), carazolol (3.2 μM), clomipramine (3.3 μM), imipramine (3.5 μM), amitriptyline (3.7 μM), doxepin (4.7 μM), chlorpromazine (5.3 μM), desipramine (8.4 μM), and nortriptyline (12.0 μM).

Discussion

A method to study drug-drug interactions between substrates of OATP1A2 using rosuvastatin as a probe substrate was developed and validated. Inhibition studies between the probe-substrate and different β-blockers demonstrated that carvedilol was the most potent inhibitor. The other β-blockers evaluated had little or no significant effects. Furthermore, a structure-activity relationship established from the tricyclic compounds evaluated demonstrated that the transport activity of OATP1A2 was inhibited by compounds composed of a tricyclic ring and a short aliphatic amine chain. These compounds were not found to be transported by OATP1A2.

The K_m of rosuvastatin for OATP1A2 in this study was determined as 60.2 μM, which is higher than the previously published K_m (2.6 μM) (Ho et al., 2006). Our results also show a superior efficiency (V_{max}/K_m) of 161.8 compared to 1 μl/mg protein/min (Ho et al., 2006). This discrepancy may be explained by the different in vitro models employed. Ho et al. used transiently transfected HeLa cells while a HEK293-OATP1A2 stable cell line was used in this study. The major setback with transiently transfected cells is the lack of reproducibility from one experiment to another. Variability may even arise within a single experiment from one well of transfected cells to another. A stable cell line offers uniformity within a cell population and simplicity once the cell line has been developed. Due to these advantages, a stable cell line was used in this study.

The β-blockers evaluated, with the exception of carvedilol, were not able to compete with rosuvastatin for OATP1A2 transport. These results imply that they are either not substrates of OATP1A2 as previously reported or they have a weaker affinity for the transporter than rosuvastatin. A K_m of 84.3 μM for nadolol has been reported, which supports the second explanation (Misaka et al., 2014). It remains to be determined for the other β-blockers. Carvedilol blocked OATP1A2-mediated uptake of rosuvastatin very efficiently without being transported by it. Other compounds evaluated with a similar structure also blocked OATP1A2-mediated uptake of rosuvastatin. These results suggest that compounds composed of a tricyclic ring and a short aliphatic amine chain could potentially block the transport of OATP1A2 substrates. Based on this structure-relationship finding, tricyclic antidepressants have been selected to further investigate OATP1A2 uptake and transport inhibition. Our results observed with the tricyclic antidepressants strongly suggest that these structural features appear to be determinant for the inhibition of OATP1A2 but not to mediate substrate uptake transport.

Drug-drug interaction studies involving the transporter have mainly focused on drug absorption since OATP1A2 expression has previously been detected at the duodenum by immunohistochemistry (Glaeser et al., 2007). Several publications demonstrated that fruit juices and green tea decreased the bioavailability of OATP1A2 substrates (Dresser et al., 2005; Bailey et al., 2007; Glaeser et al., 2007; Rebello et al., 2012; Misaka et al., 2014). However, other publications failed to prove in human an interaction demonstrated in a cell model (Eechoute et al., 2011). In addition, Misaka et al. showed that green tea decreased the C_{max} and AUC of nadolol and their results suggest that the interaction is in part mediated by OATP1A2 (Misaka et al., 2014). In contrast, grapefruit juice, an established inhibitor of

OATP1A2, did not have the same effect on nadolol (Misaka et al., 2013). A recent study looking at influx and efflux drug transporters in the small intestine using the LC-MS/MS demonstrated that OATP1A2 was not expressed in any segment of the intestine and other influx transporters, such as OATP2B1, PEPT1 and OCT1, may be implicated in the absorption of drugs instead (Groer et al., 2013; Drozdzik et al., 2014). This may explain the inconsistency in studies where *in vivo* and *in vitro* data do not corroborate and conflicting results among different clinical studies using the same inhibitor.

As OATP1A2 is expressed on the luminal membrane of endothelial cells from the blood-brain barrier, it may potentially be involved in the distribution of drugs to the brain. Tricyclic antidepressants must cross the blood-brain barrier to reach their site of action and we showed that these drugs are inhibitors of OATP1A2. It could be speculated that the co-administration of a tricyclic antidepressant with a CNS drug substrate for OATP1A2 may lead to a drug-drug interaction when both drugs meet at the blood-brain barrier. It could result into a loss in efficacy by limiting their penetration to the brain. Cheng et al. conducted a structure-activity relationship study using triptan structural analogs and demonstrated that an amine atom was necessary for efficient uptake through OATP1A2 and that the uptake rate was the highest for tertiary amine followed by secondary then primary amine (Cheng et al., 2012). Likewise, tricyclic antidepressants, which are also CNS active drugs, share some general similarities with triptans and β-blockers including an amine residue within their structure, a tricyclic ring and a short aliphatic chain.

Taken together, the data in this study showed that compounds composed of a tricyclic ring with a short aliphatic amine chain inhibited OATP1A2 activity. Tricyclic antidepressants are a class of medication with such structure and we demonstrated their strong inhibition on OATP1A2-mediated transport of rosuvastatin. Such an interaction may potentially be significant for CNS-active drugs that use OATP1A2 to cross the blood-brain barrier. Future work needs to be done to assess whether OATP1A2-mediated transport of CNS-active drugs can be blocked by tricyclic antidepressants. As well, the clinical relevance of such an interaction needs to be investigated further.

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We thank Dr. Markus Keiser and Dr. Werner Siegmund (Department of Clinical Pharmacology, Center of Drug Absorption and Transport, University Medicine Greifswald, Greifswald, Germany) for kindly providing the HEK293-OATP1A2 and the HEK293-VC cell lines.

Authorship Contributions

Participated in research design: Lu, Michaud, Turgeon

Conducted experiments: Lu, Guilarte Moya, Leung

Contributed new reagents or analytic tools: Gaudette

Performed data analysis: Lu, Gaudette

Wrote or contributed to the writing of the manuscript: Lu, Michaud, Turgeon

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Footnotes

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- Canadian Society of Pharmacology and Therapeutics (CSPT), Toronto ON, June 12-15 2012.
- Club de Recherches Cliniques du Quebec (CRCQ), Orford QC, October 11-13 2012.
- Centre de Recherche du CHUM (CRCHUM) 15th annual meeting, Montreal QC, December 18 2012.
- American Society for Clinical Pharmacology and Therapeutics (ASCPT) 114th Annual Meeting, Indianapolis IN, March 5-9 2013.
- Canadian Society of Pharmacology and Therapeutics (CSPT), Boston MA, April 21-24 2013.
- American Society for Clinical Pharmacology and Therapeutics (ASCPT) 115th Annual Meeting, Atlanta GA, March 19-22 2014.

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Figure Legends

Fig. 1. OATP1A2-mediated transport of rosuvastatin. (A) Uptake of 60 μM rosuvastatin in HEK293-OATP1A2 and HEK293-VC cells was assessed for 1, 2, 3, 5, and 10 min at 37°C (B) Uptake of rosuvastatin (10, 25, 50, 75, 100, and 250 μM) in HEK293-OATP1A2 and HEK293-VC cells was assessed for 2 min at 37°C. The quantity of intracellular rosuvastatin was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. K_m and V_{max} were calculated by fitting the data to the Michaelis-Menten equation. Each point represents the mean ± S.D. of triplicate from a single experiment.

Fig. 2. Inhibition of OATP1A2-mediated transport of rosuvastatin by different β-blockers. HEK293-OATP1A2 and HEK293-VC cells were coincubated with rosuvastatin (150 μM) and different β-blockers (1.5 – 100 μM; up to 200 μM for carvedilol) for 2 min at 37°C. The quantity of intracellular rosuvastatin measured was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation in GraphPad Prism. (A) (●) metoprolol; (■) propranolol; (▲) acebutolol; (B) (●) alprenolol; (■) celiprolol; (▲) nadolol; (C) (●) timolol; (■) atenolol; (▲) sotalol; (D) (●) carvedilol. Each point represents the mean ± S.D. of triplicate from a single experiment. The values in parentheses represent the 95% confidence interval.

Fig. 3. Chemical structures of compounds evaluated. Structure of carvedilol, carbazole, amitriptyline, propranolol and the list of structurally similar compounds evaluated.

Fig. 4. Intracellular concentrations of carvedilol. HEK293-OATP1A2 and HEK293-VC cells were incubated in a solution of 2 μM carvedilol for 2 min at 37°C. The quantity of intracellular carvedilol was normalized to protein content. Each column represents the mean ± S.D. of triplicate from a single experiment.

Fig. 5. Dixon plot of inhibition of OATP1A2-mediated transport of rosuvastatin by carvedilol. HEK293-OATP1A2 and HEK293-VC cells were coincubated with rosuvastatin (25, 50, 100, 250 μM) and carvedilol (0.5 – 10 μM) for 2 min at 37°C. The quantity of intracellular rosuvastatin was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. The x-axis represents the concentration of the inhibitor and the y-axis represents the reciprocal velocity (1/V). Linear regression was used to fit each set of data and the intercept of all lines represents the - K_i. The K_i was accurately calculated in GraphPad Prism. (●) 25 μM rosuvastatin; (■) 50 μM rosuvastatin; (▲) 100 μM rosuvastatin; (▼) 250 μM rosuvastatin. The values in parentheses represent the 95% confidence interval.

Table 1. IC_{50} values from the inhibition of rosuvastatin uptake through OATP1A2 by different tricyclic compounds. The values in parentheses represent the 95% confidence interval (See also Supplemental Figure 2).

Compound	IC_{50} (µM)
amitriptyline	11.7 (9.1 - 15.0)
carazolol	3.7 (2.9 – 4.8)
carvedilol	3.2 (2.7 – 3.9)
chlorpromazine	12.0 (7.5 – 19.2)
clomipramine	8.2 (5.2 – 13.0)
desipramine	37.0 (22.3 – 61.4)
doxepin	12.1 (7.1 – 20.6)
imipramine	16.9 (13.2 – 21.6)
nortriptyline	25.0 (13.2 – 47.6)
trimipramine	15.0 (8.1 – 27.8)
carbamazepine	No effect
carbazole	No effect
phenothiazine	No effect

Table 2. K_i values from the inhibition of rosuvastatin uptake through OATP1A2 by different tricyclic compounds. The values in parentheses represent the 95% confidence interval (See also Supplemental Figure 3).

Compound	K_i (μM)
amitriptyline	3.7 (2.9 – 4.6)
carazolol	3.2 (2.2 – 4.1)
carvedilol	1.1 (0.9 – 1.3)
chlorpromazine	5.3 (4.4 – 6.1)
clomipramine	3.3 (2.7 – 3.9)
desipramine	8.4 (6.7 – 10.0)
doxepin	4.7 (3.6 – 5.8)
imipramine	3.5 (2.5 – 4.5)
nortriptyline	12.0 (9.0 – 15.0)
trimipramine	2.8 (2.1 – 3.5)

Figure 1

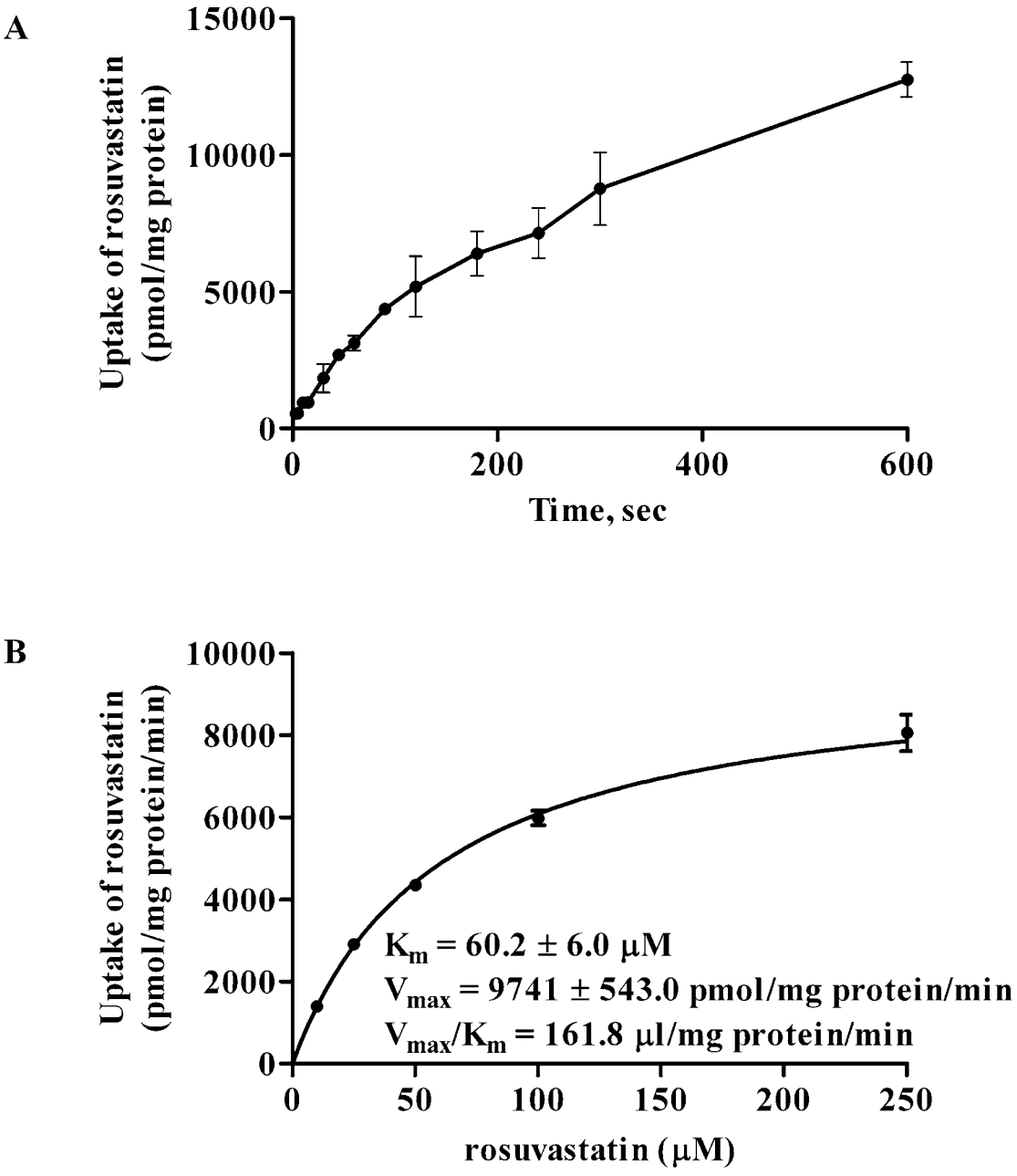

A
15000
10000
5000
0
Uptake of rosuvastatin (pmol/mg protein)
0
200
400
600
Time, sec
B
10000
8000
6000
4000
2000
0
Uptake of rosuvastatin (pmol/mg protein/min)
K_m = 60.2 ± 6.0 μM
V_{max} = 9741 ± 543.0 pmol/mg protein/min
V_{max}/K_m = 161.8 μl/mg protein/min
0
50
100
150
200
250
rosuvastatin (μM)

Figure 2

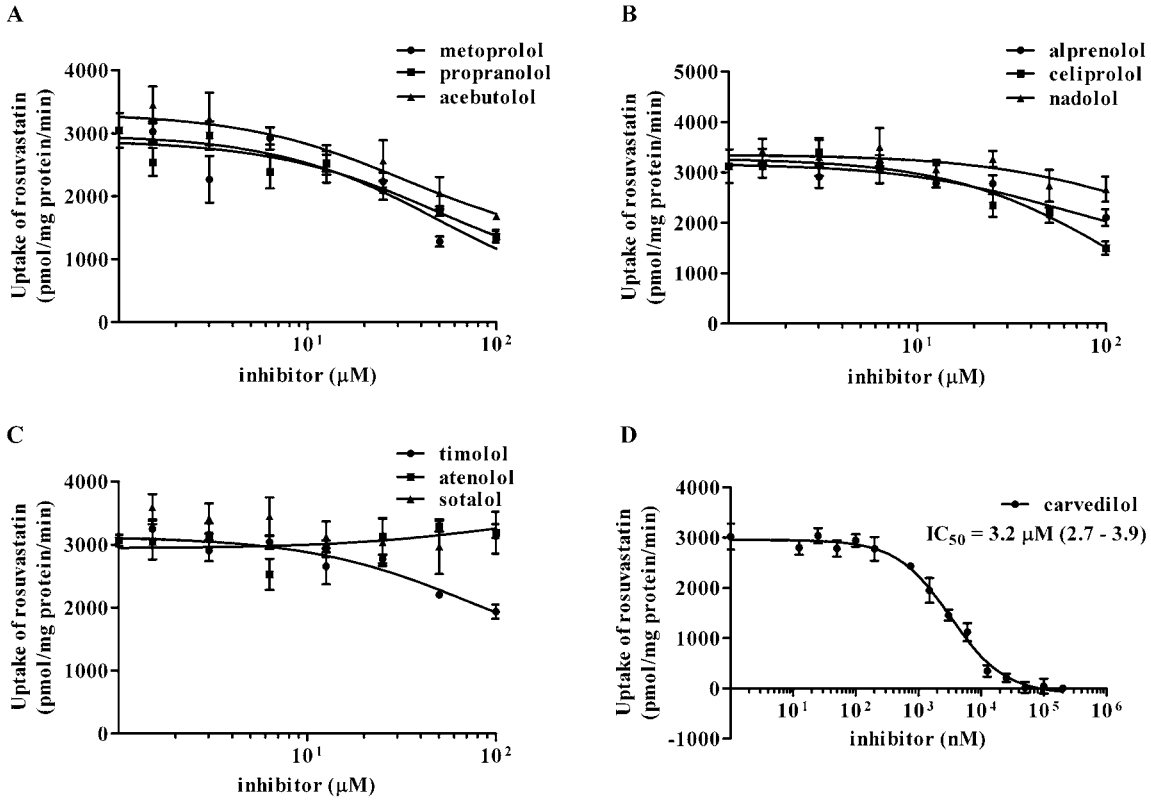
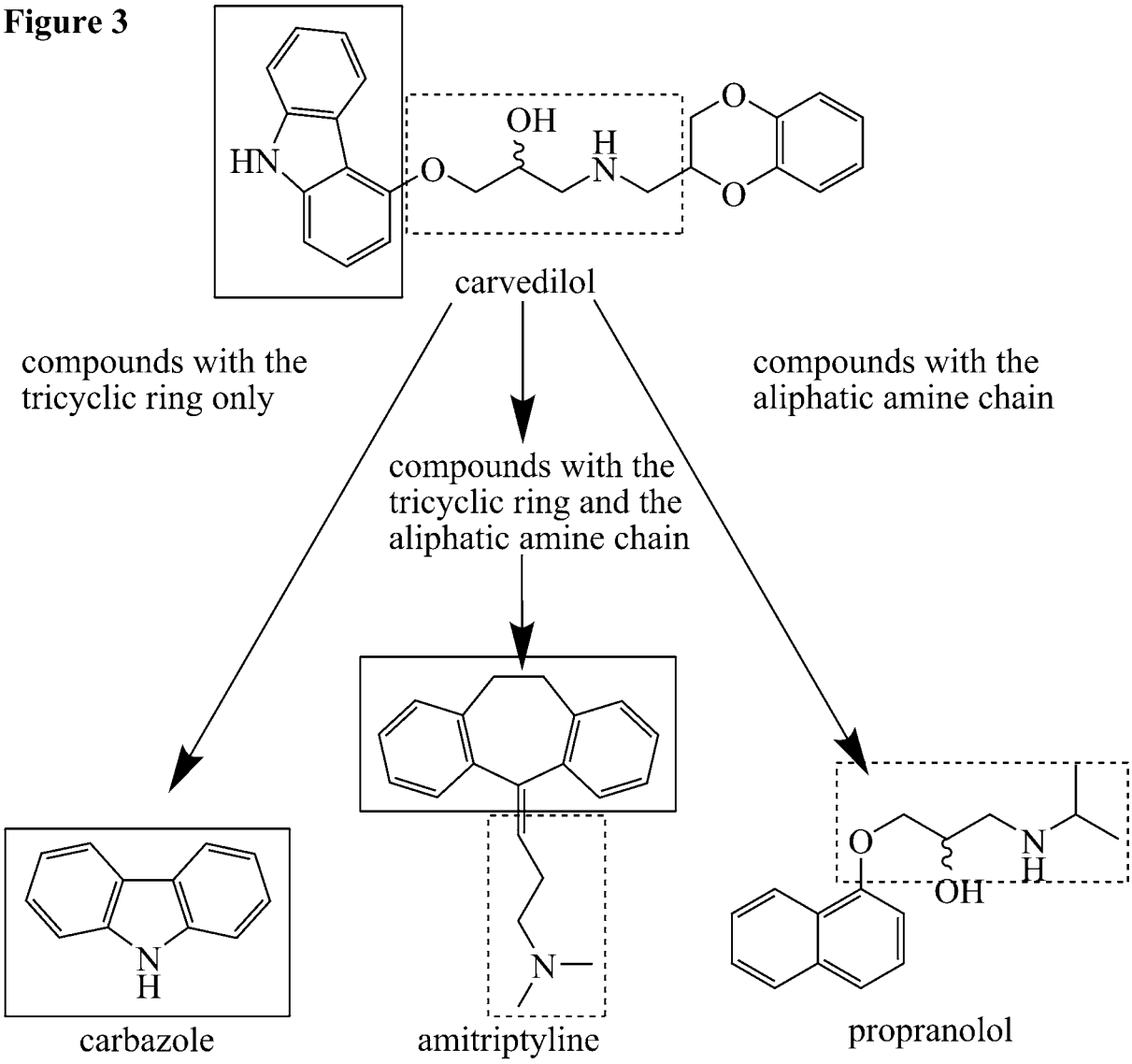

A
metoprolol
propranolol
acebutolol
Uptake of rosuvastatin (pmol/mg protein/min)
inhibitor (μM)
B
alprenolol
celiprolol
nadolol
Uptake of rosuvastatin (pmol/mg protein/min)
inhibitor (μM)
C
timolol
atenolol
sotalol
Uptake of rosuvastatin (pmol/mg protein/min)
inhibitor (μM)
D
carvedilol
IC50 = 3.2 μM (2.7 - 3.9)
Uptake of rosuvastatin (pmol/mg protein/min)
inhibitor (nM)

Figure 3



carbamazepine
phenothiazine

carazolol
chlorpromazine
clomipramine
desipramine
doxepin
imipramine
nortriptyline
trimipramine

alprenolol
metoprolol
timolol
acebutolol
nadolol
celiprolol
sotalol
atenolol

Figure 4

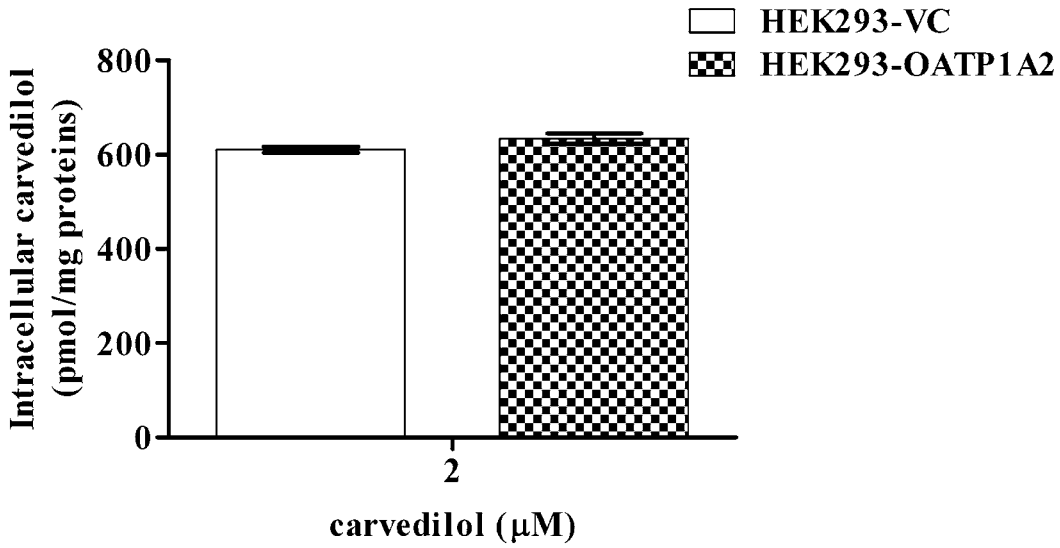
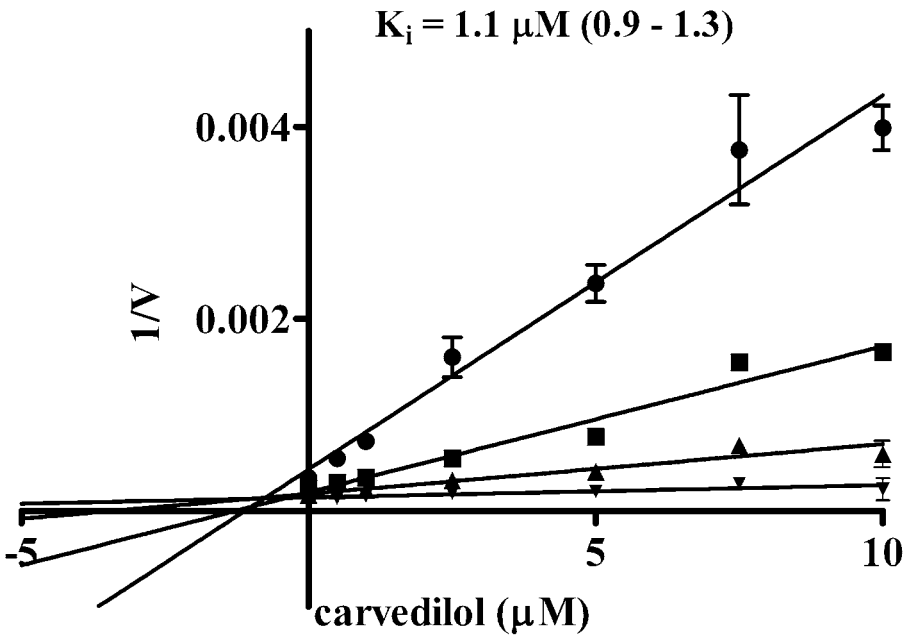

HEK293-VC
HEK293-OATP1A2
Intracellular carvedilol (pmol/mg proteins)
800
600
400
200
0
2
carvedilol (μM)

Figure 5

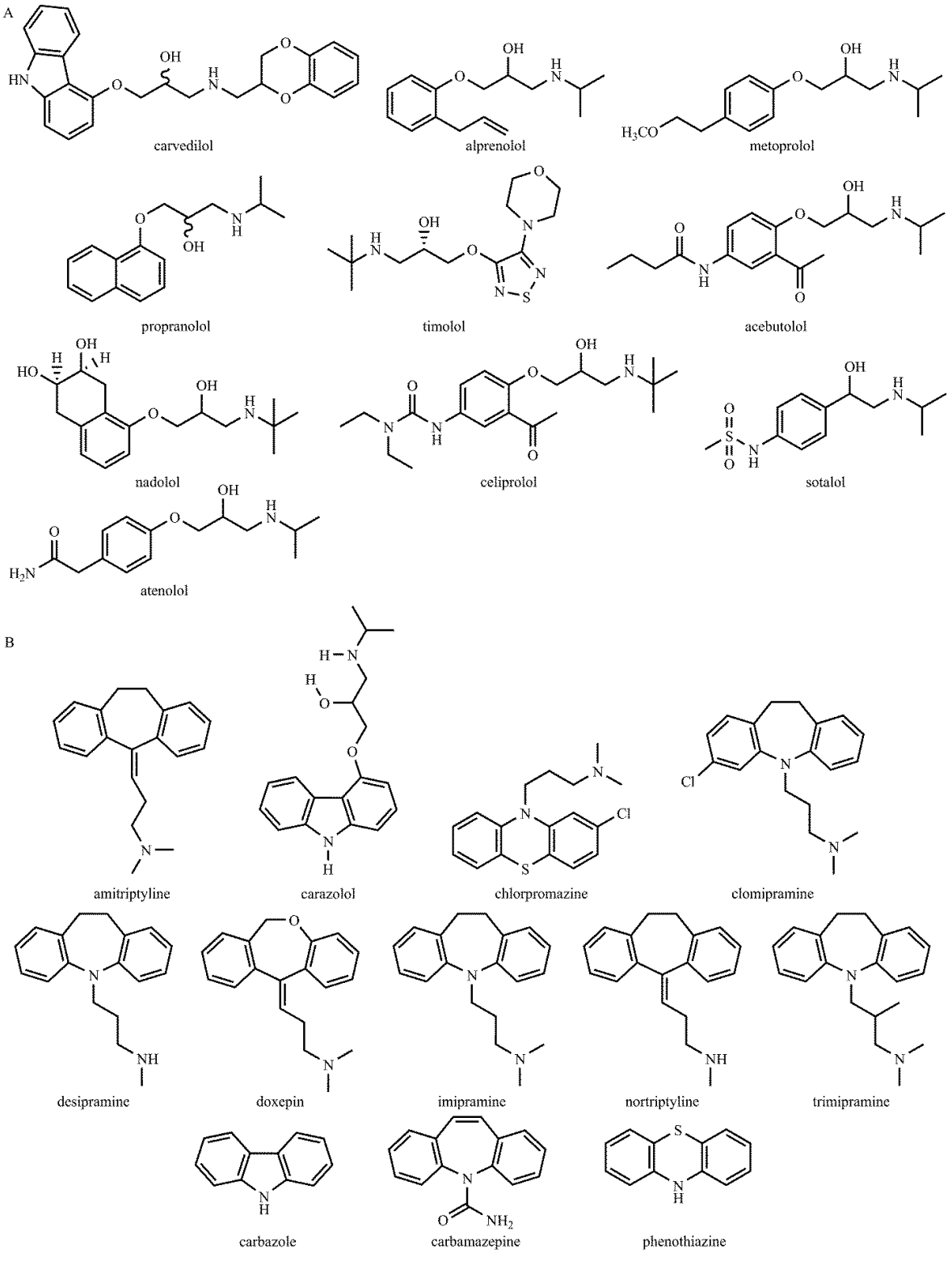


Supplemental Figures

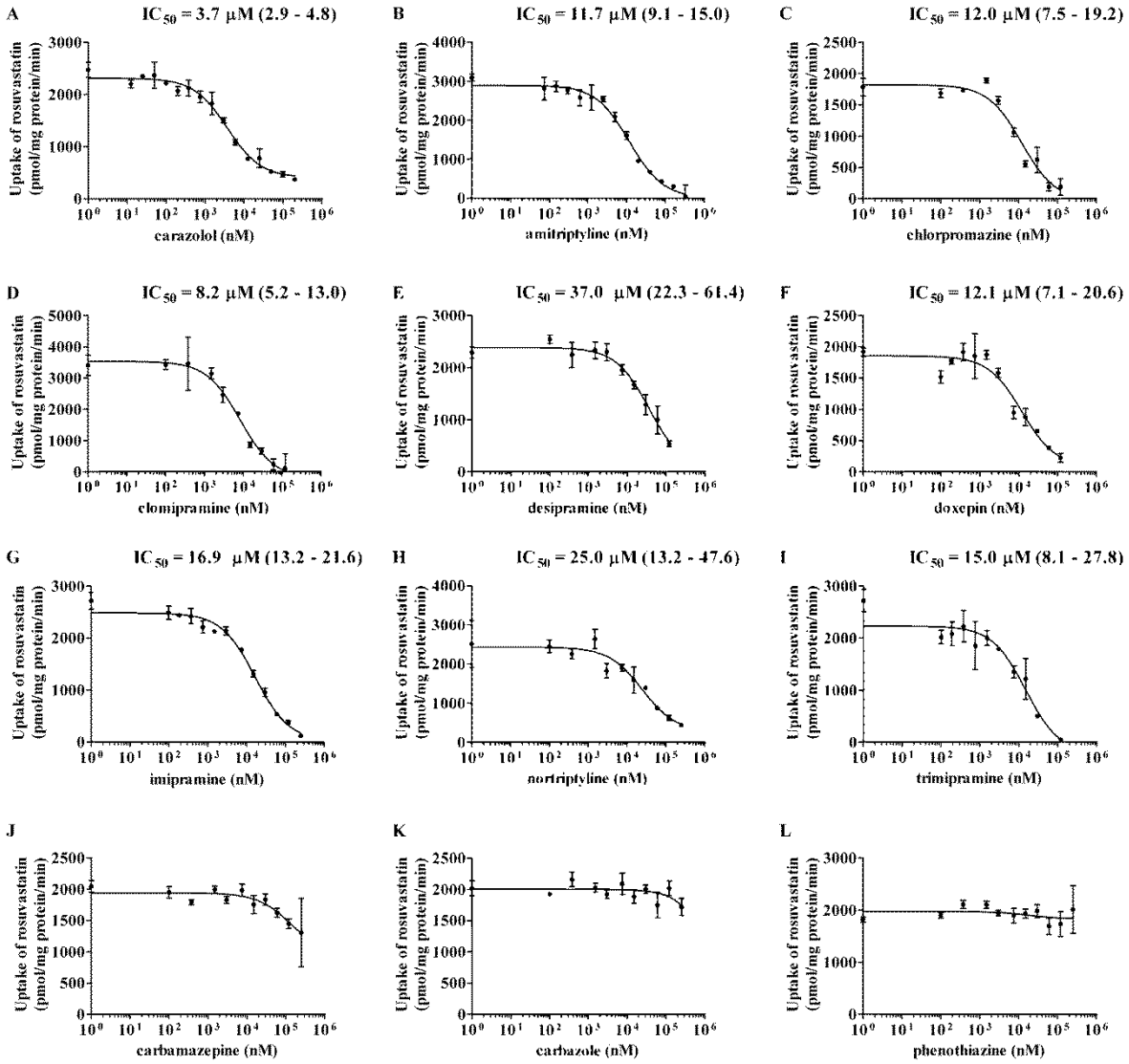
Journal of Pharmacology and Experimental Therapeutics

Effects of β-blockers and tricyclic antidepressants on the activity of human organic anion transporting polypeptide 1A2 (OATP1A2)

Jennifer Lu, Veronique Michaud, Liliam Gabriela Guilarte Moya, Fleur Gaudette, Yat Hei Leung, and Jacques Turgeon

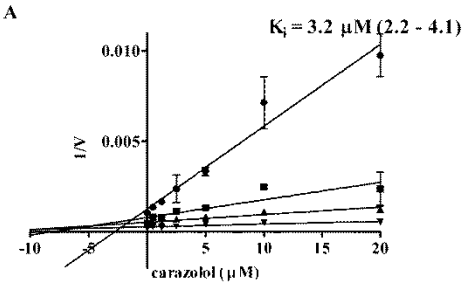


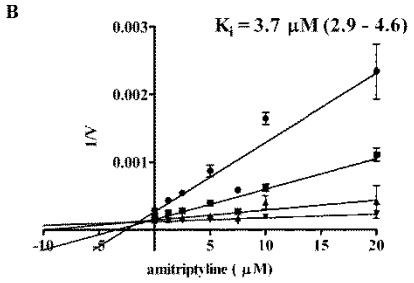
Supplemental Figure 1. Structures of (A) different β-blockers tested and (B) different tricyclic compounds tested.

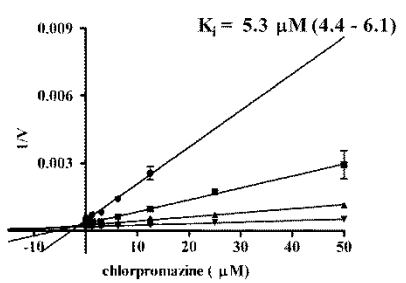


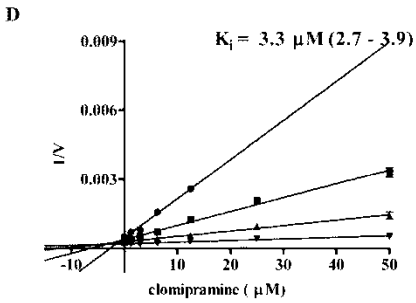
Supplemental Figure 2. Inhibition of OATP1A2-mediated transport of rosuvastatin by different tricyclic compounds. HEK293-OATP1A2 and HEK293-VC cells were coincubated with rosuvastatin (150 µM) and different tricyclic compounds (12.5 nM – 250 µM) for 2 min at 37°C. The quantity of intracellular rosuvastatin was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the

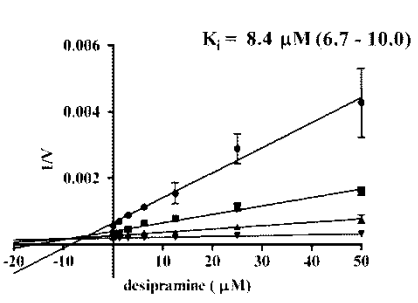
log(inhibitor) vs. response equation in GraphPad Prism. (A) carazolol; (B) amitriptyline; (C) chlorpromazine; (D) clomipramine; (E) desipramine; (F) doxepin; (G) imipramine; (H) nortriptyline; (I) trimipramine; (J) carbamazepine; (K) carbazole; and (L) phenothiazine. Each point represents the mean ± S.D. of triplicate from a single experiment. The values in parentheses represent the 95% confidence interval.

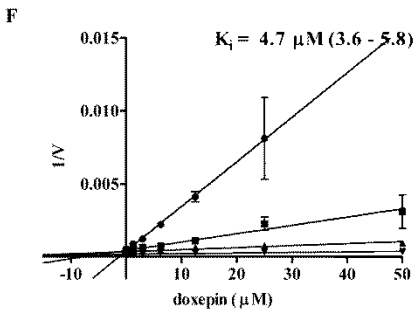

A
K_i = 3.2 µM (2.2 - 4.1)
1/V
0.010
0.005
-10
-5
5
10
15
20
carazolol (µM)

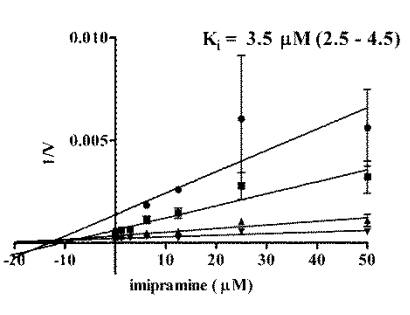

B
K_i = 3.7 µM (2.9 - 4.6)
1/V
0.003
0.002
0.001
-10
-5
5
10
15
20
amitriptyline (µM)

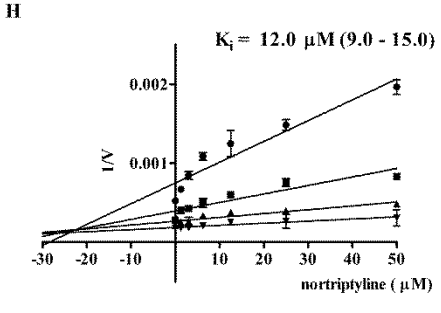

C
K_i = 5.3 µM (4.4 - 6.1)
1/V
0.009
0.006
0.003
-10
10
20
30
40
50
chlorpromazine (µM)

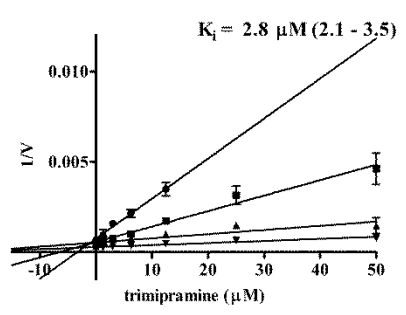

D
K_i = 3.3 µM (2.7 - 3.9)
1/V
0.009
0.006
0.003
-10
10
20
30
40
50
clomipramine (µM)


E
K_i = 8.4 µM (6.7 - 10.0)
1/V
0.006
0.004
0.002
-20
-10
10
20
30
40
50
desipramine (µM)


F
K_i = 4.7 µM (3.6 - 5.8)
1/V
0.015
0.010
0.005
-10
10
20
30
40
50
doxepin (µM)


G
K_i = 3.5 µM (2.5 - 4.5)
1/V
0.010
0.005
-20
-10
10
20
30
40
50
imipramine (µM)


H
K_i = 12.0 µM (9.0 - 15.0)
1/V
0.002
0.001
-30
-20
-10
10
20
30
40
50
nortriptyline (µM)


I
K_i = 2.8 µM (2.1 - 3.5)
1/V
0.010
0.005
-10
10
20
30
40
50
trimipramine (µM)

Supplemental Figure 3. Dixon plots of inhibition of OATP1A2-mediated transport of rosuvastatin by different tricyclic compounds. HEK293-OATP1A2 and HEK293-VC cells were coincubated with rosuvastatin (25, 50, 100, 250 μM) and different tricyclic drugs (0.5 – 50 μM) for 2 min at 37°C. The quantity of intracellular rosuvastatin was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. The x-axis represents the concentration of the inhibitor and the y-axis represents the reciprocal velocity (1/V). Linear regression was used to fit each set of data and the intercept of all lines represents the - K_i. The K_i was accurately calculated in GraphPad Prism. (●) 25 μM rosuvastatin; (■) 50 μM rosuvastatin; (▲) 100 μM rosuvastatin; (▼) 250 μM rosuvastatin. (A) carazolol; (B) amitriptyline; (C) chlorpromazine; (D) clomipramine; (E) desipramine; (F) doxepin; (G) imipramine; (H) nortriptyline; and (I) trimipramine. The values in parentheses represent the 95% confidence interval.

2.1.4 DISCUSSION

A method to study DDIs between substrates and inhibitors of OATP1A2 using rosuvastatin as a probe substrate was developed and validated. Competition between rosuvastatin and different β-blockers for transport through OATP1A2 demonstrated that carvedilol was the most potent inhibitor. Further investigations using drugs with a structure similar to carvedilol were tested for their potential to inhibit OATP1A2. A structure-activity relationship was defined using the data. It was demonstrated that drugs composed of a tricyclic ring with a short aliphatic amine chain were potent OATP1A2 inhibitors. These drugs were not transported by OATP1A2, indicating that they were solely inhibitors.

As discussed in the article and in the introduction of my thesis, a lot of attention was drawn to OATP1A2 because it was thought to mediate DDI in the intestine. This was later challenged by several groups when its presence could not be detected in the intestine using a more sensitive protein quantification method [36, 37]. Other intestinal uptake transporters, such as OATP2B1, are believed to mediate the observed effects. A recent DDI study assessed the coadministration of rosuvastatin with ronacaleret, a drug previously under development for the treatment of osteoporosis [154]. Rosuvastatin Cmax decreased by 33% and the AUC decreased by 50% in the presence of ronacaleret compared to administration of rosuvastatin alone. *In vitro* data demonstrated that this interaction can be mediated by OATP2B1. Furthermore, studies in individuals carrying the *OATP2B1* 1457C>T reduced function variant allele showed decreased exposure to fexofenadine and celiprolol after oral administration [155, 156]. These findings are indicative of an important role for OATP2B1 in oral absorption of clinically relevant drugs.

Although OATP1A2 is not expressed in the intestine, its presence at the BBB is well established. The second article presented will study OATP1A2 in this context.

SECTION 2.2

ARTICLE #2

Effects of tricyclic compounds on the transport of anti-migraine triptans through human organic anion transporting polypeptide 1A2 (OATP1A2)

2.2.1 OBJECTIVES

The first objective was to characterize the uptake of different triptans through OATP1A2. The second objective was to determine whether the tricyclic drugs evaluated in the first article inhibited OATP1A2-mediated transport of triptans. The third objective was to repeat the inhibition studies using clinically relevant drug concentrations.

2.2.2 INTRODUCTION

As presented in the introduction of my thesis, the BBB is well known for its role in protecting the brain from external aggressions, including pathogens, toxins, the majority of endogenous substances, and clinically relevant drugs. Yet, some drugs are able to penetrate the brain as demonstrated by their ability to exert a positive pharmacological effect when their site of action is located at the CNS and other drugs are associated to neurological adverse events. Drug transporters may be the gateway across the BBB for those pharmaceuticals. Efflux transporters may limit the entrance of drugs and influx transporters may facilitate their penetration to the brain. OATP1A2 expression at the human BBB is well established. Previous immunofluorescence studies demonstrated its presence at the luminal membrane of endothelial cells making up the BBB [21, 23, 30, 86]. This localisation strongly suggests a role for OATP1A2 in mediating the entrance of drugs to the CNS.

Most drugs capable of penetrating the BBB are small and lipid soluble [157, 158]. A compound's lipophilicity or membrane permeability is characterized by its logP value. LogP refers to the partition coefficient of the compound between two immiscible phases at equilibrium, which are usually n-octanol and water. It's a measure of the ratio of the concentrations of the non-ionized compound between these two phases as shown by the following formula:

$$\log P = \log \left(\frac{[\text{solute}]_{\text{non-ionized in n-octanol}}}{[\text{solute}]_{\text{non-ionized in water}}} \right)$$

A logP value above 1 is characteristic of a molecule more soluble in octanol (more lipophilic); whereas, a logP value below 1 is characteristic of a molecule more soluble in water (more hydrophilic). The distribution coefficient (logD) value is another measure of permeability of a drug and it takes into account ionized and non-ionized forms of the molecule as shown in the following formula:

$$\log D = \log \left(\frac{[\text{solute}]_{\text{non-ionized in n-octanol}} + [\text{solute}]_{\text{ionized in n-octanol}}}{[\text{solute}]_{\text{non-ionized in water}} + [\text{solute}]_{\text{ionized in water}}} \right)$$

Given that the ionization state of a molecule varies with the pH, the logD value also varies accordingly. The logD value is more relevant when studying the permeability of a drug under physiological conditions.

Triptans, a class of medications commonly used in treating acute migraine attacks, are believe to act at both peripheral and central sites. In order for triptans to access their central targets, they need to cross the BBB. Compared to most CNS-active drugs, triptans have poor passive permeability and are unlikely to cross by passive diffusion [159]. The logD values at pH 7.4 are -2.1, +0.5, -1.0, -0.2, -0.7, -1.5, and -1.0 for almotriptan, eletriptan, frovatriptan, naratriptan, rizatriptan, sumatriptan, and zolmitriptan, respectively [160]. Based on their logD value, eletriptan appears to be able to enter the BBB by passive diffusion. The more hydrophilic triptans may enter by facilitated transport and OATP1A2 offers an interesting access point.

2.2.3 ARTICLE

The authors' specific contributions were as follow:

- Participated in research design: Jennifer Lu, Veronique Michaud, Jacques Turgeon
- Conducted experiments: Jennifer Lu
- Contributed new reagents or analytic tools: Jennifer Lu, Alexia Grangeon, Fleur Gaudette
- Performed data analysis: Jennifer Lu, Fleur Gaudette

- Wrote or contributed to the writing of the manuscript: Jennifer Lu, Veronique Michaud, Jacques Turgeon

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Effects of Tricyclic Compounds on the Transport of Anti-migraine Triptans through Human Organic Anion Transporting Polypeptide 1A2 (OATP1A2)

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Effects of tricyclic compounds on the transport of anti-migraine triptans through human organic anion transporting polypeptide 1A2 (OATP1A2)

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Abstract

OATP1A2 is a membrane drug-transporter expressed at the human blood-brain barrier (BBB) that may potentially mediate penetration of drugs in the brain. Triptans, hydrophilic antimigraine drugs, are substrates of OATP1A2. It is believed that triptans should cross the BBB to reach their site of action. Thus, OATP1A2 can limit brain penetration of triptans and may consequently influence their antimigraine drug action. We have previously demonstrated that compounds composed of a tricyclic ring with a short aliphatic amine chain, such as tricyclic antidepressants and carvedilol, inhibited OATP1A2-mediated rosuvastatin uptake. The main objective of this study was to determine whether triptans transport via OATP1A2 is affected by tricyclic compounds. First, we confirmed that triptans were substrates of OATP1A2 but not OATP2B1 using HEK293 stable cell lines. The tricyclic drugs evaluated were able to inhibit OATP1A2-mediated uptake of triptans. Carvedilol was the most potent inhibitor. Potential inhibition was assessed with a range of total plasma concentrations of the drugs. Carvedilol and nortriptyline lowered the uptake of both almotriptan and zolmitriptan whereas clomipramine diminished the uptake of almotriptan only. Our data suggest that these three drugs may limit the penetration of triptans to the brain by modulating OATP1A2 transport at clinically relevant concentrations.

Keywords: Drug transporter, OATP1A2, drug-drug interaction, triptans, blood-brain barrier

Abbreviations: CNS, central nervous system; HEK, human embryonic kidney; OATP, organic anion transporting polypeptide; PBS, phosphate-buffered saline.

Introduction

Migraines are an important cause of disability in Canada, affecting 8.3% of the population (2.7 millions) [1]. Triptan drugs are typically used in the treatment of acute migraine attacks. Triptans are selective agonists of the serotonin receptors 5-HT_{1B} and 5-HT_{1D} located on smooth muscle cells of intracranial and extracerebral blood vessels as well as on trigeminal sensory neurons [2-4]. Their mechanisms of action are believed to imply inhibition of activated trigeminal neurons, inhibition of neuropeptides release, interruption of pain transmission, and perhaps selective vasoconstriction of cranial blood vessels [5]. It appears that triptans are required to cross the blood-brain barrier (BBB) to reach their target site in the central nervous system (CNS). However, these drugs are hydrophilic, limiting their penetration through the BBB. Thus, transport mechanisms must exist to facilitate their entrance into the brain. Many membrane drug transporters are expressed at the BBB to limit or facilitate the access of drugs to the brain. Among those involved in drug influx, OATP1A2 and OATP2B1 proteins are expressed on the luminal membrane of the endothelial cells making up the BBB [6-9]. Their physiological roles at the BBB may implicate the distribution of thyroid hormones (triiodothyronine and thyroxine) to the CNS by OATP1A2 and the transport of conjugated neuroactive steroids (pregnenolone sulfate and dehydroepiandrosterone-3-sulfate) to the brain by OATP2B1 [10,11].

OATP1A2 and OATP2B1 transport a wide spectrum of endogenous compounds and xenobiotics while having overlapping substrate selectivity. Recently, a study screened 36 CNS-active drugs for transport through OATP1A2 and has shown that triptans are OATP1A2 substrates [12]. Using triptan structural analogs, a structure-activity relationship was established where an amine residue was essential for transport through OATP1A2 and the uptake rate was the highest for

tertiary amine followed by secondary and then primary amines. These findings are interesting as it would suggest that OATP1A2 may facilitate permeation of triptans to the brain.

We have previously demonstrated that the transport of rosuvastatin through OATP1A2 can be inhibited by compounds composed of a tricyclic ring and a short aliphatic amine chain, such as tricyclic antidepressants and carvedilol [13]. The objectives of this study were to 1) confirm triptans as OATP1A2 substrates in our human embryonic kidney (HEK293)-OATP1A2 stable cell line; 2) determine whether triptans are OATP2B1 substrates using a HEK293-OATP2B1 stable cell line; 3) determine whether compounds composed of a tricyclic ring and a short aliphatic amine chain inhibit the transport of triptans through OATP1A2; and 4) determine whether tricyclic compounds can inhibit OATP1A2-mediated uptake of triptans at total plasma concentrations. The consequence of such an interaction in humans would be a diminishment or abolishment in antimigraine efficiency by a limited delivery of triptans into the brain.

Materials and Methods

Reagents

Amitriptyline hydrochloride, carbamazepine, carbazole, chlorpromazine hydrochloride, clomipramine hydrochloride, desipramine hydrochloride, imipramine hydrochloride, naratriptan hydrochloride, nortriptyline hydrochloride, phenothiazine, rizatriptan benzoate, sumatriptan succinate, trimipramine maleate salt, zolmitriptan were purchased from Sigma-Aldrich (St-Louis, MO, USA). Almotriptan hydrochloride, carazolol hydrochloride, carvedilol, doxepin hydrochloride, eletriptan hydrobromide were purchased from Toronto Research Chemicals (Toronto, ON, Canada). All chemicals and solvents were obtained from Sigma-Aldrich, Fisher Scientific (Fair Lawn, NJ, USA) or J.T. Baker (Center Valley, PA, USA).

Cell culture

HEK293-OATP1A2, HEK293-OATP2B1, and HEK293-VC cells were kindly provided by Dr. Markus Keiser and Dr. Werner Siegmund (Department of Clinical Pharmacology, Center of Drug Absorption and Transport, University Medicine Greifswald, Greifswald, Germany). The cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1x nonessential amino acids, and 1x sodium pyruvate at 37°C and 5% CO_2. Cell culture media and supplements were purchased from Multicell Wisent Inc. (St-Jean-Baptiste, QC, Canada); whereas, fetal bovine serum was obtained from HyClone Thermo Scientific (Logan, UT, USA).

Uptake assays and competition assays

Reproducibility of our HEK293-OATP1A2 cell model was assessed with 2-3 different cell batches and comparable K_m values were obtained. The uptake assays were performed as previously described [13]. Briefly, tissue culture plates (6-well or 12-well) were first treated with poly-L-lysine (Sigma-Aldrich, St-Louis, MO, USA) before seeding the HEK293-OATP1A2,

HEK293-OATP2B1, and HEK293-VC cells. The number of cells seeded in 6-well and 12-well plates was 1.5 x 10^6 cells/well and 7.5 x 10^5 cells/well, respectively. After 24 hours, the culture media was replaced with warm transport buffer (142 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3) and pre-incubated at 37°C for 5 minutes. Following the pre-incubation period, the cells were incubated with warm transport buffer containing the substrate in the presence or absence of an inhibitor at 37°C. After incubation, the cells were washed twice with phosphate-buffered saline (PBS) containing 10% acetonitrile followed by a final wash with PBS.

Time-dependent uptake experiments through OATP1A2 were done in six-well plates by incubating HEK293-OATP1A2 and HEK293-VC cells with drugs at determined K_m, *i.e.* almotriptan (5 μM), eletriptan (1 μM), naratriptan (20 μM), rizatriptan (43 μM), sumatriptan (94 μM), or zolmitriptan (21 μM). The K_m and V_{max} of the different triptans transport through OATP1A2 was determined by incubating HEK293-OATP1A2 and HEK293-VC cells in six-well plates with almotriptan (0.375 to 25 μM), eletriptan (0.125 to 5 μM), naratriptan (0.625 to 100 μM), rizatriptan (0.75 to 250 μM), sumatriptan (1.5 to 500 μM), and zolmitriptan (0.75 to 250 μM). To determine whether a compound can block OATP1A2-mediated transport of triptans, HEK293-OATP1A2 and HEK293-VC cells were seeded in 12-well plates and co-incubated with almotriptan (15 μM), eletriptan (3 μM), naratriptan (60 μM), rizatriptan (130 μM), sumatriptan (300 μM), or zolmitriptan (65 μM) in the absence or presence of different tricyclic compounds (0.15–150 μM). In the inhibition studies, a concentration of triptan at three times the K_m value was selected in order to saturate the OATP1A2 transporter with the substrate. An incubation time of 2 minutes was chosen for almotriptan, naratriptan, rizatriptan, sumatriptan, and zolmitriptan; whereas 1 minute was chosen for eletriptan. Time-dependent uptake of triptans at clinically

relevant concentrations was done in six-well plates by incubating HEK293-OATP1A2 and HEK293-VC cells with almotriptan (50 ng/mL) or zolmitriptan (3 ng/mL). To determine whether clinically relevant concentrations of tricyclic compounds can inhibit OATP1A2-mediated transport of triptans, HEK293-OATP1A2 and HEK293-VC cells were seeded in six-well plates and co-incubated with either almotriptan (50 ng/mL) or zolmitriptan (3 ng/mL) for 1 or 2 minutes, respectively, in the absence or presence of different tricyclic compounds (10 – 200 ng/mL).

To determine whether triptans are transported by OATP2B1, HEK293-OATP2B1 and HEK293-VC cells were seeded in six-well plates and incubated with almotriptan (5 and 25 μM), eletriptan (1 and 3.75 μM), naratriptan (20 and 60 μM), rizatriptan (40 and 120 μM), sumatriptan (100 and 300 μM), or zolmitriptan (21 and 65 μM). The concentrations chosen for each substrate correspond to its K_m and 3-times K_m value determined in HEK293-OATP1A2 cells.

The protein concentration was measured using the Pierce BCA protein assay kit from Thermo Scientific (Rockford, IL, USA). Three wells of each cell line were lyzed with 1% SDS + 0.2 N NaOH and the average value was used to normalize intracellular triptan concentrations.

Quantification of Triptans by High-Performance Liquid Chromatography-UV

The quantity of triptans transported in the cells was measured by high performance liquid chromatography with UV detection. The instrumentation consisted of a SpectraSystem P4000 pump, SpectraSystem AS3000 autosampler, Finnigan SpectraSystem UV6000 ultraviolet detector and SpectraSystem SN4000 system controller from Thermo Electron Corporation (San Jose, CA, USA). ChromQuest Version 4.2.34 software was used for data acquisition (Thermo Electron Corporation). The samples were separated on a Phenomenex Luna 3μm PFP(2) column

(150 x 4.6 mm, 3µM; Phenomenex, Torrance, CA, USA). Table 1 summarizes the details for each method.

Since the cell lysate affected the absorbance of the analytes, calibration curves and quality controls samples were prepared in the cell lysate. Linear regressions (weighted 1/concentration) were judged to produce the best fit for the concentration-detector relationship for all triptans. The coefficients of correlation (r^2) were greater than 0.997 for all compounds in all batches. The reproducibility of each method was evaluated by analyzing six replicates of lysate samples fortified at LLOQ, low, mid and high concentrations in three individual runs. Precisions were better than 11.3% and accuracies were in the 96.0 - 110% range. The inter- and intra-batch precision and accuracy statistical results for all compounds are shown in Table 2.

After the final wash with PBS, the samples were processed as previously described [13]. Briefly, the cells were lyzed with methanol containing the IS (100 ng/ml). The cell lysate was transferred to a 1.7 mL microtube and the samples were spun down at maximum speed for 10 minutes at room temperature. The supernatant was transferred to a culture borosilicate glass tube, evaporated to dryness, and reconstituted in 100 µl of reconstitution solution. The reconstitution solution consisted of a mixture of ddH_2O and methanol in the following proportions: almotriptan (70:30 v/v), eletriptan (50:50 v/v), naratriptan (70:30 v/v), rizatriptan (70:30 v/v), sumatriptan (70:30 v/v), and zolmitriptan (70:30 v/v). A volume of 20 µl per sample was injected.

Quantification of Almotriptan and Zolmitriptan by Liquid Chromatography-Tandem Mass Spectrometry

The quantity of almotriptan and zolmitriptan transported in the cells when incubated at clinically relevant concentrations was measured by liquid chromatography-tandem mass spectrometry. The instrumentation consisted of a TSQ Quantiva Triple Quadrupole mass spectrometer interfaced

with an Ultimate 3000XRS UHPLC system using pneumatic assisted heated electrospray ion source from Thermo Scientific (San Jose, CA, USA). Xcalibur 3.0.63 software was used for data acquisition and analysis (San Jose, CA, USA). The samples were separated on a Phenomenex Luna PFP(2) column (150 x 3.0 mm, 3µM; Phenomenex, Torrance, CA, USA) coupled with a Phenomenex PFP security guard cartridge (4 x 2.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 10 mM ammonium formate, pH 3, and acetonitrile in the following proportions: almotriptan (70:30 v/v) and zolmitriptan (80:20 v/v). The flow rate was set at 0.3 ml/min and the column was heated at 40°C for almotriptan. The flow rate was set at 0.4 ml/min and the column was heated at 50°C for zolmitriptan. $^{2}H_{6}$-almotriptan and $^{2}H_{6}$-zolmitriptan were used as IS and the retention times are 4.4 and 2.8 minutes for almotriptan and zolmitriptan, respectively. MS detection was performed in positive ion mode, using selected reaction monitoring. The precursor-ion reactions for the analytes were set at 336.2 → 291.1 for almotriptan and 288.3 → 167.1 for zolmitriptan.

The analytical range was set at 37.5 to 25,000 pg/ml for almotriptan and set at 75.0 to 25,000 pg/ml for zolmitriptan. A linear regression (weighted 1/concentration) was judged to produce the best fit for the concentration-detector relationship for almotriptan and zolmitriptan. The r^2 was greater than 0.998 for almotriptan and 0.996 for zolmitriptan. The reproducibility of the method was evaluated by analyzing three replicates of lysate samples fortified at low, mid and high concentrations in three individual runs. Precisions were better than 13% and accuracies were in the 92 -103% range. The intra and inter batch precision and accuracy statistical results are shown in Supplemental Table 1.

After the final wash with PBS, the samples were processed as follows. The cells were lyzed with 1 ml methanol containing the IS (2 ng/ml $^{2}H_{6}$-almotriptan or 0.5 ng/ml $^{2}H_{6}$-zolmitriptan). The

cell lysate was transferred to a 1.7 ml microtube and the samples were spun down at maximum speed for 10 minutes at room temperature. The supernatant was transferred to a culture borosilicate glass tube, evaporated to dryness at 10 psi with N_2 at 40°C, and reconstituted in 200 µl of reconstitution solution. The reconstitution solution consisted of a mixture of 10 mM ammonium formate, pH 3, and methanol (70:30 v/v) for almotriptan and H_2O and methanol (95:5 v/v) for zolmitriptan. A volume of 10 µl per sample for almotriptan and 5 µl per sample for zolmitriptan was injected.

Data analysis

The net transport of triptan through OATP1A2 was calculated by subtracting the value in the VC cells from the value in the OATP1A2 cells. Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Each data point is expressed as the mean ± S.D. K_m and V_{max} were calculated by fitting the data to the Michaelis-Menten equation. IC_{50} values were calculated by fitting the data to the log(inhibitor) versus response equation, and the range given represents the 95% confidence interval.

Results

Transport of Triptans through OATP1A2

A cell model stably expressing OATP1A2 in HEK293 cells was used to study OATP1A2-mediated transport of the different triptans. Time-dependent uptake was assessed up to 60 minutes with the exception of eletriptan, up to 15 minutes, due to its higher lipophilicity compared to other triptans (Fig. 1). All triptans evaluated showed time-dependent saturable transport by OATP1A2. An incubation time of 2 minutes was chosen for further experiments with almotriptan, naratriptan, rizatriptan, sumatriptan, and zolmitriptan and 1 minute was chosen for eletriptan as these time-points remain in their linear range. All triptans evaluated also showed concentration-dependent saturable transport via OATP1A2 (Fig. 2; Table 3). Eletriptan showed the highest affinity for OATP1A2 and sumatriptan has the lowest affinity as the K_m were calculated to be 0.8 ± 0.2 µM and 94.5 ± 9.9 µM, respectively. OATP1A2 transport velocity was the lowest for almotriptan and the highest was observed with sumatriptan as the V_{max} were 1265 ± 54.4 pmol/mg protein per minute and 8072 ± 300.2 pmol/mg protein per minute, respectively. The intrinsic clearance (CL_{int}) was the lowest for sumatriptan (85.4 µl/mg protein per minute) and the highest for eletriptan (2042.5 µl/mg protein per minute).

Transport of Triptans through OATP2B1

Transport of triptans through OATP2B1 was evaluated, using a HEK293 cell model stably expressing this transporter, as OATP2B1 is also found at the BBB and has overlapping substrates with OATP1A2. Two concentrations of each triptan were assessed. The concentrations chosen for each substrate correspond to its K_m and 3-times K_m value determined for OATP1A2. A slightly greater intracellular concentration of eletriptan and sumatriptan was observed in HEK293-OATP2B1 cells compared to HEK293-VC cells (Fig. 3). No transport by OATP2B1

was noticed when incubations were performed with almotriptan, naratriptan, rizatriptan, and zolmitriptan. The small difference, less than 21%, observed between the two cell lines with eletriptan and sumatriptan was considered non-significant and likely due to the variability of experiments.

Effect of Tricyclic Compounds on OATP1A2-mediated Uptake of Triptans

To determine whether compounds composed of a tricyclic ring and a short aliphatic amine chain inhibit OATP1A2-mediated uptake of triptans, competition studies were performed (Supplemental Figs. 1, 2, 3, and 4; Table 4). Carvedilol showed the strongest inhibition on the uptake of all six triptans with an IC_{50} of 0.5, 0.7, 2.1, 2.2, 3.5, and 3.8 μM for eletriptan, almotriptan, sumatriptan, zolmitriptan, rizatriptan, and naratriptan, respectively. Carazolol was the second strongest inhibitor with an IC_{50} of 1.6, 4.6, and 5.5 μM for almotriptan, zolmitriptan, and naratriptan, respectively. Amitriptyline, chlorpromazine, clomipramine, desipramine, doxepin, imipramine, nortriptyline, and trimipramine demonstrated slightly lower inhibition potencies than carvedilol and carazolol. Carbamazepine, carbazole, and phenothiazine exerted no significant effect on the transport of almotriptan, naratriptan, and zolmitriptan.

Studies in the range of total plasma concentrations

As the IC_{50} studies were carried out with the concentration of substrates at saturation, it does not reflect the interaction at the blood-brain barrier in clinical settings. Thus, a study using range of total plasma concentrations of triptans and inhibitors were carried out with almotriptan and zolmitrptan. These two triptans were selected based on their greater hydrophilic profile in the cell model used. In Canada, almotriptan is typically given in a 12.5 mg dose tablet and zolmitriptan is typically given in a 2.5 mg dose tablet, 2.5 to 5 mg dose nasal spray or 2.5 mg dose orally disintegrating tablet [14]. Pharmacokinetic studies have shown that a single dose of

almotriptan results in a C_{max} of 50 ng/mL and a single dose of zolmitriptan in any of the dosage forms corresponds to a C_{max} of 3-7 ng/mL [15-19].

Time-dependent uptake of almotriptan and zolmitriptan was re-assessed as lower concentrations might affect the kinetics. Almotriptan (50 ng/mL) and zolmitriptan (3 ng/mL) showed a time-dependent saturable transport with a similar profile as when incubated at K_m (Fig. 4A and 5A). An incubation time of 1 and 2 minutes was chosen for the competition experiments for almotriptan and zolmitriptan, respectively. The concentrations of inhibitors correspond to the plasma concentrations measured at half C_{max}, C_{max}, and 2-times C_{max} for a given dose. The reported peak plasma concentrations (C_{max}) after an oral dose are: 33.5 ng/ml for amitriptyline 50 mg ; 35 ng/ml for carvedilol 12.5 mg ; 50 ng/ml for chlorpromazine 100 mg ; 63 ng/ml for clomipramine 25 mg ; 18 ng/ml for desipramine 50 mg ; 25 ng/ml for doxepin 75 mg ; 63 ng/ml for imipramine 100 mg; 50-150 ng/ml for nortriptyline 25-50 mg; and 22 ng/ml for trimipramine 75 mg [20-28]. Among the inhibitors evaluated, carvedilol and nortriptyline lowered the uptake of both almotriptan and zolmitriptan whereas clomipramine diminished the uptake of almotriptan only. The other tricyclic compounds had no significant effects on OATP1A2-mediated uptake of almotriptan and zolmitriptan at clinically relevant concentrations.

Discussion

Using stable cell lines overexpressing selected transporters, we confirmed that triptans are substrates for OATP1A2 but not OATP2B1. Inhibition studies demonstrated that compounds composed of a tricyclic ring and a short aliphatic amine chain inhibited OATP1A2-mediated uptake of triptans. The IC_{50} values of the inhibitors determined in this study followed the same pattern as those previously published when using rosuvastatin as the probe substrate for OATP1A2 [13]. Carvedilol and carazolol were the strongest inhibitors followed by amitriptyline, chlorpromazine, clomipramine, desipramine, doxepin, imipramine, nortriptyline, and trimipramine. Inhibition studies conducted in the range of total plasma concentrations showed that carvedilol, clomipramine and nortriptyline were able to diminish the transport of triptans through OATP1A2.

The K_m values of almotriptan, eletriptan and zolmitriptan for OATP1A2 determined in this study (5.1, 0.8, and 21.4 μM, respectively) are in line with those previously published (4.8, 1.3, and 15.1 μM, respectively) [12]. However, the K_m values of rizatriptan and sumatriptan are higher in this study (42.9 and 94.5 versus 6.0 and 27.0 μM). The drug's solubility in the solvent used to dissolve or the incubation buffer may account for this discrepancy. In fact, when a drug is incompletely dissolved, the shape of the K_m V_{max} curve is changed when compared to the situation where the drug is completely dissolved at all concentrations. The V_{max} values for the substrates are higher in this study. This variability may be explained by the differences in the in vitro model used: the quantity of OATP1A2 protein expressed at the cell surface, the quantity of functional proteins expressed, or the quantity of transporters exposed to the media and available for drug uptake. Although the CL_{int} values (V_{max}/K_m) are different in the two studies, they both

follow the same order of magnitude: eletriptan > zolmitriptan > almotriptan > rizatriptan > sumatriptan. Naratriptan could not be compared as the previous publication did not assess it.

There are evidences supporting a mechanism of action in the CNS for triptans in addition to their peripheral effects: 1) 5-HT_{1B} and 5-HT_{1D} receptors proteins are found on trigeminal sensory neurons; and 2) activation of the trigeminal nucleus neurons by electrical stimulation is inhibited after administration of a triptan in animal models [2,3,29-31]. CNS adverse events, such as dizziness, vertigo, and ataxia are indirect indications that triptans have the potential to access the brain [32]. Using positron emission tomography (PET), two studies demonstrated that zolmitriptan can penetrate the brain at therapeutic doses and can bind to their receptors located in the CNS [33,34]. As triptans are hydrophilic, thus cannot cross the blood-brain barrier by passive diffusion, OATP1A2 may play a role in facilitating the transport of triptans to their site of action. Our data suggest that the co-administration of carvedilol, clomipramine, or nortriptyline with a triptan may limit the entrance of triptans to the CNS by inhibiting OATP1A2. The drug concentration might fall below its therapeutic window in the brain. Consequently, the antimigraine activity may be abolished.

Of interest, one third of migraineurs receiving triptan therapy do not achieve headache relief and the most common reason for the discontinuation of these medications is the lack efficacy [32,35,36]. With the purpose of understanding the causes behind this lack of efficacy, a few studies have looked at polymorphisms found in genes involved in the pharmacokinetic and pharmacodynamic response to triptans. Associations have been reported for the genes encoding the serotonin transporter, monoamine oxidase A, and CYP1A2 [37]. Polymorphisms in the gene

encoding for OATP1A2 and their ability to transport triptans should also be investigated. However, pharmacogenomics alone may not explain the lack of efficacy of triptans in all non-responders. Drug-drug interactions may explain inter-subject variability in antimigraine efficacy for cases where gene polymorphisms are not involved. Interestingly, migraine is often diagnosed in patients with mood disorders, such as depression, anxiety, panic disorder, and bipolar disorder [38,39]. As a result, treatments for both conditions are commonly prescribed. Tricyclic antidepressants are not only prescribed for depression but also for other off-label uses such as obsessive-compulsive disorder, panic disorder, chronic pain, insomnia, premenstrual symptoms and bulimia. In addition, β-blockers and antidepressants, especially amitriptyline, are occasionally prescribed for the prevention of migraine attacks [40]. These observations indicate that the co-prescription of a triptan with a tricyclic antidepressant is not unusual.

Taken together, we demonstrated that compounds composed of a tricyclic ring and a short aliphatic amine chain inhibited the OATP1A2-mediated uptake of triptans. Our data suggest that carvedilol, clomipramine, and nortriptyline may limit the penetration of triptans to the brain by modulating OATP1A2 transport. Although an *in vitro* cell model permits to study the transport of a drug through a specific transporter, this experimental model is also associated with limitations when extrapolating in vitro findings to in vivo settings. Thus, emphasizing the need to confirm these results in humans. Indeed, the impact of concomitant administration of triptans with a potent OATP1A2 inhibitor on their antimigraine efficiency needs to be investigated further in clinical studies.

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Declaration of interest

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Table 1: HPLC-UV quantification methods details

	Almotriptan	**Eletriptan**	**Naratriptan**
Buffer	10 mM AF pH3.0	10 mM AF pH3.0	10 mM AF pH3.0
Solvent	Methanol	Methanol	Methanol
% Buffer/Solvent	65/35	48/52	68/32
Run time (min)	22	30	20
Flow (ml/min)	0.5	0.5	0.5
Wavelength (nm)	283	272	284
Temperature (°C)	40	50	40
t_R (min)	19.0	15.7	18.1
t_R IS (min)	15.2 (IS: naratriptan)	27.5 (IS: doxepin)	8.8 (IS: sumatriptan)

	Rizatriptan	**Sumatriptan**	**Zolmitriptan**
Buffer	10 mM AF pH3.0	10 mM AF pH3.0	10 mM AF pH3.0
Solvent	Methanol	Methanol	Methanol
% Buffer/Solvent	71/29	68/32	80/20
Run time (min)	21	20	24
Flow (ml/min)	0.5	0.5	0.5
Wavelength (nm)	282	283	283
Temperature (°C)	50	40	50
t_R (min)	9.0	8.9	21.7
t_R IS (min)	17.9 (IS: naratriptan)	18.5 (IS: naratriptan)	17.8 (IS: rizatriptan)

AF: ammonium formate; t_R: retention time; IS: internal standard

Table 2: Validation of HPLC-UV quantification methods of triptans

Compound	Concentration (ng/mL)	Intra (n=6) Mean ± SD (ng/mL)	Intra (n=6) CV (%)	Intra (n=6) Nominal (%)	Inter (n=18) Mean ± SD (ng/mL)	Inter (n=18) CV (%)	Inter (n=18) Nominal (%)
Almotriptan	25.0	22.0 ± 4.5	20.0	-11.9	23.9 ± 2.7	11.3	-4.5
	100	97.5 ± 2.8	3.0	-7.0	96.1 ± 3.4	3.5	-3.9
	500	484 ± 9.7	2.0	-3.2	494 ± 12.9	2.6	-1.2
	5000	5229 ± 77.7	1.5	4.6	5102 ± 124	2.4	2.0
Eletriptan	100	106 ± 14.4	13.5	14.8	111 ± 9.0	8.1	11.1
	250	268 ± 10.0	3.7	7.0	258 ± 9.3	3.6	3.4
	500	508 ± 16.3	3.2	-2.1	496 ± 16.0	3.2	-0.8
	5000	5326 ± 163	3.1	6.5	5123 ± 188	3.7	2.5
Naratriptan	50.0	54.9 ± 2.4	4.4	15.8	55.0 ± 2.4	4.4	10.0
	100	103 ± 5.8	5.6	4.8	104 ± 3.9	3.8	4.0
	500	491 ± 20.6	4.2	-5.4	480 ± 15.8	3.3	-4.0
	5000	5162 ± 170	3.3	3.2	5089 ± 130	2.6	1.8
Rizatriptan	25.0	27.5 ± 0.9	3.4	12.2	26.8 ± 1.6	6.2	7.1
	100	98.5 ± 3.7	3.7	-1.7	99.2 ± 2.7	2.8	-0.8
	500	510 ± 10.5	2.1	-6.5	484 ± 21.0	4.3	-3.1
	5000	5173 ± 97.3	1.9	3.4	5024 ± 130	2.6	0.5
Sumatriptan	25.0	26.0 ± 2.0	7.5	13.8	26.6 ± 2.0	7.4	6.3
	100	103 ± 2.9	2.8	-5.2	99.2 ± 4.2	4.2	-0.8
	500	498 ± 15.0	3.0	-3.9	490 ± 14.4	2.9	-2.1
	2500	2480 ± 93.0	3.8	-2.2	2480 ± 70.4	2.8	-0.8
Zolmitriptan	50.0	47.0 ± 2.9	6.1	8.7	51.5 ± 4.2	8.1	3.0
	100	103 ± 9.4	9.2	5.7	102 ± 7.0	6.8	2.3
	500	498 ± 12.8	2.6	3.8	492 ± 11.8	2.4	-1.5
	5000	5113 ± 110	2.2	2.3	5052 ± 91.9	1.8	1.0

SD: standard deviation; CV: coefficient of variation

Table 3. K_m, V_{max} and CL_{int} values for the transport of triptans through OATP1A2. The values were calculated by fitting the data to the Michaelis-Menten equation (± S.D.). CL_{int} was calculated by dividing the V_{max} by K_m. (See also Fig. 2.)

	K_m (μM)	V_{max} (pmol/mg protein per minute)	CL_{int} (μl/mg protein per minute)
Almotriptan	5.1 ± 0.6	1265 ± 54	248
Eletriptan	0.8 ± 0.2	1634 ± 93	2042
Naratriptan	20.3 ± 1.0	3871 ± 70	191
Rizatriptan	42.9 ± 5.7	4798 ± 234	112
Sumatriptan	94.5 ± 9.9	8072 ± 300	85
Zolmitriptan	21.4 ± 1.4	5764 ± 110	269

Table 4. IC_{50} values from the inhibition of triptans uptake through OATP1A2 by different tricyclic compounds. The values in parentheses represent the 95% confidence interval (see also Supplemental Fig. 1, 2, 3, and 4).

	Almotriptan	Naratriptan	Zolmitriptan
Inhibitors	μM		
Amitriptyline	4.6 (2.4−8.9)	13.2 (7.2−24.2)	6.4 (4.0−10.1)
Carazolol	1.6 (0.9−2.8)	5.5 (3.3−9.2)	4.6 (3.1−6.8)
Carvedilol	0.7 (0.3−1.4)	3.8 (3.0−4.8)	2.2 (1.6−2.9)
Chlorpromazine	8.7 (4.9−15.6)	20.3 (12.1−34.1)	16.9 (10.2−27.8)

Clomipramine	6.1 (3.7−10.2)	19.6 (11.4−33.9)	13.5 (8.8−20.9)
Desipramine	16.2 (7.5−35.0)	19.8 (11.1−35.3)	18.4 (12.1−28.1)
Doxepin	2.5 (1.7−3.9)	12.9 (8.4−19.8)	6.8 (2.9−16.1)
Imipramine	4.3 (2.8−6.7)	7.4 (3.2−17.1)	10.3 (4.7−22.4)
Nortriptyline	4.5 (2.6−7.8)	19.1 (6.8−53.7)	13.0 (9.4−17.9)
Trimipramine	7.6 (3.5−16.4)	20.0 (12.2−32.6)	13.6 (9.4−19.7)
Carbamazepine	No effect	No effect	No effect
Carbazole	No effect	No effect	No effect
Phenothiazine	No effect	No effect	No effect

	Eletriptan	Rizatriptan	Sumatriptan
Inhibitors	μM		
Amitriptyline	N/A	12.6 (6.8−23.4)	9.5 (5.2−17.4)
Carvedilol	0.5 (0.2−1.6)	3.5 (2.2−5.6)	2.1 (1.2−3.6)
Doxepin	N/A	4.8 (2.2−10.8)	5.9 (3.4−10.2)
Imipramine	11.1 (2.7−46.1)	N/A	N/A
Nortriptyline	81.0 (9.9−662.2)	N/A	N/A

N/A: Not available (The inhibition assay was not evaluated)

Figure Legends

Fig. 1. Time-dependent OATP1A2-mediated transport of triptans. Uptake of triptans at 37°C in HEK293-OATP1A2 and HEK293-VC cells was conducted as follows. (A) 5 μM almotriptan uptake was assessed for 0.5, 0.75, 1, 2, 3, 5, 10, 20, 30, and 60 minutes. (B) 1 μM eletriptan uptake was assessed for 1, 3, 5, 10, 15, and 30 seconds followed by 1, 2, 3, 4, 5, 10, and 15 minutes. (C) 20 μM naratriptan uptake was assessed for 0.5, 0.75, 1, 2, 3, 5, 10, 20, 30, and 60 minutes. (D) 43μM rizatriptan uptake was assessed for 1, 3, 5, 10, 15, and 30 seconds followed by 1, 2, 3, 4, 5, 15, 30, and 60 minutes. (E) 94 μM sumatriptan uptake was assessed for 0.5, 0.75, 1, 2, 3, 5, 10, 20, 30, and 60 minutes. (F) 21 μM zolmitriptan uptake was assessed for 0.5, 0.75, 1, 2, 3, 5, 10, 20, 30, and 60 minutes. The quantity of intracellular triptan was normalized to protein content. The net transport was calculated by subtracting the values measured in the VC cells from the values measured in OATP1A2 cells. Each point represents the mean ± S.D. of triplicate from a single experiment.

Fig. 2. K_m and V_{max} of OATP1A2-mediated transport of triptans. Uptake of (A) almotriptan (0.375, 0.75, 1.5, 3, 6.25, 12.5, and 25 μM); (B) eletriptan (0.125, 0.25, 0.5, 1, 2, 3, 4, and 5 μM); (C) naratriptan (0.625, 1.25, 2.5, 5, 7.5, 15, 25, 50, and 100 μM); (D) rizatriptan (0.75, 1.5, 3, 7.5, 15, 30, 62.5, 125, and 250 μM); (E) sumatriptan (1.5, 3, 7.5, 15, 30, 62.5, 125, 250, and 500 μM); and (F) zolmitriptan (0.75, 1.5, 3, 7.5, 15, 30, 62.5, 125, and 250 μM) was assessed at 37°C in HEK293-OATP1A2 and HEK293-VC cells. The transport was assessed for 2 minutes for all triptans except for eletriptan which was assessed for 1 minute. The quantity of intracellular triptan was normalized to protein content. The net transport was calculated by subtracting the values measured in the VC cells from the values measured in OATP1A2 cells.

K_m and V_{max} were calculated by fitting the data to the Michaelis-Menten equation. Each point represents the mean ± S.D. of triplicate from a single experiment.

Fig. 3. OATP2B1-mediated transport of triptans. Uptake of (A) almotriptan (5 and 25 μM); (B) eletriptan (1 and 3.75 μM); (C) naratriptan (20 and 60 μM); (D) rizatriptan (40 and 120 μM); (E) sumatriptan (100 and 300 μM); and (F) zolmitriptan (21 and 65 μM) was assessed at 37°C in HEK293-OATP2B1 and HEK293-VC cells. The transport was assessed for 2 minutes for all triptans except for eletriptan which was assessed for 1 minute. The quantity of intracellular triptan was normalized to protein content. Each point represents the mean ± S.D. of triplicate from a single experiment.

Fig. 4. Inhibition of OATP1A2-mediated transport of almotriptan by various tricyclic compounds at range of total plasma concentrations of the drugs. (A) The uptake of almotriptan (50 ng/ml) was assessed for 0.5, 0.75, 1, 2, 3, 4, 5, and 10 minutes at 37°C in HEK293-OATP1A2 and HEK293-VC cells. (B) HEK293-OATP1A2 and HEK293-VC cells were coincubated with almotriptan (50 ng/ml) and different tricyclic compounds (10 – 200 ng/ml) for 1 min at 37°C. The quantity of intracellular almotriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. Each point represents the mean ± S.D. of triplicate from a single experiment.

Fig. 5. Inhibition of OATP1A2-mediated transport of zolmitriptan by various tricyclic compounds at range of total plasma concentrations of the drugs. (A) The uptake of

zolmitriptan (3 ng/ml) was assessed for 0.5, 0.75, 1, 2, 3, 5, 10, and 20 minutes at 37°C in HEK293-OATP1A2 and HEK293-VC cells. (B) HEK293-OATP1A2 and HEK293-VC cells were coincubated with zolmitriptan (3 ng/ml) and different tricyclic compounds (10 – 200 ng/ml) for 2 min at 37°C. The quantity of intracellular zolmitriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. Each point represents the mean ± S.D. of triplicate from a single experiment.

Figure 1

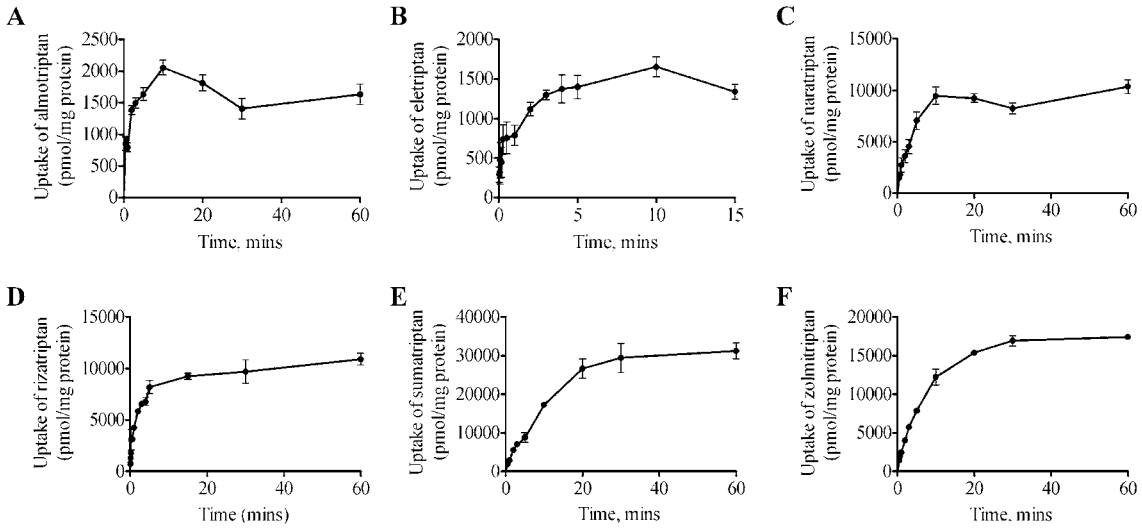

A
Uptake of almotriptan (pmol/mg protein)
2500
2000
1500
1000
500
0
0
20
40
60
Time, mins
B
Uptake of eletriptan (pmol/mg protein)
2000
1500
1000
500
0
0
5
10
15
Time, mins
C
Uptake of naratriptan (pmol/mg protein)
15000
10000
5000
0
0
20
40
60
Time, mins
D
Uptake of rizatriptan (pmol/mg protein)
15000
10000
5000
0
0
20
40
60
Time (mins)
E
Uptake of sumatriptan (pmol/mg protein)
40000
30000
20000
10000
0
0
20
40
60
Time, mins
F
Uptake of zolmitriptan (pmol/mg protein)
20000
15000
10000
5000
0
0
20
40
60
Time, mins

Figure 2

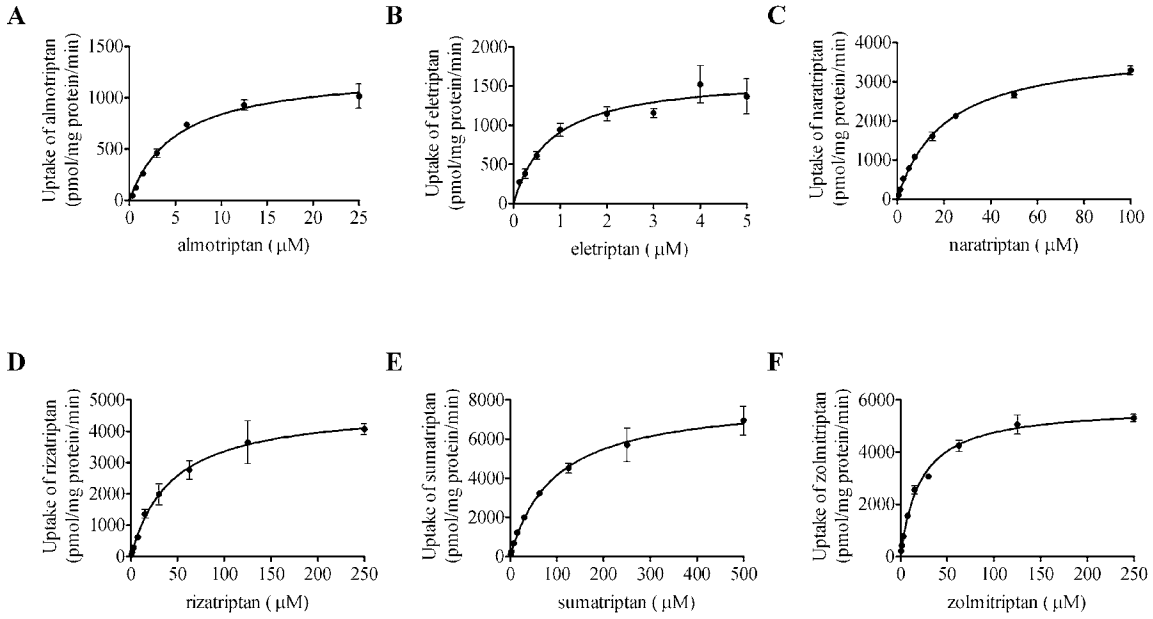

A
Uptake of almotriptan (pmol/mg protein/min)
1500
1000
500
0
0 5 10 15 20 25
almotriptan (μM)
B
Uptake of eletriptan (pmol/mg protein/min)
2000
1500
1000
500
0
0 1 2 3 4 5
eletriptan (μM)
C
Uptake of naratriptan (pmol/mg protein/min)
4000
3000
2000
1000
0
0 20 40 60 80 100
naratriptan (μM)
D
Uptake of rizatriptan (pmol/mg protein/min)
5000
4000
3000
2000
1000
0
0 50 100 150 200 250
rizatriptan (μM)
E
Uptake of sumatriptan (pmol/mg protein/min)
8000
6000
4000
2000
0
0 100 200 300 400 500
sumatriptan (μM)
F
Uptake of zolmitriptan (pmol/mg protein/min)
6000
4000
2000
0
0 50 100 150 200 250
zolmitriptan (μM)

Figure 3

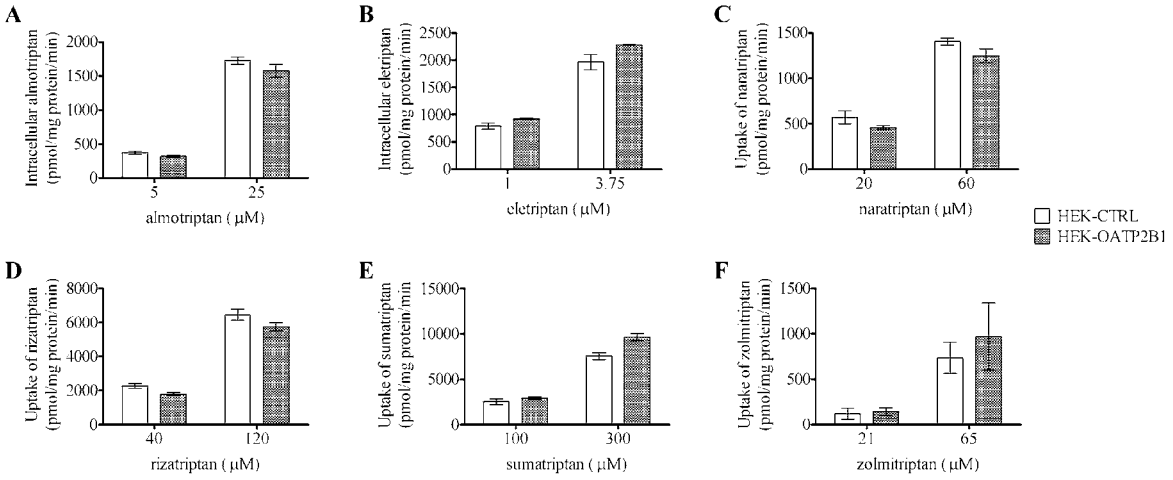
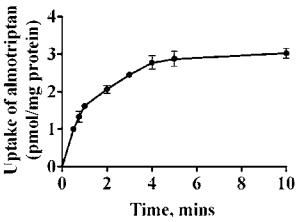

A
Intracellular almotriptan (pmol/mg protein/min)
2000
1500
1000
500
0
5
25
almotriptan (μM)
B
Intracellular eletriptan (pmol/mg protein/min)
2500
2000
1500
1000
500
0
1
3.75
eletriptan (μM)
C
Uptake of naratriptan (pmol/mg protein/min)
1500
1000
500
0
20
60
naratriptan (μM)
HEK-CTRL
HEK-OATP2B1
D
Uptake of rizatriptan (pmol/mg protein/min)
8000
6000
4000
2000
0
40
120
rizatriptan (μM)
E
Uptake of sumatriptan (pmol/mg protein/min
15000
10000
5000
0
100
300
sumatriptan (μM)
F
Uptake of zolmitriptan (pmol/mg protein/min)
1500
1000
500
0
21
65
zolmitriptan (μM)

Figure 4

A


Uptake of almotriptan (pmol/mg protein)
0
1
2
3
4
0
2
4
6
8
10
Time, mins

B

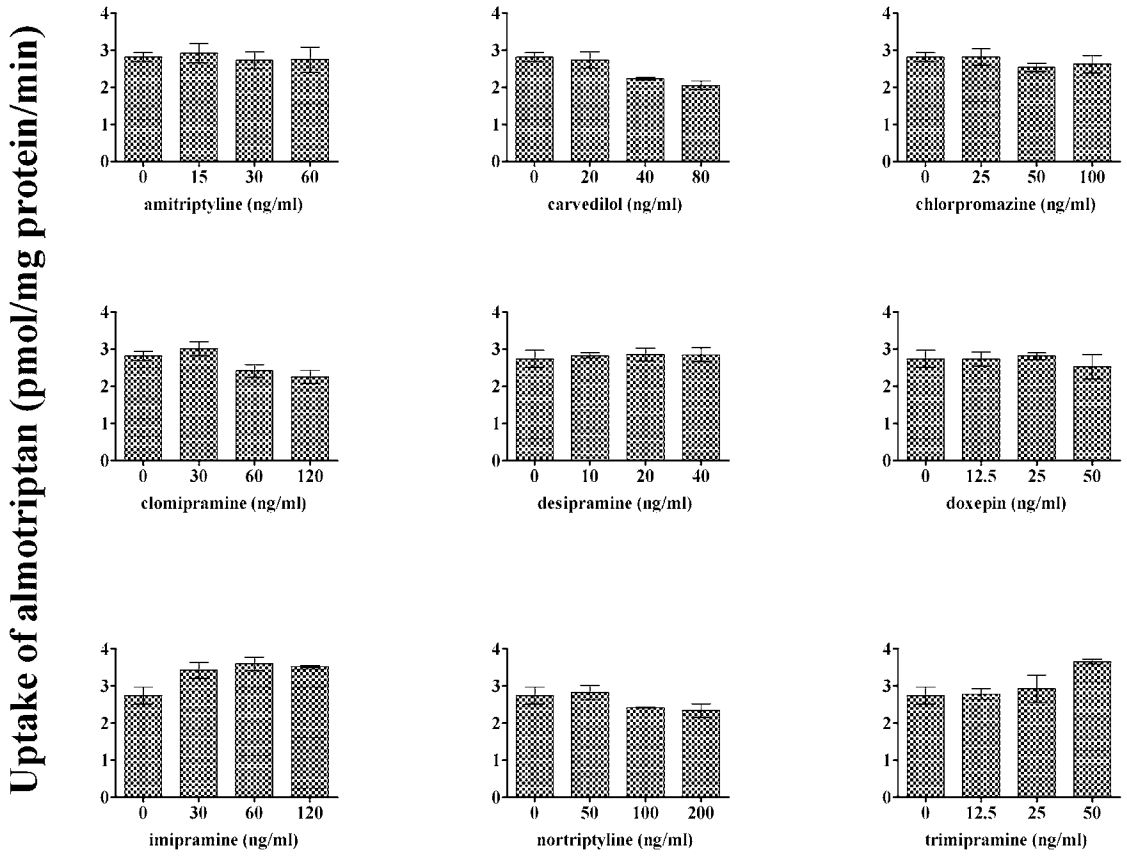
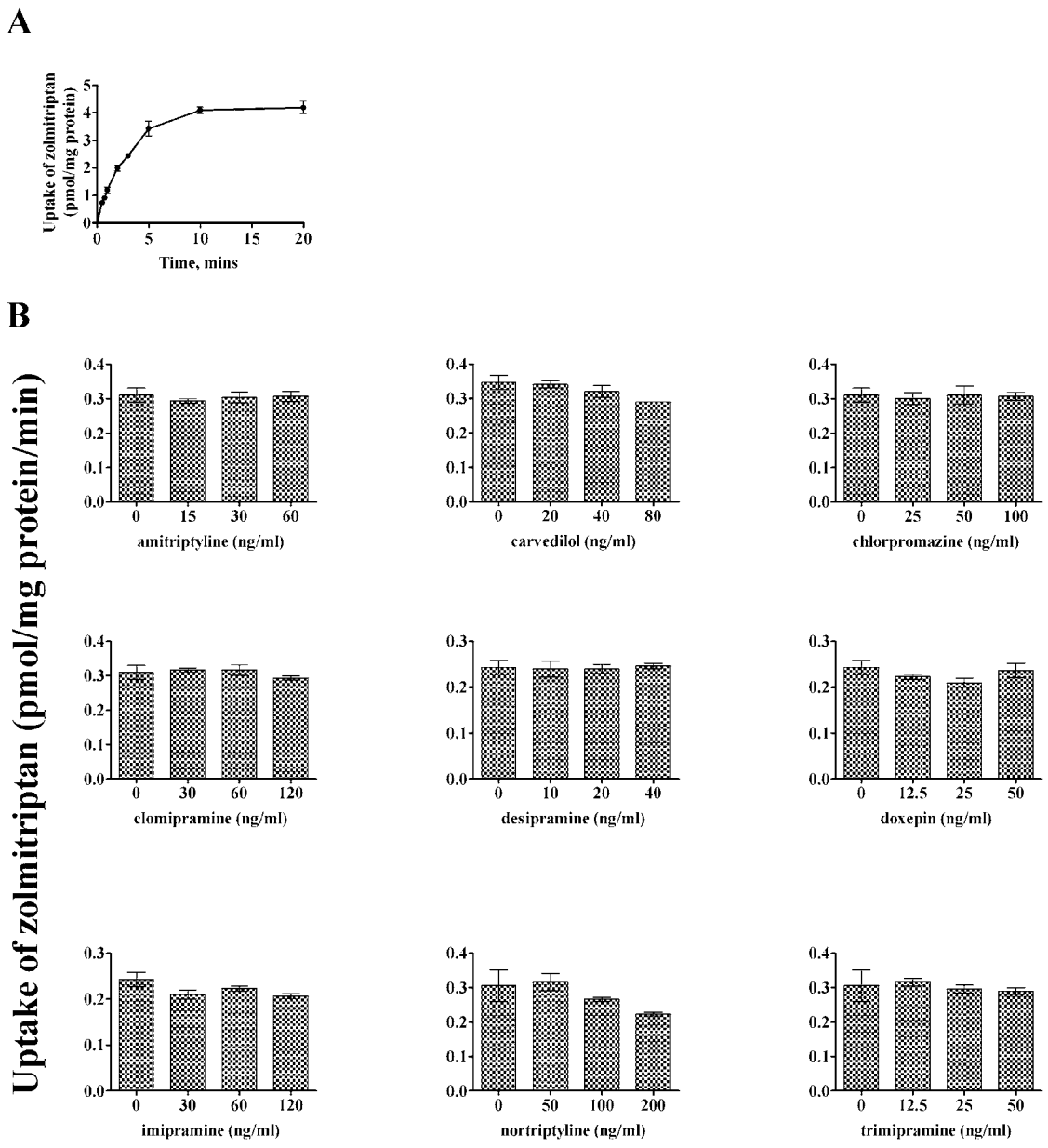

Uptake of almotriptan (pmol/mg protein/min)
amitriptyline (ng/ml)
0
15
30
60
carvedilol (ng/ml)
0
20
40
80
chlorpromazine (ng/ml)
0
25
50
100
clomipramine (ng/ml)
0
30
60
120
desipramine (ng/ml)
0
10
20
40
doxepin (ng/ml)
0
12.5
25
50
imipramine (ng/ml)
0
30
60
120
nortriptyline (ng/ml)
0
50
100
200
trimipramine (ng/ml)
0
12.5
25
50

Figure 5


A
Uptake of zolmitriptan (pmol/mg protein)
Time, mins
B
Uptake of zolmitriptan (pmol/mg protein/min)
amitriptyline (ng/ml)
carvedilol (ng/ml)
chlorpromazine (ng/ml)
clomipramine (ng/ml)
desipramine (ng/ml)
doxepin (ng/ml)
imipramine (ng/ml)
nortriptyline (ng/ml)
trimipramine (ng/ml)

Supplemental Figures

Journal of Pharmacokinetics and Experimental Therapeutics

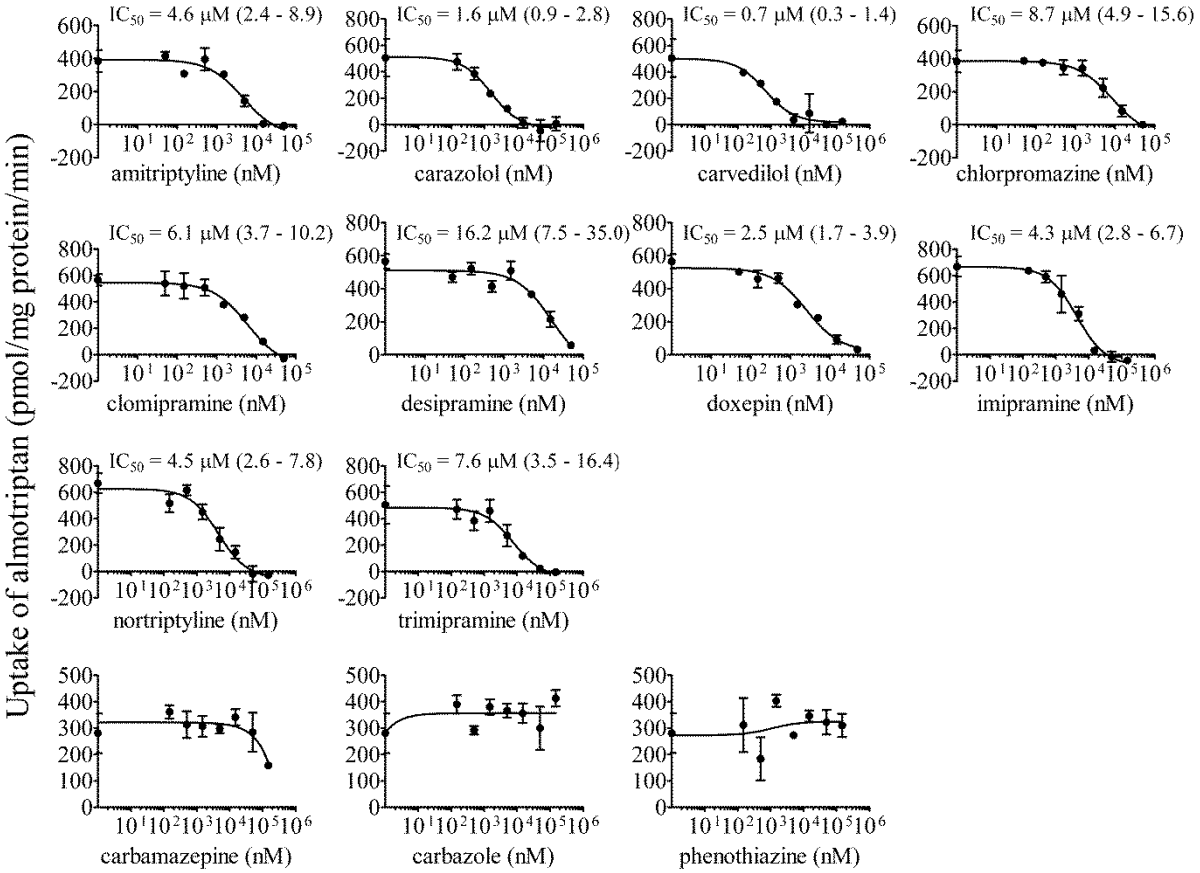
Effects of tricyclic compounds on the transport of anti-migraine triptans through human organic anion transporting polypeptide 1A2 (OATP1A2)

Jennifer Lu, Alexia Grangeon, Fleur Gaudette, Jacques Turgeon, and Veronique Michaud

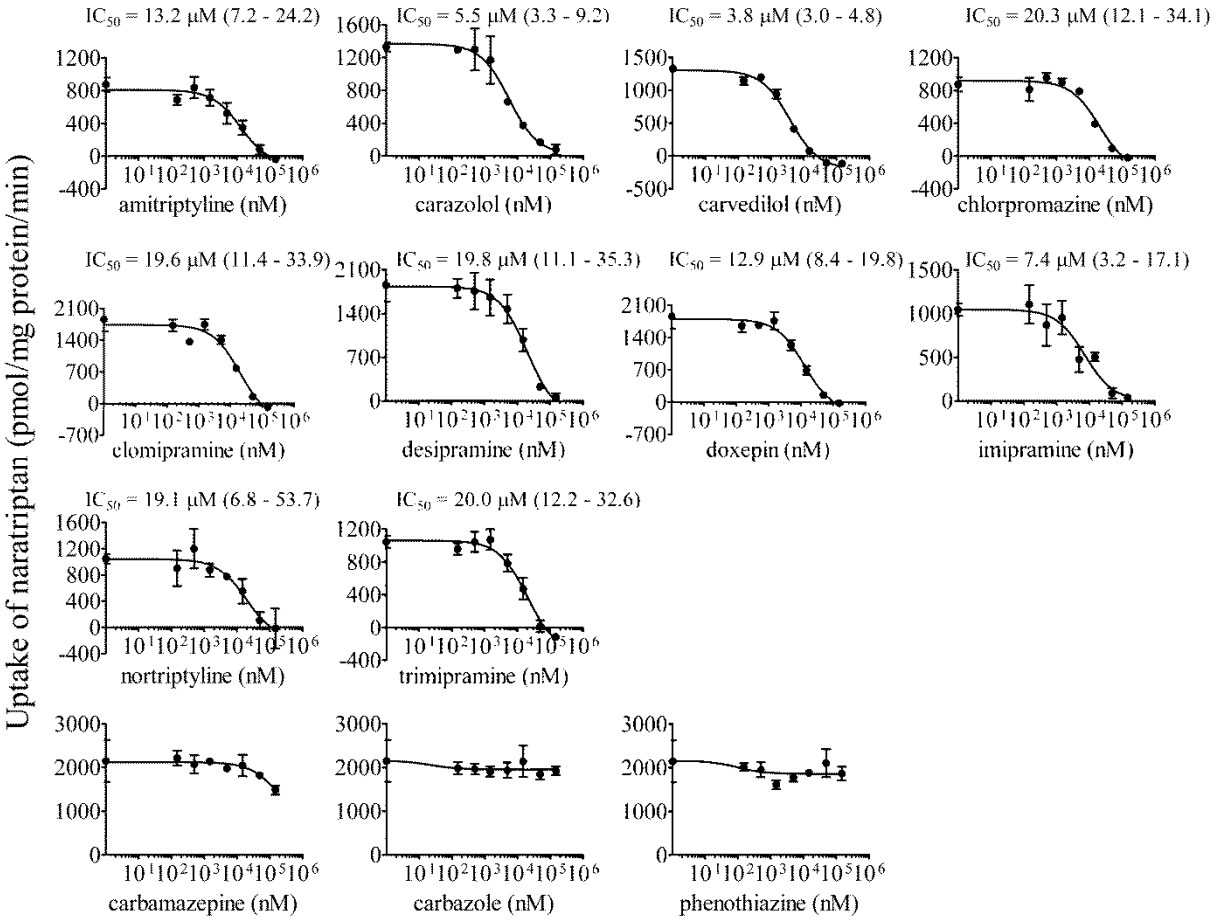
Supplemental Table 1: Validation of LC-MS/MS quantification methods of triptans

Compound	Concentration (pg/mL)	Intra (n=3) Mean ± SD (pg/mL)	Intra (n=3) CV (%)	Intra (n=3) Nominal (%)	Inter (n=9) Mean ± SD (pg/mL)	Inter (n=9) CV (%)	Inter (n=9) Nominal (%)
Almotriptan	75.0	72.4 ± 3.7	5.1	-3.5	76.2 ± 9.9	13.0	1.5
	1250	1203 ± 28	2.3	-3.8	1189 ± 32	2.7	-4.9
	15000	15424 ± 382	2.5	2.8	15013 ± 470	3.1	0.1
Zolmitriptan	300	275 ± 2.9	1.1	-8.4	282.8 ± 11.3	4.0	-5.7
	5000	4633 ± 234	5.1	-7.3	4814 ± 225	4.7	-3.7
	20000	19322 ± 57	0.3	-3.4	20045 ± 790	3.9	0.2

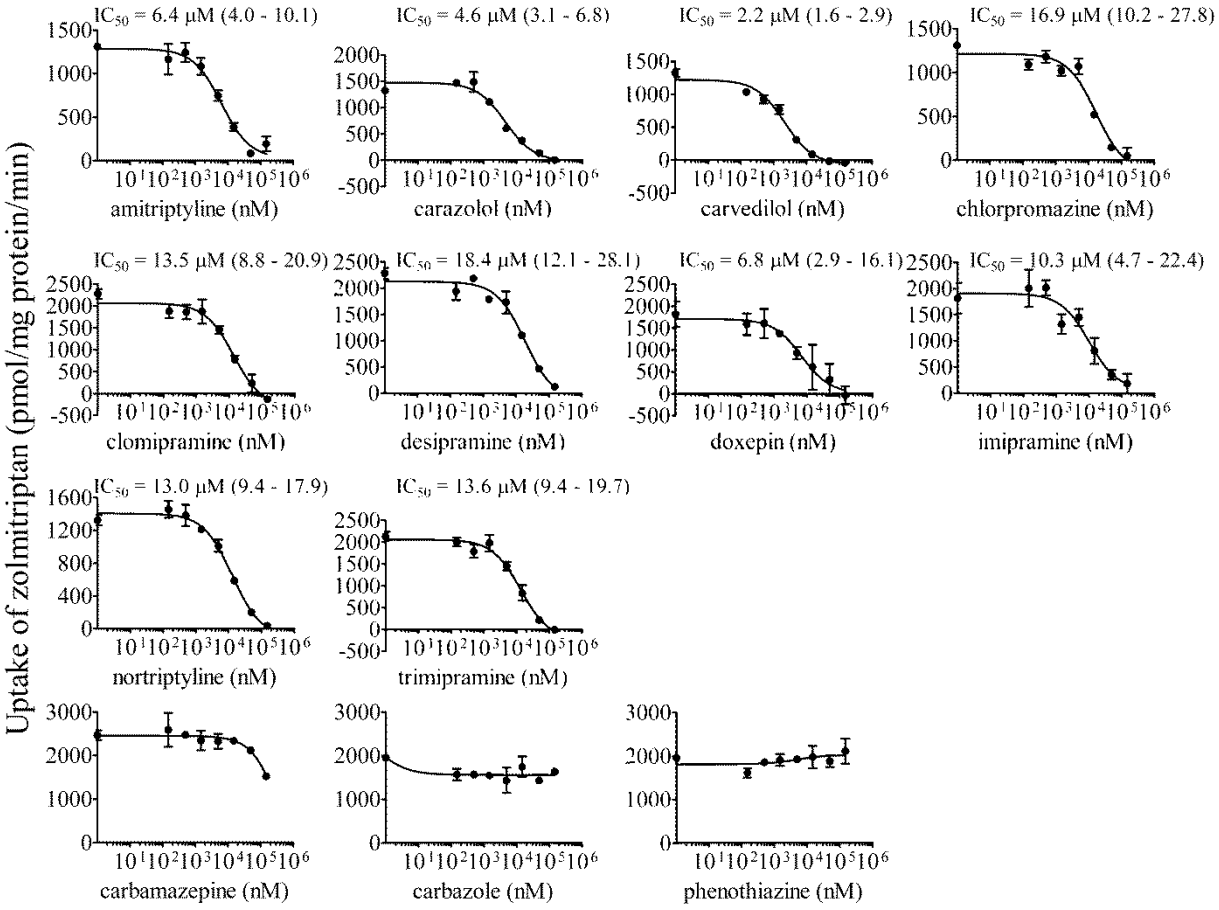
SD: standard deviation; CV: coefficient of variation



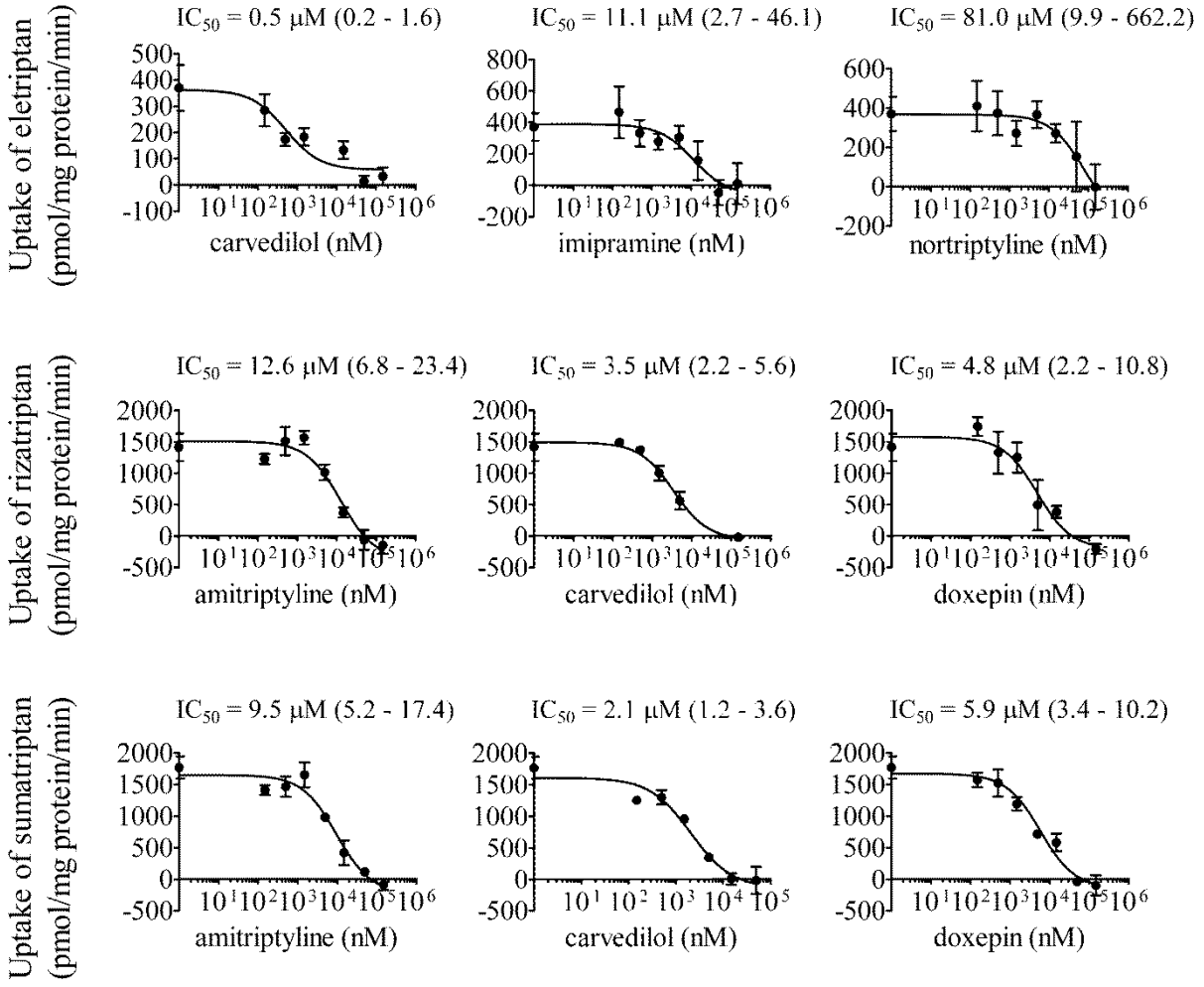
Supplemental Figure 1. Inhibition of OATP1A2-mediated transport of almotriptan by different tricyclic compounds. HEK293-OATP1A2 and HEK293-VC cells were coincubated with almotriptan (15 μM) and different tricyclic compounds (150 nM – 150 μM) for 2 min at 37°C. The quantity of intracellular almotriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation in GraphPad Prism. Each point represents the mean ± S.D. of triplicate from a single experiment. The values in parentheses represent the 95% confidence interval.



Supplemental Figure 2. Inhibition of OATP1A2-mediated transport of naratriptan by different tricyclic compounds. HEK293-OATP1A2 and HEK293-VC cells were coincubated with naratriptan (60 μM) and different tricyclic compounds (150 nM – 150 μM) for 2 min at 37°C. The quantity of intracellular naratriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation in GraphPad Prism. Each point represents the mean ± S.D. of triplicate from a single experiment. The values in parentheses represent the 95% confidence interval.



Supplemental Figure 3. Inhibition of OATP1A2-mediated transport of zolmitriptan by different tricyclic compounds. HEK293-OATP1A2 and HEK293-VC cells were coincubated with zolmitriptan (65 μM) and different tricyclic compounds (150 nM – 150 μM) for 2 min at 37°C. The quantity of intracellular zolmitriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation in GraphPad Prism. Each point represents the mean ± S.D. of triplicate from a single experiment. The values in parentheses represent the 95% confidence interval.



Supplemental Figure 4. Inhibition of OATP1A2-mediated transport of eletriptan, rizatriptan and sumatriptan by different tricyclic compounds. HEK293-OATP1A2 and HEK293-VC cells were coincubated with eletriptan (3 μM), rizatriptan (130 μM) or sumatriptan (300 μM) and different tricyclic compounds (150 nM – 150 μM). The incubation time for eletriptan was 1 min; whereas, rizatriptan and sumatriptan were incubated for 2 min at 37°C. The quantity of intracellular triptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor)

vs. response equation in GraphPad Prism. Each point represents the mean ± S.D. of triplicate from a single experiment. The values in parentheses represent the 95% confidence interval.

2.2.4 DISCUSSION

The transport of six triptans (almotriptan, eletriptan, naratriptan, rizatriptan, sumatriptan, and zolmitriptan) through OATP1A2 was characterized. Frovatriptan was not easily accessible and was not evaluated in this study. The tricyclic compounds evaluated in the previous article inhibited OATP1A2-mediated triptans uptake. At total plasma concentrations of the drugs, DDIs were still observed between a few substrate and inhibitor combinations. As mentioned in the article, there are evidences in the literature supporting the entrance of triptans to the brain. Our data suggest that the hydrophilic triptans may use OATP1A2 to cross the BBB.

As mentioned earlier, eletriptan's logD value at physiological pH predicts its passage to the brain by passive diffusion. However, eletriptan is a substrate of P-gp, an efflux transporter highly expressed on the apical membrane of the BBB. *In vitro* studies using polarized cells overexpressing P-gp on the apical membrane showed that the rate of transport of eletriptan from the basolateral to apical side is greater than the rate of transport from the apical to basolateral. In addition, animal studies showed that *Mdr1a*$^{+/+}$ mice have a 40-fold reduction in brain exposure to eletriptan than *Mdr1a*$^{-/-}$ mice [161]. This is a clear indication of eletriptan efflux from the BBB by P-gp. As exemplified with eletriptan, designing small lipophilic molecules do not guarantee transport to the CNS as they are also good substrates for P-gp [162].

Efflux transporters (P-gp, BCRP, MRP1, MRP4, and MRP5) play an important role in limiting the entrance of drugs to the brain. Owing to the fact that they transport an exceptionally wide range of structurally diverse substrates together, it is believed that very few drugs bypass their surveillance, contributing to the difficulty in targeting the CNS in drug development. Studies in epileptic patients refractory to anti-epileptic drugs showed that they have markedly increased level of P-gp and/or MRP1 at the brain [163]. Other studies demonstrated a link between *ABCB1* polymorphisms and the response to anti-epileptic drugs [164]. These are clear evidences on the function of efflux transporters at the BBB.

The BBB is a complex regulatory interface that serves as obstacle to external aggressions. It is believed that the barrier could somehow be circumvented by exploiting influx transporters for drug delivery [157]. An endogenous substrate uptake rate across the BBB through a transporter is approximately 10 times greater than by passive diffusion [165]. Thus, exploiting transporters in drug development offers high uptake rates and specific targeting of drugs to inaccessible regions, such as the CNS [157]. To go forward in this direction, further characterization of influx drug transporters expressed at the BBB is needed. An important aspect involves understanding how genetic variability in the genes encoding for drug transporters plays a role in causing interindividual variability in drug response. The next article will characterize two protein variants of OATP1A2.

SECTION 2.3

ARTICLE #3

Impact of single nucleotide polymorphisms found in human organic anion transporting polypeptide 1A2 (OATP1A2) on triptans transport

2.3.1 OBJECTIVES

The objectives were to characterize the transport of triptans through two OATP1A2 proteins variants (OATP1A2*2 and *3) and to determine whether the tricyclic compounds maintained their inhibition on the variants.

2.3.2 INTRODUCTION

As presented in the introduction on my thesis, interindividual variability in drug response is a big challenge in the clinic. DDIs and SNPs found in genes encoding for drug transporters may affect the pharmacokinetics of a drug. The two previous articles demonstrated instances of DDIs affecting the uptake of rosuvastatin and triptans through OATP1A2. The third article evaluated the activity of two OATP1A2 protein variants, *2 (T38C, I13T) and *3 (A516C, E172D), found at more than 1% in allelic frequency in the population [23, 44, 166, 167].

Given that OATP1A2 may be involved in the penetration of hydrophilic triptans across the BBB, genetic variants influencing OATP1A2's activity may also interfere with the distribution of triptans to the brain and ultimately the anti-migraine effect is affected. In addition, as the effect of a polymorphism on the transporter is substrate-dependant, it may also be inhibitor-dependent. Thus, the tricyclic drugs that inhibited wild-type OATP1A2 were evaluated.

2.3.3 ARTICLE

The authors' specific contributions were as follow:

- Participated in research design: Jennifer Lu, Veronique Michaud, Jacques Turgeon
- Conducted experiments: Jennifer Lu
- Contributed new reagents or analytic tools: Jennifer Lu, Fleur Gaudette
- Performed data analysis: Jennifer Lu, Fleur Gaudette

- Wrote or contributed to the writing of the manuscript: Jennifer Lu, Veronique Michaud, Jacques Turgeon

Impact of single nucleotide polymorphisms found in human organic anion transporting polypeptide 1A2 (OATP1A2) on triptans transport

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Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM), Montreal, QC, Canada, H2X 0A9 (J.L., F.G., and V.M.)

Faculty of Pharmacy, Université de Montréal, Montreal, QC, Canada, H3T 1J4 (J.L., J.T., and V.M.)

Running Title: Impact of OATP1A2 SNPs on triptans transport

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Text Pages: 25

Number of Tables: 2 (+1 supplemental table)

Number of Figures: 1 (+2 supplemental figures)

Number of References: 18

Number of words in Abstract: 256

Number of words in Introduction: 522

Number of words in Discussion: 556

Abbreviations: CNS, central nervous system; HEK, human embryonic kidney; OATP, organic anion transporting polypeptide; PBS, phosphate-buffered saline; SNP, single nucleotide polymorphism

Abstract

Migraines are an important cause of disability. Triptan drugs have been successful in treating acute migraine attacks. However, a significant proportion of patients fail to respond to triptans. This failure is partially explained by single nucleotide polymorphisms (SNPs) found in the genes associated to the neurotransmitter system and drug metabolism. As these drugs are believed to act at the central nervous system (CNS) as well as at the periphery, an additional mechanism for response failure may be provided by their inability to cross to the blood-brain barrier (BBB). Triptans are hydrophilic molecules and require facilitated transport to cross the BBB. Previous studies demonstrated triptans as substrates of OATP1A2, a drug transporter located at the BBB, suggesting a potential role in the entry of drugs to the CNS. Several genetic variants are known to affect OATP1A2 activity. The main objective of this study was to characterize triptans transport via the protein variants OATP1A2*2 (T38C, I13T) and *3 (A516C, E172D). First, the variants activity was evaluated with the prototypical substrate estrone-3-sulfate. The K_m for each triptan was comparable between the wild-type (WT) and the variants with the exception of sumatriptan. The rate of transport and the intrinsic clearance for a specific substrate was variable between WT, *2, and *3. The tricyclic compounds, previously determined as OATP1A2 inhibitors, inhibited the uptake of triptans through *2 and *3 with the same potency as the WT. Our data suggest that genetic polymorphisms in the *SLCO1A2* gene may provide an additional explanation to the lack of triptan efficacy observed in unresponsive patients.

Introduction

Triptans are a class of medication commonly used in the abortive treatment of acute migraine attacks. A meta-analysis study compared 133 randomized controlled trials to demonstrate that 42 – 76% of patients achieved headache relief within two hours following the administration of a triptan (Cameron *et al.*, 2015). Despite their rate of success, a proportion of migraineurs fail to reach headache relief. In clinical practice, more than one third of triptan users discontinue these drugs and the main reason is the failure to relief pain (Holland *et al.*, 2013; Wells *et al.*, 2014). The lack of efficacy in triptan treatment may be in part explained by polymorphisms found in the genes involved in the pharmacological response to triptans. Up to now, studies have concentrated on genes associated to the neurotransmitter system and drug metabolism. Studies have found associations with an altered response to triptans and single nucleotide polymorphisms (SNPs) in the genes encoding the serotonin transporter, monoamine oxidase A, and CYP1A2 (Gentile *et al.*, 2010; Terrazzino *et al.*, 2010).

Another reason for triptan failure may be attributed to the drug's inability to reach its site of action located at the central nervous system (CNS). As they are hydrophilic, drug transporters are needed to facilitate their entrance into the brain. Previous studies suggested that OATP1A2 may be responsible for their permeation as it is located at the blood-brain barrier (BBB) and triptans are substrates of this transporter (Bronger *et al.*, 2005; Cheng *et al.*, 2012; Gao *et al.*, 2000; Gao *et al.*, 2014; Lee *et al.*, 2005). Genetic variations in the SLCO1A2 gene (encoding the OATP1A2 protein) modifying the transporter activity or its level of expression at the cell surface may affect the passage of triptans across the BBB. Several synonymous and non-synonymous SNPs have been identified and characterized in the literature. The variants OATP1A2*2 (T38C, I13T) and

*3 (A516C, E172D) are found at high allelic frequency (>1%) in the population (Badagnani *et al.*, 2006; Boso *et al.*, 2014; Laitinen and Niemi, 2011; Lee *et al.*, 2005). Ethnical differences exist for these two variants as they are most frequently observed in European-Americans, followed by Hispanics and African-Americans but undetected in Asian-Americans. *In vitro* evaluations generated inconsistent data on OATP1A2*2 activity. Lee *et al.* reported the uptake of estrone-3-sulfate, deltorphin II, and [D-Pen2,5]Enkephalin (DPDPE) to be unchanged in OATP1A2*2 but Badagnani et al. reported increased transport of estrone-3-sulfate and methotrexate (Badagnani *et al.*, 2006; Lee *et al.*, 2005). OATP1A2*3 transport activity with all substrates evaluated was reduced in both studies and this decrease was attributed to its lower trafficking to the cell surface.

In addition to SNPs, drug-drug interactions via OATP1A2 may modulate the transport of triptans across the BBB. We have previously demonstrated that compounds composed of a tricyclic ring and a short aliphatic amine chain, such as carvedilol and tricyclic antidepressants, inhibit the transport of our probe drug, rosuvastatin, through OATP1A2 (Lu *et al.*, 2015). We have also established that the transport of triptans through OATP1A2 is inhibited by these compounds (Lu *et al.*, 2016).

Since genetic variation may play an important role in interindividual variation in triptans disposition and response, the transport of these drugs through OATP1A2*2 and *3 variants were investigated further. The objectives of this study were to: 1) determine whether the transport kinetics of our probe drug, rosuvastatin, in OATP1A2*2 and *3 differs from the wild-type (WT), 2) determine whether the transport of triptans is affected in OATP1A2*2 and *3 variants, and 3)

determine whether the inhibition of rosuvastatin and triptans uptake by different tricyclic compounds is maintained in the variants.

Materials and Methods

Reagents

Amitriptyline hydrochloride, estrone-3-sulfate potassium salt, naproxen, naratriptan hydrochloride, rizatriptan benzoate, sumatriptan succinate, zolmitriptan were purchased from Sigma-Aldrich (St-Louis, MO, USA). Almotriptan hydrochloride, carvedilol, doxepin hydrochloride, eletriptan hydrobromide, rosuvastatin calcium salt were purchased from Toronto Research Chemicals (Toronto, ON, Canada). [^{3}H]Estrone-3-sulfate, ammonium salt (45.6 μCi/nmol) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). All chemicals and solvents were obtained from Sigma-Aldrich, Fisher Scientific (Fair Lawn, NJ, USA) or J.T. Baker (Center Valley, PA, USA).

Cell culture

HEK293-OATP1A2 WT, HEK293-OATP1A2*2, HEK293-OATP1A2*3, and HEK293-VC cells were kindly provided by Dr. Markus Keiser and Dr. Werner Siegmund (Department of Clinical Pharmacology, Center of Drug Absorption and Transport, University Medicine Greifswald, Greifswald, Germany). Cell passage was done with 0.25% trypsin/EDTA. The cells were cultured in minimum essential medium with Earle's salts and L-Glutamine supplemented with 10% fetal bovine serum, 1x nonessential amino acids, and 1 mM sodium pyruvate. They were maintained in a 37°C and 5% CO_2 incubator. Cell culture media, trysin/EDTA, and supplements were purchased from Multicell Wisent Inc. (St-Jean-Baptiste, QC, Canada).

Uptake assays and competition assays

The transport (K_m V_{max} and IC_{50}) of triptans and rosuvastatin was assessed as previously described (Lu *et al.*, 2015). Briefly, HEK293-OATP1A2 WT, *2, *3 and HEK293-VC cells were seeded in poly-L-lysine (Sigma-Aldrich) treated tissue culture plates one day before the

experiment. On the experiment day, the culture media was removed and the cells were pre-incubated with warm transport buffer at 37°C for 5 minutes. Transport was initiated at 37°C by replacing the buffer with a solution of substrate diluted in transport buffer. In the IC_{50} studies, the replacement solution contained a mix of substrate and inhibitor in transport buffer. After the incubation period, the cells were washed twice with cold phosphate-buffered saline (PBS) containing 10% acetonitrile followed by a final wash with cold PBS.

Transport kinetics of the six triptans and rosuvastatin through OATP1A2 variants was determined by incubating HEK293-OATP1A2 WT, *2, *3 and HEK293-VC cells in six-well plates with almotriptan (0.375, 0.75, 1.5, 3, 6.25, 12.5, and 25 μM), eletriptan (0.25, 0.5, 1, 2, 3, 4, and 5 μM), naratriptan (2.5, 5, 7.5, 15, 25, 50, and 100 μM), rizatriptan (3, 7.5, 15, 30, 62.5, 125, and 250 μM), sumatriptan (7.5, 15, 30, 62.5, 125, 250, and 500 μM), zolmitriptan (3, 7.5, 15, 30, 62.5, 125, and 250 μM), and rosuvastatin (7.5, 15, 30, 62.5, 125, and 250 μM). Inhibition studies were conducted by co-incubating HEK293-OATP1A2 WT, *2, *3 and HEK293-VC cells with almotriptan (15 μM), naratriptan (60 μM), zolmitriptan (65 μM) or rosuvastatin (150 μM) in the absence or presence of different tricyclic compounds (0.15–150 μM). In order to saturate the transporter with the substrate, the concentrations of triptans selected equal to three times their K_m value determined in HEK293-OATP1A2 WT cells. The transport of estrone-3-sulfate was assessed in a similar fashion as the other substrates. With the following exceptions: the cells (1.2 x 10^6 cells/plate) were seeded in 35 mm poly-L-lysine treated culture plates and incubated with estrone-3-sulfate (2.5, 5, 10, 20, 40, 80, and 160 μM). A mix of 11 nM [^{3}H]estrone-3-sulfate (0.5 μCi/ml) and various concentrations of nonradiolabeled estrone-3-sulfate was used in the incubation. An incubation time of 2 minutes was chosen for almotriptan, estrone-3-sulfate,

naratriptan, rizatriptan, rosuvastatin, sumatriptan, and zolmitriptan; whereas 1 minute was chosen for eletriptan as these time-points remained within the linear range of their uptake curve.

To measure the protein concentration, three extra wells or plates of each cell line was prepared. They were lyzed with 1% SDS + 0.2 N NaOH and the Pierce BCA protein assay kit from Thermo Scientific (Rockford, IL, USA) was used to measure the protein concentration. The average value was used to normalize intracellular concentrations of substrates.

Quantification of Triptans and Rosuvastatin by High-Performance Liquid Chromatography-UV

The quantity of triptans and rosuvastatin transported in the cells was measured by high performance liquid chromatography with UV detection (HPLC-UV). The sample processing methods and HPLC-UV detection methods have been previously described (Lu *et al.*, 2016; Lu *et al.*, 2015).

Quantification of Radiolabeled Estrone-3-Sulfate

After the final wash in PBS, the estrone-3-sulfate samples were processed differently than the other substrates. The cells were lyzed with 0.5 ml of 1% SDS + 0.2 N NaOH and the lysate was homogenized with a 27G x ½" needle coupled to a 1 ml syringe. A volume of 0.4 ml cell lysate was mixed in 5 ml of liquid scintillation cocktail from MP Biomedicals (Solon, OH, USA). The samples were analyzed using a Tri-Carb® 2100TR liquid scintillation counter from PerkinElmer Life Sciences (Boston, MA, USA).

Data analysis

The net transport of substrate through OATP1A2 variants was calculated by subtracting the value in the VC cells from the value in the OATP1A2 WT, *2, or *3 cells. Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Each data point is expressed as the

mean ± S.D. K_m and V_{max} were calculated by fitting the data to the Michaelis-Menten equation. IC_{50} values were calculated by fitting the data to the log(inhibitor) versus response equation, and the range given represents the 95% confidence interval.

Results

*Transport of Triptans and Rosuvastatin through OATP1A2 WT, *2, and *3*

First, to assess how the transport activity found in HEK293 cell lines stably expressing OATP1A2*2 and *3 compares to the WT, the cells were incubated with the prototypical substrate estrone-3-sulfate. The transport kinetic of OATP1A2*2 is similar to the WT; whereas, *3 is diminished compared to the WT (Supplemental Figure 1 and Supplemental Table 1). The uptake in both variants was saturable, indicating that the transporter is functional. Then, we determined whether the transport kinetics of triptans and rosuvastatin is altered in OATP1A2*2 and *3. All seven substrates demonstrated concentration-dependent saturable transport via OATP1A2 WT, *2, and *3 (Figure 1 and Table 1). The K_m for each substrate was comparable between the WT and the variants with the exception of sumatriptan, where the K_m was higher in the *2 and *3 than the WT. The rate of transport for all substrates was either unchanged or decreased in the variants compared to the WT with the exception of eletriptan, where *3 was higher than the WT and *2. The V_{max} of eletriptan, sumatriptan, and rosuvastatin transport through OATP1A2*2 was unchanged but it was reduced for almotriptan, naratriptan, rizatriptan, and zolmitriptan. The rate of transport via OATP1A2*3 was unchanged for almotriptan and rosuvastatin, reduced for naratriptan, rizatriptan, sumatriptan, and zolmitriptan, and surprisingly increased for eletriptan. The intrinsic clearance (CL_{int}) of almotriptan, rizatriptan, sumatriptan, and zolmitriptan was reduced in the variants; whereas, it was unchanged for naratriptan. The CL_{int} of eletriptan through OATP1A2*2 was higher than the WT but OATP1A2*3 was lower.

Effect of Tricyclic Compounds on OATP1A2 WT, *2, *3-mediated Uptake of Triptans

To determine whether compounds composed of a tricyclic ring and a short aliphatic amine chain inhibit uptake of triptans through the two variants, competition studies were performed (Table 2 and Supplemental Figure 2). The three inhibitors tested inhibited the uptake of almotriptan, naratriptan, zolmitriptan, and rosuvastatin through OATP1A2 WT, *2, and *3. The IC_{50} values are consistent between the WT and the two variants for a given inhibitor and substrate. As expected, carvedilol showed the strongest inhibition on the uptake of the four substrates.

Discussion

Drug transporters are important determinants in drug absorption, distribution, elimination, efficacy and toxicity. The numerous drug transporters expressed at the BBB are responsible for limiting or facilitating the entrance of xenobiotics to the brain. Up to now, most studies focused on efflux transporters at the BBB as they are believed to contribute to the failure of many CNS-active drugs. Recently, we came to appreciate the role of influx transporters at the BBB in drug delivery (Urquhart and Kim, 2009). Notably, OATP1A2 is expressed on the luminal membrane of the endothelial cells constituting the BBB and transports a broad spectrum of substrates. We believe that OATP1A2 is involved in the transport of triptans to its site of action at the CNS.

A SNP modifying the activity of a drug transporter implicated in the fate of a medication can lead to changes in the drug's pharmacokinetics and pharmacodynamics. Consequently, we characterized the transport activity of two variants of the OATP1A2 protein, *2 (T38C, I13T) and *3 (A516C, E172D), for their uptake of the anti-migraine drugs triptans. The affinity (K_m) of each substrate does not differ much between the WT and its two variants, with the exception of sumatriptan. It suggests that the binding site of these substrates, except for sumatriptan, is not affected by the mutations and that sumatriptan may bind differently to OATP1A2 than the other triptans. By comparing to our previous results, the K_m values are consistent but not the V_{max} values (Lu *et al.*, 2016). The cells batch-to-batch variability in the level of transporter expression accounts for the V_{max} differences.

Previous studies showed that OATP1A2*2 transport activity is either unchanged or increased compared to the WT. However, we observed either a decrease or unchanged in transport depending on the substrate. The literature reported a decrease in OATP1A2*3 activity, which is not consistently observed with all substrates evaluated in this study. Yet, the uptake of the prototypical substrate, estrone-3-sulfate, was unchanged in *2 and decreased in *3, which is in line with the data published by Lee *et al* (2005). How the variant affects the transporter activity seems to be substrate-dependent. The CL_{int} values seem to indicate a reduced clearance of almotriptan, eletriptan (*3 only), rizatriptan, sumatriptan and zolmitriptan through the variants. The IC_{50} values do not vary between the WT, *2, and *3 for a given substrate and a given inhibitor; suggesting that the mutations do not change the inhibitors binding site on the transporter.

In vivo assessment of OATP1A2 function has unfortunately yield a negative correlation between drug response and the *2 and *3 variants. *SLCO1A2* genotype did not correlate with plasma concentrations of lopinavir in HIV patients (Hartkoorn *et al.*, 2010). In addition, steady-state plasma concentrations of imatinib is not affected in cancer patients with the *SLCO1A2*-516A>C genotype even if *in vitro* data showed a clear abolition in transport (Eechoute *et al.*, 2011). This discrepancy may be explained by incorrect assumptions on OATP1A2's location as the hypothesis was based on its presence in the intestine. Later studies could not detect OATP1A2 in the intestine.

Taken together, our data suggest that individuals with the OATP1A2*2 and *3 variants may have a reduced capacity in directing most triptan drugs across the BBB. By limiting their transport to the CNS, we can speculate that the response to triptan treatment in migraine may be diminished or even abolished. The clinical significance of these variants in their role regarding triptan failure needs to be investigated.

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Authorship Contributions

Participated in research design: J. Lu, J. Turgeon, V. Michaud

Conducted experiments: J. Lu

Contributed new reagents or analytic tools: J. Lu, F. Gaudette

Performed data analysis: J. Lu, F. Gaudette

Wrote or contributed to the writing of the manuscript: J. Lu, J. Turgeon, V.Michaud

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Footnotes

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Figure Legends

Figure 1. Transport of triptans and rosuvastatin through OATP1A2 variants. Uptake of (A) almotriptan (0.375 to 25 μM), (B) eletriptan (0.25 to 5 μM), (C) naratriptan (2.5 to 100 μM), (D) rizatriptan (3.0 to 250 μM), (E) sumatriptan (7.5 to 500 μM), (F) zolmitriptan (3.0 to 250 μM), and (G) rosuvastatin (7.5 to 250 μM) was assessed at 37°C. The transport was assayed for 2 minutes for all substrates except for eletriptan which was assayed for 1 minute. The intracellular quantity of substrate was normalized to protein content. The net transport was calculated by subtracting the values measured in the VC cells from the values measured in OATP1A2 WT, *2, and *3 expressing cells. Each point represents the mean ± S.D. of triplicate from a single experiment. Each experiment was performed twice.

Table 1. K_m, V_{max}, and CL_{int} values of different substrates uptake through OATP1A2 WT, *2, and *3. The values were calculated by fitting the data to the Michaelis-Menten equation using GraphPad Prism 5 (± standard error mean).

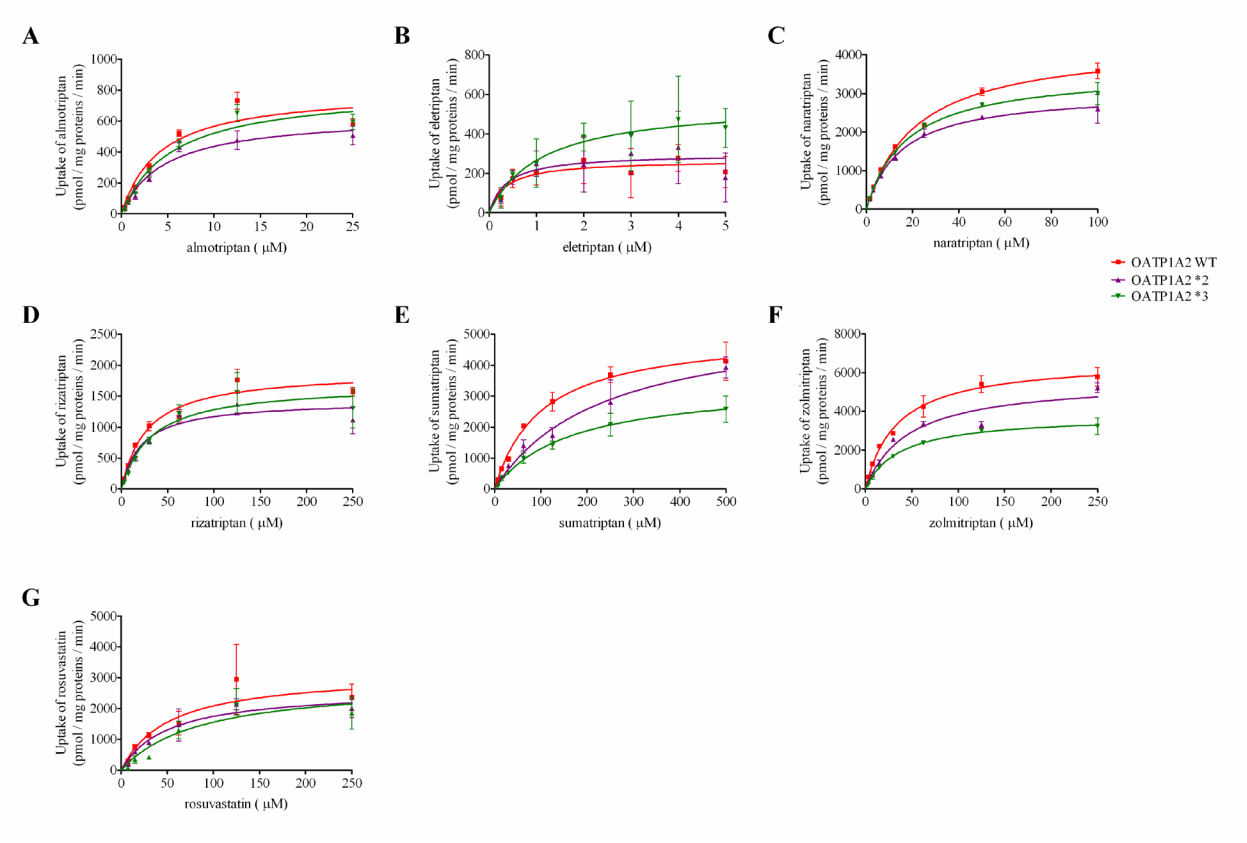
		K_m (μM)	V_{max} (pmol/mg proteins per minute)	CL_{int} (μl/mg proteins per minute)
Almotriptan	WT	4.1 ± 0.9	801.2 ± 62.3	195.4 ± 45.5
	*2	4.6 ± 0.9	638.1 ± 41.9	138.7 ± 28.6
	*3	5.5 ± 1.2	809.7 ± 62.4	147.2 ± 34.1
Eletriptan	WT	0.4 ± 0.2	266.5 ± 38.8	666.2 ± 347.0
	*2	0.4 ± 0.3	298.4 ± 51.3	746.0 ± 574.0
	*3	1.1 ± 0.6	561.0 ± 93.5	510.0 ± 290.9
Naratriptan	WT	21.5 ± 1.3	4318 ± 99.1	200.8 ± 13.0
	*2	15.2 ± 1.6	3052 ± 104.9	200.8 ± 22.2
	*3	17.3 ± 1.2	3579 ± 87.6	206.9 ± 15.2
Rizatriptan	WT	27.6 ± 4.4	1904 ± 98.4	69.0 ± 11.6
	*2	21.9 ± 4.8	1426 ± 89.5	65.1 ± 14.8
	*3	32.1 ± 8.4	1691 ± 140.9	52.7 ± 14.5
Sumatriptan	WT	101.4 ± 13.3	5071 ± 240.9	50.0 ± 7.0
	*2	233.4 ± 54.4	5594 ± 646.8	24.0 ± 6.2
	*3	163.2 ± 30.4	3418 ± 266.9	20.9 ± 4.2
Zolmitriptan	WT	33.8 ± 3.6	6648 ± 231.5	196.7 ± 22.0
	*2	45.7 ± 8.2	5612 ± 355.6	122.8 ± 23.4

	*3	36.8 ± 3.7	3784 ± 126.2	102.8 ± 10.9
Rosuvastatin	WT	51.4 ± 20.0	3164 ± 452.8	61.6 ± 25.5
	*2	50.4 ± 13.5	2630 ± 252.6	52.2 ± 14.8
	*3	85.5 ± 38.6	2895 ± 554.2	33.8 ± 16.6

Table 2. IC_{50} values from the inhibition of triptans and rosuvastatin uptake through OATP1A2 WT, *2, and *3 by different tricyclic compounds. IC_{50} values were calculated by fitting the data to the log(inhibitor) versus response equation in GraphPad Prism 5. The values in parentheses represent the 95% confidence interval (see also Supplemental Figure 2).

		IC_{50} (μM)		
		Amitriptyline	Carvedilol	Doxepin
Almotriptan	WT	3.7 (2.4 – 5.8)	1.0 (0.7 – 1.7)	2.2 (1.2 – 4.0)
	*2	3.9 (1.8 – 8.5)	0.7 (0.4 – 1.3)	3.8 (1.7 – 8.6)
	*3	11.2 (3.0 – 41.5)	1.7 (1.0 – 2.7)	4.5 (2.2 – 9.2)
Naratriptan	WT	11.8 (8.5 – 16.4)	3.2 (2.6 – 4.1)	14.9 (9.4 – 23.5)
	*2	14.8 (9.4 – 23.4)	3.0 (1.9 – 4.8)	13.5 (9.6 – 19.1)
	*3	11.4 (5.8 – 22.7)	4.2 (2.5 – 7.1)	13.4 (7.6 – 23.8)
Zolmitriptan	WT	9.8 (7.6 – 12.7)	2.4 (2.0 – 2.9)	9.5 (7.2 – 12.5)
	*2	7.6 (5.6 – 10.5)	2.5 (1.7 – 3.5)	8.8 (5.5 – 14.2)
	*3	14.0 (11.6 – 17.0)	2.9 (1.9 – 4.3)	9.1 (5.8 – 14.3)
Rosuvastatin	WT	7.7 (4.6 – 12.8)	4.1 (2.6 – 6.4)	16.8 (8.4 – 33.5)
	*2	8.2 (5.2 – 12.8)	3.8 (2.3 – 6.2)	12.5 (5.6 – 27.8)
	*3	1.8 (0.4 – 7.2)	9.0 (4.4 – 18.5)	19.2 (7.4 – 49.7)

Figure 1

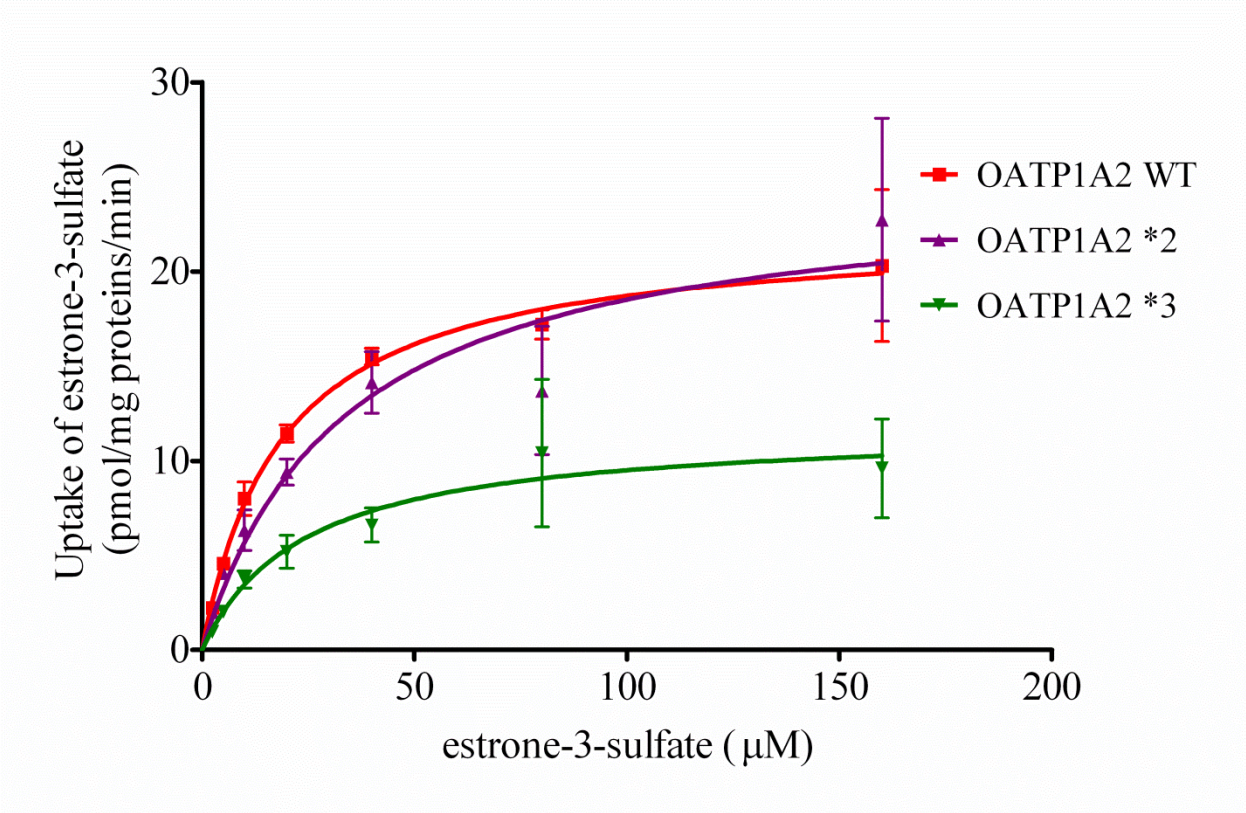

A
Uptake of almotriptan (pmol / mg proteins / min)
almotriptan (μM)
B
Uptake of eletriptan (pmol / mg proteins / min)
eletriptan (μM)
C
Uptake of naratriptan (pmol / mg proteins / min)
naratriptan (μM)
OATP1A2 WT
OATP1A2 *2
OATP1A2 *3
D
Uptake of rizatriptan (pmol / mg proteins / min)
rizatriptan (μM)
E
Uptake of sumatriptan (pmol / mg proteins / min)
sumatriptan (μM)
F
Uptake of zolmitriptan (pmol / mg proteins / min)
zolmitriptan (μM)
G
Uptake of rosuvastatin (pmol / mg proteins / min)
rosuvastatin (μM)

Supplemental Figures

Molecular Pharmacology

Impact of single nucleotide polymorphisms found in human organic anion transporting polypeptide 1A2 (OATP1A2) on triptans transport

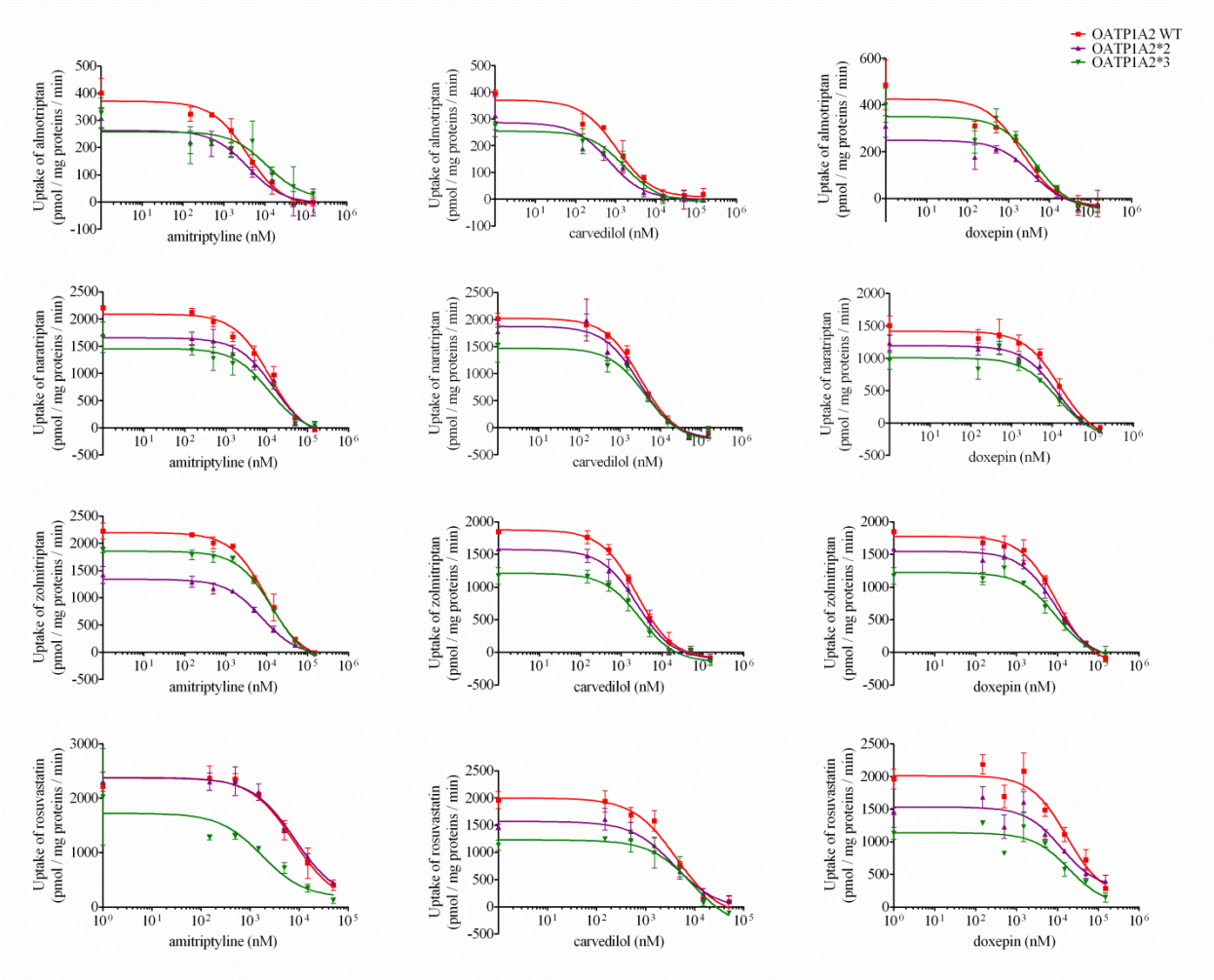
Jennifer Lu, Fleur Gaudette, Jacques Turgeon, and Veronique Michaud



Supplemental Figure 1. Uptake of estrone-3-sulfate through OATP1A2 variants. Uptake of estrone-3-sulfate (2.5 to 160 μM) was assessed for 2 min at 37°C. The quantity of intracellular estrone-3-sulfate was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2 variants-expressing cells. Each point represents the mean ± S.D. of triplicate from a single experiment.

Supplemental Table 1. K_m, V_{max}, and CL_{int} values of estrone-3-sulfate uptake through OATP1A2 WT, *2, and *3. The values were calculated by fitting the data to the Michaelis-Menten equation using GraphPad Prism 5 (± standard error mean).

		K_m (μM)	V_{max} (pmol/mg proteins/minute)	CL_{int} (μl/mg proteins/minute)
Estrone-3-sulfate	WT	18.8 ± 2.6	22.2 ± 1.0	1.2
	*2	33.6 ± 9.8	24.7 ± 2.6	0.7
	*3	24.2 ± 8.0	11.8 ± 1.3	0.5



Supplemental Figure 2. Inhibition of almotriptan, naratriptan, zolmitriptan, and rosuvastatin transport via OATP1A2 variants by amitriptyline, carvedilol, and doxepin. Inhibition of almotriptan (15 µM), naratriptan (60 µM), zolmitriptan (65 µM), and rosuvastatin (150 µM) by different tricyclic compounds (150 nM – 150 µM) was assessed for 2 min at 37°C. The intracellular quantity of substrate was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in the transporter expressing cells. Each point represents the mean ± S.D. of triplicate from a single experiment.

2.3.4 DISCUSSION

The effect of the two genetic polymorphisms studied on the activity of OATP1A2 was substrate-dependant. On the other hand, the inhibitors potency did not differ between the wild-type, *2, and *3 for a given substrate. The data suggest that the transport of several triptans to the CNS may be limited through the variants.

Genetic polymorphisms found in the *SLCO1A2* gene have been associated to variability in drug disposition. The clearance of the anti-cancer drug imatinib is higher in patients with the *SLCO1A2* - 361G>A genotype (p=0.005) and lower in patients with the *SLCO1A2* - 1105G>A and the *SLCO1A2* - 1032G>A genotype (p=0.075) [168]. Thus, further evaluation of OATP1A2 involvement in drug disposition and the effect of SNPs found in *SLCO1A2* is warranted.

SECTION 3: CONCLUSION

3. CONCLUSION

The extent to which a drug is able to exert its therapeutic effects depends on the drug concentration accumulating within its site of action. Drug transporters are the gatekeepers that may facilitate or hinder the entry of drugs into the tissue. It is easy to conceive that factors influencing the drug transporters activity, such as interactions with concomitant medications and polymorphisms in the genes encoding drug transporters, may alter drug disposition and thus affecting its therapeutic and toxic effects. Therefore, it is important to characterize the transporters known to be involved in drug disposition.

In this thesis, the membrane drug transporter OATP1A2 was characterized in regards to its substrates, inhibitors, and proteins variants. We demonstrated that compounds composed of a tricyclic ring and a short aliphatic amine chain, such as carvedilol and tricyclic anti-depressants, were potent OATP1A2 inhibitors. To the best of our knowledge, this has not been reported in the literature before. Triptans were confirmed as OATP1A2 substrates and tricyclic drugs inhibited their uptake. Furthermore, competition experiments at concentrations observed in patients treated with these drugs showed that the inhibition of almotriptan and zolmitriptan uptake is maintained with carvedilol and nortriptyline; whereas, clomipramine inhibited only the transport of almotriptan. Finally, there appeared to be a substrate-dependant effect from the two OATP1A2 protein variants studied.

A previous study, using *Xenopus laevis* oocytes, demonstrated that several β-blockers were OATP1A2 substrates [49]. In our studies, we found that most β-blockers evaluated were weak competitors for rosuvastatin uptake, indicating that they are either not transported by OATP1A2, as previously reported, or they have a weaker affinity than rosuvastatin. Similar experiments were repeated with triptans and also concluded β-blockers as weak competitors for triptans uptake (Table 8, Figure 2, Figure 3, and Figure 4). The second explanation is more plausible as a K_m of 84.3 µM for nadolol was reported by Misaka *et al.*, whereas a K_m of 60.2,

5.1, 20.3, and 21.4 μM was reported for rosuvastatin, almotriptan, naratriptan, and zolmitriptan, respectively [34].

Additionally, rifampicin, also known as rifampin, inhibited the uptake of triptans through OATP1A2 (Table 8, Figure 2, Figure 3, and Figure 4). This is interesting because rifampicin is used as first-line therapy to treat CNS tuberculosis and its logD value at pH7.4 is below 1.0 [169, 170]. Rifampicin has been shown to distribute to the cerebrospinal fluid, a surrogate marker for the penetration of drugs to the CNS [171, 172]. Our data suggest that OATP1A2 may potentially mediate the uptake of hydrophilic rifampicin across the BBB. It would be interesting to determine whether rifampicin is an OATP1A2 substrate.

Furthermore, we found that tricyclic antidepressants were not substrates of OATP1A2, which is also in line with the literature [55]. These drugs are relatively lipophilic; therefore, they do not require a transporter to cross the BBB. They can nonetheless interact with transporters to inhibit them as shown by our data. Triptans were found to be substrates of OATP1A2, in line with data published by Cheng *et al* [55].

We found that the effect from the genetic polymorphisms on OATP1A2 transport was substrate-dependent. Other transporters also exhibit substrate specific effects with nonsynonymous SNPs. For example, the transport of estrone-3-sulfate and estradiol 17β-glucuronide through OATP1B1 *5 (T521C, V174A) is not affected; whereas, it is decreased with pravastatin, atorvastatin [173, 174]. OCTN1 (C1672T, L503F) showed enhanced function with tetrabutylammonium, tetraethylammonium, and tetrapentylammonium, but reduced function with carnitine, choline, cimetidine, lidocaine, N-methylnicotinamide, and verapamil [175]. The two polymorphisms evaluated in OATP1A2 were not related to changes in substrate affinity; thus, the substrate binding site is not affected. Since a variant may show functional effects only with specific substrates, it emphasizes the need to evaluate the variant with specific substrates instead of drawing a general conclusion with probe-substrates when characterizing the transporter.

In vitro cell models for the study of drug transporter offer several advantages: the effect observed is attributable to the overexpressed transporter when a proper control is used, the experimental set-up can be controlled, the simplicity of their use, and the absence of specific probe substrates and inhibitors does not prevent their evaluation. Despite their advantages, the main limitation with *in vitro* cells models is the difficulty in extrapolating the findings to *in vivo* settings. Conclusions drawn from *in vitro* models, whether they are overexpression cell models, vesicular membrane preparations, or polarized Caco-2 cells, always need to be confirmed in humans.

A limitation with conducting a conventional ADME study to test drug disposition is where the drug can be sampled. The commonly used measurements are blood draws, total urine and feces collection. This does not allow us to study drug distribution to specific tissues and organs and whether the drug is directed to its postulated site of action or not. A drug's profile in tissue concentrations may differ from its profile in blood and rapid equilibrium in the body cannot be assumed. Therefore, a drug's response cannot simply be predicted based on its concentration-time profile in plasma. Lately, several sophisticated noninvasive *in vivo* imaging techniques have been employed to study how efficiently drugs reach their target organs, their retention, their distribution in the organ, to predict adverse drug reactions, and to study DDIs [176, 177]. Positron-emission tomography (PET), single-photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI) are imaging techniques used in the drug development field. PET offers the advantage over other imaging techniques of using customizable radiolabeled molecules as isotopes (^{11}C, ^{13}N, ^{15}O, and ^{18}F) are directly introduced in the drug structure. Immediately following intravenous injection of the radiolabeled drug, its distribution throughout the body can be monitored by a PET scanner. Imaging studies will also help in understanding the *in vivo* effects of genetic polymorphisms on drug transporters and provide insights into interindividual variability in the response to drugs.

As discussed earlier, there are situations where a DDI has been observed *in vitro* as well as *in vivo* but the effect observed is not necessarily attributed to the transporter studied. For example, the grapefruit juice and fexofenadine interaction in the intestine is now known to not implicate OATP1A2. Conventional approaches to study DDIs *in vivo* do not allow us to pin point the effect to a transporter in particular. Also, DDIs can become quite complex when multiple transporters are involved and when there is interplay with CYP450 enzymes. To improve this field, better and more standardized *in vitro* methods to assess and predict DDIs through transporters will need to be developed. The difficulty in studying drug transporters arises from the lack of specific and even selective probe substrates and inhibitors. Future work is needed in finding proper probe molecules. We also need to move towards imaging techniques to have a clear image of how and where the DDIs occur in humans.

Unfortunately, no animal has yet proven to be a good model for the study of the human OATP1A2. Rat Oatp1a4, which has 72% homology with OATP1A2, is expressed at the BBB but it does not transport triptans [178]. *In vitro* models of the BBB, such as freshly isolated mouse brain microvascular endothelial cells and animal or human immortalized endothelial cell lines, can give us insights into drug transport to the brain [179, 180]. The major difficulty in investigating drug transport using these models is that they do not necessarily reflect the human BBB. The specific structure of the BBB owing to the presence of astrocytes, the proteins making up the tight junctions, the numerous receptors and transporters expressed render the *in vitro* replication very challenging. The main disadvantages encountered with primary cells include the low yield, high batch-to-batch variability, and low-throughput; whereas immortalized cell lines are not restrictive enough for permeability studies and they do not maintain transporter and enzymatic functions [179].

A certain percentage of migraineur fail to obtain relief even when triptan drugs are used properly. Studies conducted on genes involved in the pharmacokinetics and pharmacodynamics response to triptans reported associations with the serotonin transporter, monoamine oxidase A, and CYP1A2 in a number of refractory patients [181]. It is possible

that other cases may be explained by the inability of the triptan drugs to reach their site of action at the CNS due to either DDIs or SNPs in the drug transporters involved in triptan disposition.

Future studies would ultimately involve testing whether triptans can cross the BBB when coadministered with either carvedilol or a tricyclic antidepressant and evaluating whether subjects with polymorphisms in the *SLCO1A2* gene have an altered distribution of triptans to the brain. These studies would typically be done by PET using a radiolabeled triptan and following its distribution from the blood to the brain. As previously discussed, the *in vitro* findings may not be reflected *in vivo*. In this specific study, we may not observe an DDI *in vivo* as sumatriptan, naratriptan, rizatriptan, and zolmitriptan are also substrates of the organic cation transporter 1 (OCT1), a transporter equally expressed at the BBB [85, 182]. It would be interesting to determine whether the tricyclic compounds inhibit OCT1.

In overall, the data presented in this thesis helped characterize the OATP1A2 drug transporter. As we recently came to appreciate the role OATP1A2 may play in transporting CNS-active drugs through the BBB, investigating this transporter further can help us exploit OATP1A2 for drug delivery to the brain. The impermeability of the BBB makes drug targeting to the brain very difficult. Many drugs developed to act at the brain fail to get on the market because they do not produce a desirable effect. As mentioned previously, designing drugs to be more lipophilic renders them good substrate for P-gp. An interesting alternative would be to exploit influx transporters, such as OATP1A2, for drug delivery.

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APPENDICES

APPENDIX 1

LIST OF PUBLICATIONS AND ABSTRACTS

PUBLICATIONS

Original Manuscripts

1. Lu J, Michaud V, Moya LG, Gaudette F, Leung YH, Turgeon J. Effects of β-blockers and tricyclic antidepressants on the activity of human organic anion transporting polypeptide 1A2 (OATP1A2). *J Pharmacol Exp Ther.* 2015 Mar;352(3):552-8.
2. Huguet J, Lu J, Gaudette F, Chiasson JL, Hamet P, Michaud V, Turgeon J. No effects of pantoprazole on the pharmacokinetics of rosuvastatin in healthy subjects. *Eur J Clin Pharmacol.* 2016 Aug;72(8):925-31.

Manuscripts Accepted for Publication

1. Lu J, Grangeon A, Gaudette F, Turgeon J, Michaud V. Effects of tricyclic compounds on the transport of anti-migraine triptans through human organic anion transporting polypeptide 1A2 (OATP1A2). *Journal of Pharmacokinetics & Experimental Therapeutics*. Accepted on November 20 2016.

In Preparation

1. Lu J, Gaudette F, Turgeon J, Michaud V. Impact of single nucleotide polymorphisms found in human organic anion transporting polypeptide 1A2 (OATP1A2) on triptans transport. (*Molecular Pharmacology*)
2. Leung YH, Lu J, Armstrong C, Bélanger F, Turgeon J, Michaud V. Effects of a series of acidic drugs on L-lactic acid transport by the monocarboxylate transporters MCT1 and MCT4. (*Xenobiotica*)

ABSTRACTS

1. Lu J, Armstrong C, Bélanger F, Gaudette F, Turgeon J. Development of an in vitro system for the functional study of the OATP1B1 transporter of statins. Canadian Society of Pharmacology and Therapeutics (CSPT). Montreal QC, May 24-26 2010.
2. Lu J, Gaudette F, Bélanger F, Turgeon J. Development of an in vitro system for the functional study of the OATP1A2 transporter in the uptake of statins. American

College of Clinical Pharmacology (ACCP). 40th Annual Meeting of the College. Chicago IL, September 11-13 2011.

3. Leung YH, Armstrong C, Bélanger F, Lu J, Turgeon J. The use of cancer cell line to dissect the functional activities of two membrane transporters: MCT1 and MCT4. American College of Clinical Pharmacology (ACCP). 40th Annual Meeting of the College. Chicago IL, September 11-13 2011.

4. Lu J, Gaudette F, Bélanger F, Turgeon J. Développement d'un système in vitro pour étude fonctionnelle des transporteurs de statines OATP. Club de recherches Cliniques du Québec (CRCQ). 53e réunion annuelle du CRCQ. Mont-Gabriel QC, September 22-24 2011.

5. Leung YH, Armstrong C, Bélanger F, Lu J, Turgeon J. Transport d'acide lactique par MCT1 et MCT4 dans des lignées cellulaires cancéreuses. Club de recherches Cliniques du Québec (CRCQ). 53e réunion annuelle du CRCQ. Mont-Gabriel QC, 22-24 Septembre 2011. (Oral presentation)

6. Lu J, Gaudette F, Leung YH, Turgeon J. Interactions médicamenteuses entre rosuvastatine et des β-bloqueurs via le transporteur membranaire OATP1A2. 14e Congrès annuel des étudiants et stagiaires du CRCHUM. Hôpital Notre-Dame, Montreal QC, December 13 2011.

7. Leung YH, Armstrong C, Bélanger F, Lu J, Turgeon J. Transport d'acide lactique par MCT1 et MCT4 dans des lignées cellulaires cancéreuses. 14e congrès annuel des étudiants du CRCHUM. Montreal QC, December 13 2011. (Oral presentation)

8. Lu J, Leung YH, Gaudette F, Turgeon J. Drug-drug interactions between rosuvastatin and β-blockers through the OATP1A2 transporter. American Society for Clinical Pharmacology and Therapeutics (ASCPT). 113th Annual Meeting. Washington DC, March 14-17 2012.

9. Leung YH, Armstrong C, Bélanger F, Lu J, Turgeon J. Drug-transporter interactions: inhibition of MCT1 and MCT4 by statins. Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics (ASCPT). National Harbour MD. March 14-17 2012

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11. Lu J, Guilarte Moya LG, Leung YH, Gaudette F, Turgeon J. Tricyclic drugs inhibit the uptake of rosuvastatin through the OATP1A2 transporter. Canadian Society of Pharmacology and Therapeutics (CSPT). Modern Therapeutics 2012. Toronto ON, June 12-15 2012.

12. Leung YH, Lu J, Bélanger F, Turgeon J, Michaud V. Drug-transporter interactions: inhibition of MCT1 and MCT4 by statins and other acidic drugs. Canadian Society of Pharmacology and Therapeutics (CSPT). Toronto ON, June 12-15 2012.

13. Lu J, Guilarte Moya LG, Leung YH, Gaudette F, Turgeon J. Interactions médicamenteuses entre la rosuvastatine et des β-bloqueurs par le transporteur OATP1A2. Club de Recherches Cliniques du Québec (CRCQ). Orford QC, October 11-13 2012. (Oral presentation)

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16. Lu J, Guilarte Moya LG, Leung YH, Gaudette F, Turgeon J. Inhibition du transporteur OATP1A2 par des composés tricycliques. 15^{e} Congrès Annuel des Étudiants et Stagiaires du CRCHUM. Montreal QC, December 18 2012. (Oral presentation)

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21. Lu J, Grangeon A, Gaudette F, Michaud V, Turgeon J. Transport des triptans par le transporteur membranaire OATP1A2. 16^{e} Congrès annuel des étudiants et stagiaires du CRCHUM. Montreal QC, December 5 2013.

22. Lu J, Guilarte Moya LG, Leung YH, Gaudette F, Keiser M, Michaud V, Turgeon J. Inhibition of the OATP1A2 transporter by tricyclic compounds. American Society for Clinical Pharmacology and Therapeutics (ASCPT) 115th Annual Meeting. Atlanta GA, March 18-22 2014.

23. Lu J, Grangeon A, Gaudette F, Keiser M, Michaud V, Turgeon J. Effects des composés tricycliques sur le transport de l'almotriptan par OATP1A2. Colloque Annuel CRCHUM 2014. Montreal QC, October 15 2014.

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25. Lu J, Grangeon A, Gaudette F, Keiser M, Michaud V, Turgeon J. Effects des composés tricycliques sur le transport du zolmitriptan par OATP1A2. 17e Congrès annuel des étudiants et stagiaires du CRCHUM. Montreal QC, December 5 2014.

26. Lu J, Grangeon A, Gaudette F, Keiser M, Michaud V, Turgeon J. Effects of tricyclic compounds on naratriptan transport through OATP1A2. American Society for Clinical Pharmacology and Therapeutics (ASCPT) 116th Annual Meeting. New Orleans LA, March 5-7 2015.

27. Lu J, Gaudette F, Keiser M, Michaud V, Turgeon J. Effects of tricyclic compounds on zolmitriptan transport through OATP1A2. Colloque Annuel CRCHUM 2015. Montreal QC, October 15 2015.

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APPENDIX 2

ADDITIONAL RESULTS

Objective

The objective is to present additional data mentioned in the overall discussion characterizing OATP1A2 that do not integrate into the theme of the three articles presented previously.

Introduction

Other drugs (β-blockers and rifampicin) were tested in competition with almotriptan, naratriptan, and zolmitriptan for transport through OATP1A2.

Material and Methods

The material and experimental methods are the same as outlined in the articles.

Results

Table 8: IC_{50} values from the inhibition of almotriptan, naratriptan, and zolmitriptan uptake through OATP1A2 by various compounds

The values in parentheses represent the 95% confidence interval (see also Figure 2, Figure 3, and Figure 4.

	Almotriptan	**Naratriptan**	**Zolmitriptan**
Inhibitors	**IC_{50} (µM)**		
Acebutolol	87.1 (32.6 – 232.8)	> 150	70.3 (28.8 – 171.5)
Alprenolol	26.4 (11.1 – 62.8)	79.3 (22.4 – 280.5)	145.2 (45.8 – 460.2)
Atenolol	No effect	No effect	No effect
Celiprolol	27.8 (7.9 – 97.7)	> 150	76.4 (38.7 – 150.7)
Metoprolol	76.1 (10.9 – 532.3)	137.2 (21.9 – 859.2)	> 150
Nadolol	46.5 (4.6 – 467.6)	No effect	> 150
Propranolol	16.2 (8.5 – 31.2)	91.0 (37.5 – 221.0)	44.7 (23.8 – 84.0)

Rifampicin	30.2 (11.8 – 77.4)	78.6 (39.6 – 156.3)	35.7 (16.7 – 76.3)
Sotalol	No effect	No effect	No effect
Talinolol	22.0 (8.2 – 58.6)	75.7 (32.6 – 175.6)	38.9 (22.0 – 68.8)
Timolol	53.9 (14.2 – 204.4)	118.5 (23.4 – 599.3)	> 150

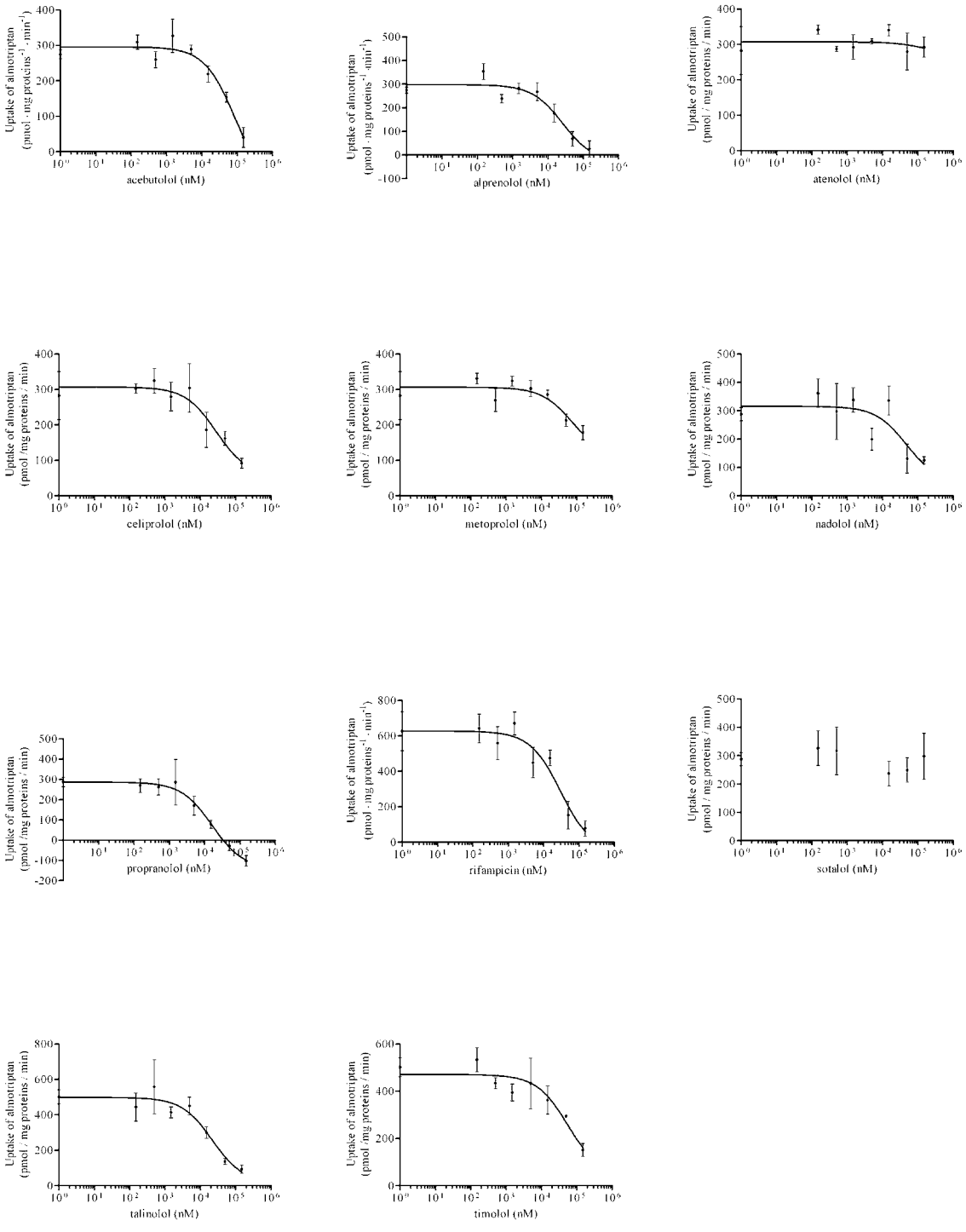

Figure 2: Inhibition of OATP1A2-mediated transport of almotriptan by different compounds.

HEK293-OATP1A2 and HEK293-VC cells were coincubated with almotriptan (15 μM) and different tricyclic compounds (150 nM – 150 μM) for 2 min at 37°C. The quantity of intracellular almotriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation in GraphPad Prism. Each point represents the mean ± S.D. of triplicate from a single experiment.

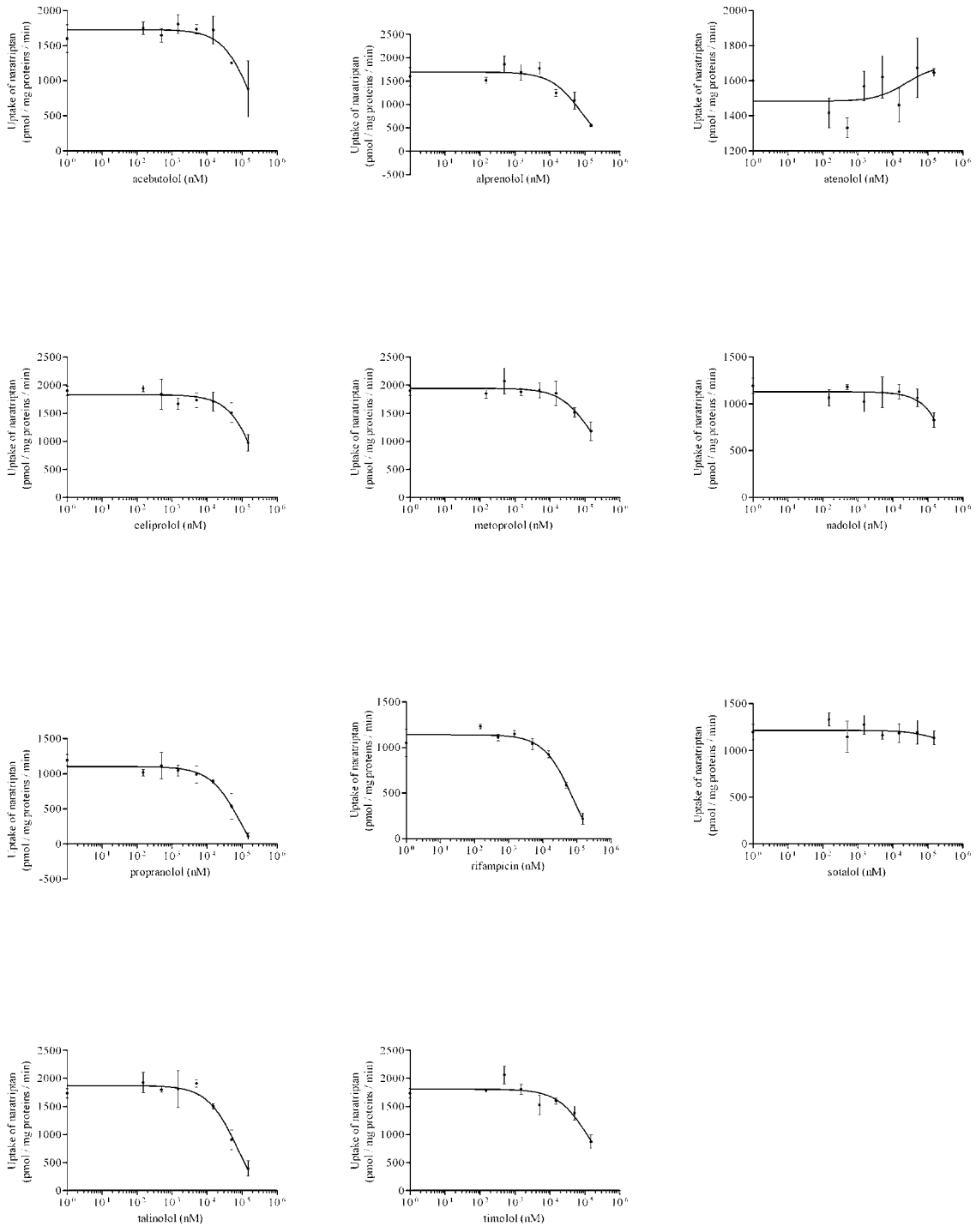

Figure 3: Inhibition of OATP1A2-mediated transport of naratriptan by different compounds.

HEK293-OATP1A2 and HEK293-VC cells were coincubated with naratriptan (60 μM) and different tricyclic compounds (150 nM – 150 μM) for 2 min at 37°C. The quantity of intracellular naratriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation in GraphPad Prism. Each point represents the mean ± S.D. of triplicate from a single experiment.

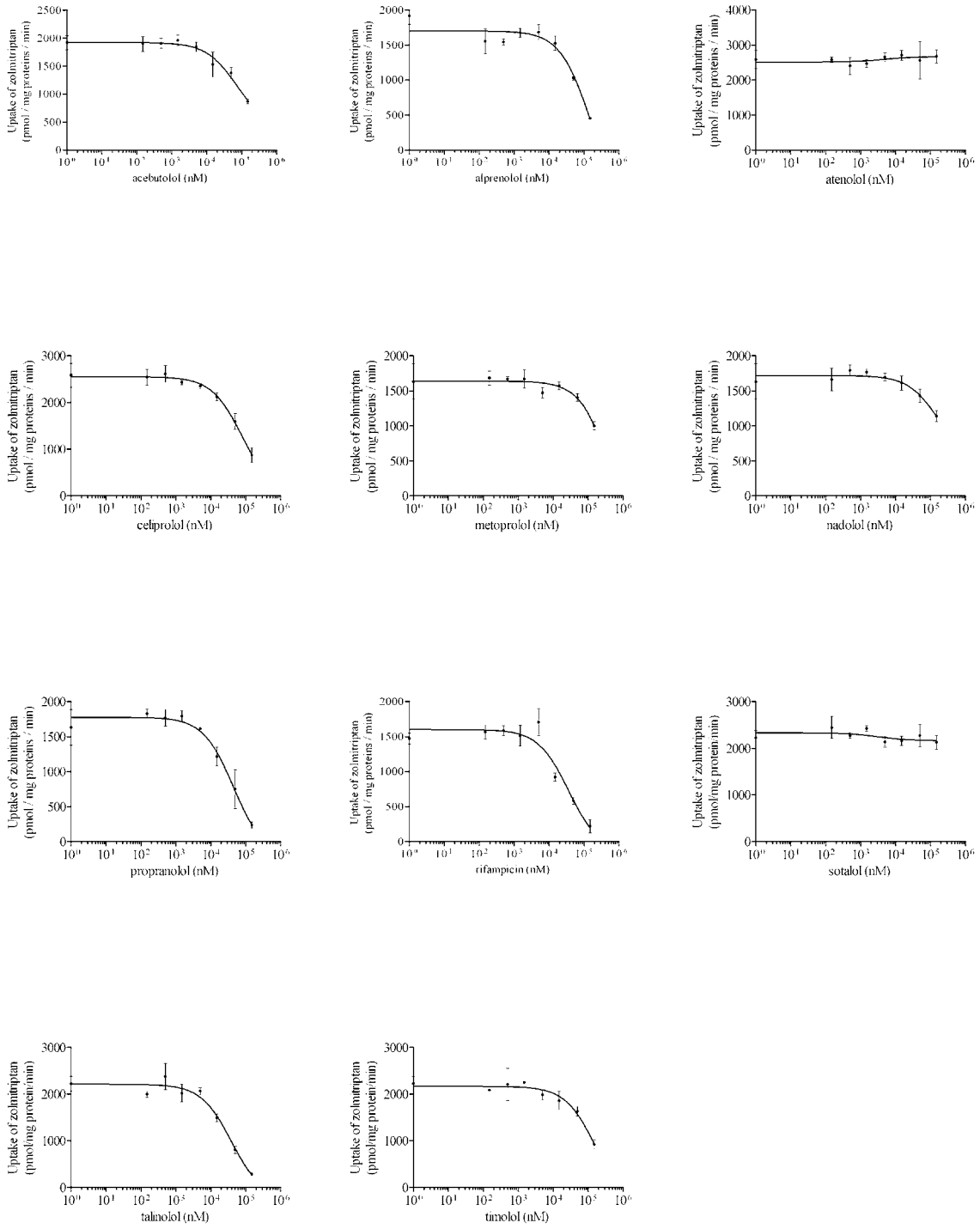

Figure 4: Inhibition of OATP1A2-mediated transport of zolmitriptan by different compounds.

HEK293-OATP1A2 and HEK293-VC cells were coincubated with zolmitriptan (65 μM) and different tricyclic compounds (150 nM – 150 μM) for 2 min at 37°C. The quantity of intracellular zolmitriptan was normalized to protein content. To obtain the net transport, values measured in the VC cells were subtracted from the values measured in OATP1A2-expressed cells. IC_{50} values were calculated by fitting the data to the log(inhibitor) vs. response equation in GraphPad Prism. Each point represents the mean ± S.D. of triplicate from a single experiment.