Membrane transporters in drug development

*The International Transporter Consortium**

Abstract | Membrane transporters can be major determinants of the pharmacokinetic, safety and efficacy profiles of drugs. This presents several key questions for drug development, including which transporters are clinically important in drug absorption and disposition, and which *in vitro* methods are suitable for studying drug interactions with these transporters. In addition, what criteria should trigger follow-up clinical studies, and which clinical studies should be conducted if needed. In this article, we provide the recommendations of the International Transporter Consortium on these issues, and present decision trees that are intended to help guide clinical studies on the currently recognized most important drug transporter interactions. The recommendations are generally intended to support clinical development and filing of a new drug application. Overall, it is advised that the timing of transporter investigations should be driven by efficacy, safety and clinical trial enrolment questions (for example, exclusion and inclusion criteria), as well as a need for further understanding of the absorption, distribution, metabolism and excretion properties of the drug molecule, and information required for drug labelling.

**A full list of authors appears in Box 1.*
Correspondence to Kathleen M. Giacomini, Shiew-Mei Huang and Donald J. Tweedie.
K.M.G. is at the Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, 513 Parnassus Avenue, California 94143-0912, USA.
S.-M.H. is at the Office of Clinical Pharmacology, Office of Translational Sciences, Center for Drug Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, Maryland 20993-0002, USA.
D.J.T. is at Boehringer Ingelheim Pharmaceuticals, 900 Ridgebury Road, PO Box 368, Ridgefield, Connecticut 06877, USA.
e-mails: kathy.giacomini@ucsf.edu; ShiewMei.Huang@fda.hhs.gov; donald.tweedie@boehringer-ingelheim.com
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Recent progress has been made in understanding the role of membrane transporters in drug safety and efficacy[1]. In particular, more than 400 membrane transporters in two major superfamilies — ATP-binding cassette (ABC; for review see REFS 1–5) and solute carrier (SLC; for review see REFS 1,3,6,7) — have been annotated in the human genome. Many of these transporters have been cloned, characterized and localized to tissues and cellular membrane domains in the human body. In drug development, particular attention has been paid to transporters expressed in epithelia of the intestine, liver and kidney, and in the endothelium of the blood–brain barrier. As a result there is now an enormous body of literature that focuses on the interaction of drugs and their metabolites with mammalian transporters present in epithelial and endothelial barriers.

Numerous studies have suggested that transporters play a part *in vivo* in drug disposition, therapeutic efficacy and adverse drug reactions. The *in vivo* role of transporters is demonstrated in several animal species, including knockout mice[8,9], and by loss-of-function genetic variants in humans[4,10,11]. These studies have provided considerable information on the *in vivo* role of many ABC and SLC transporters. Clinical pharmacokinetic drug–drug interaction (DDI) studies have suggested that transporters often work together with drug-metabolizing enzymes (DMEs) in drug absorption and elimination.

A major goal of preclinical drug evaluation is to propose clinical studies that are needed to appropriately label a drug for safe and effective use. For example, *in vitro* studies of drug interactions with metabolizing enzymes may lead to the design and conduct of DDI studies, or investigations in individuals with genetic polymorphisms of DMEs[12,13]. In fact, for drug interactions with metabolizing enzymes, the US Food and Drug Administration has developed guidances to assist drug development scientists in conducting informative *in vitro* and follow-up clinical studies[14,15]. By contrast, for drug interactions with transporters, recommendations about the appropriate conduct of *in vitro* and *in vivo* studies are not generally available, with the exception of drug interactions with multidrug resistance P-glycoprotein (P-gp; also known as MDR1, ABCB1)[16]. Many questions from pharmaceutical scientists involved in drug development are now being raised. In particular, which transporters are clinically important in drug absorption and disposition (distribution and elimination), and therefore could mediate DDIs? Which methods are suitable for studying *in vitro* drug interactions with

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Box 1 | **The International Transporter Consortium**

- Kathleen M. Giacomini*: Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, 513 Parnassus Avenue, California 94143-0912, USA
- Shiew-Mei Huang*: Office of Clinical Pharmacology, Office of Translational Sciences, Center for Drug Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, Maryland 20993-0002, USA
- Donald J. Tweedie*: Boehringer Ingelheim Pharmaceuticals, 900 Ridgebury Road, PO Box 368, Ridgefield, Connecticut 06877, USA
- Leslie Z. Benet: Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, 513 Parnassus Avenue, California 94143-0912, USA
- Kim L. R. Brouwer: Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, CB #7355, Chapel Hill, North Carolina 27599-7355, USA
- Xiaoyan Chu: Drug Metabolism and Pharmacokinetics, Merck & Co., 126 East Lincoln Avenue, Rahway, New Jersey 07065-464, USA
- Amber Dahlin: Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, 513 Parnassus Avenue, California 94143-0912, USA
- Raymond Evers: Drug Metabolism and Pharmacokinetics, Merck & Co., 126 East Lincoln Avenue, Rahway, New Jersey 07065-464, USA
- Volker Fischer: Drug Metabolism, Pharmacokinetics and Bioanalysis, Abbott, 100 Abbott Park, Illinois 60068, USA
- Kathleen M. Hillgren: Drug Disposition, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Illinois 46285, USA
- Keith A. Hoffmaster: Novartis Institutes for BioMedical Research, Metabolism and Pharmacokinetics, 250 Massachusetts Avenue, Cambridge, Massachusetts, USA
- Toshihisa Ishikawa: Omics Science Center, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan
- Dietrich Keppler: German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
- Richard B. Kim: Department of Medicine, University of Western Ontario, London, Ontario N6A 5C1, Canada
- Caroline A. Lee: Pharmacokinetics, Metabolism and Dynamics, Pfizer Global Research and Development, La Jolla Laboratories, 10724 Science Center Drive, San Diego, California 92121, USA
- Mikko Niemi: Department of Clinical Pharmacology, University of Helsinki and Helsinki University Central Hospital, PO Box 340, FIN-00029 HUS, Helsinki, Finland
- Joseph W. Polli: Drug Metabolism and Pharmacokinetics, PO Box 13398, GlaxoSmithKline, Research Triangle Park, North Carolina 27709, USA
- Yuichi Sugiyama: Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan
- Peter W. Swaan: Department of Pharmaceutical Science, University of Maryland, 20 Penn Street, Rm 621, Baltimore, Maryland 21201-1075, USA
- Joseph A. Ware: Clinical Pharmacology, Genentech, 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA
- Stephen H. Wright: Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona 85724, USA
- Sook Wah Yee: Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, 513 Parnassus Avenue, California 94143-0912, USA
- Maciej J. Zamek-Gliszczynski: Drug Disposition, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Illinois 46285, USA
- Lei Zhang: Office of Clinical Pharmacology, Office of Translational Sciences, Center for Drug Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, Maryland 20993-0002, USA

The International Transporter Consortium considers this report as a work in progress, and is highly interested in obtaining feedback. Please send any comments, including areas that have not been included in this report but should be considered in the next version as well as controversial concepts, to the corresponding authors (highlighted by asterisk).

important transporters? What criteria should be used to trigger follow-up clinical studies? What follow-up clinical studies should be conducted?

Against this backdrop, we formed the International Transporter Consortium (ITC) (BOX 1) comprising industrial, regulatory and academic scientists with expertise in drug metabolism, transport and pharmacokinetics. The ITC met by conference calls between the spring of 2007 and the summer of 2009, and held a workshop in Bethesda, Maryland, USA, in October 2008, which was co-sponsored by the Critical Path Initiative of the US Food and Drug Administration and by the Drug Information Association. The focus of the workshop was to identify which transporters, based on current knowledge, are well-established determinants of pharmacokinetics; discuss methodologies to characterize drug–transporter interactions using *in vitro* and *in vivo* studies; and propose recommendations that are important for drug development scientists in guiding preclinical and clinical studies of transporter-mediated drug interactions. For this latter point, the key consideration was that *in vitro* studies of drug–transporter interactions, if positive, would lead to, or inform, clinical studies that are relevant to drug safety or efficacy.

Here, we present the recommendations of the ITC regarding the conduct of transporter assays and data interpretation. This manuscript is divided into three major sections. In Section 1, the key transporters that have a role in drug disposition and response are described. We focused on transporters that have a compelling body of evidence from published studies demonstrating a role of the transporter in pharmacokinetics and DDIs. Many transporters could have been considered in this group; however, after ample discussions, and realizing that the field is dynamic and will change in the future, the ITC decided to focus on seven selected transporters for which all members agreed there is compelling evidence that they are involved in drug absorption, disposition and/or DDIs. In Section 2, current methods for studying drug–transporter interactions are presented together with comments about the limitations of each approach. In Section 3, recommendations and examples of guidelines that should be considered in drug development are presented. The ITC did not reach consensus on every issue, and in this article we have highlighted areas of disagreement or in need of further study. Transporter pharmacology is a rapidly emerging field in drug discovery and development with challenges of overlapping substrate or inhibitor specificities across the transporters, and recommendations based on strong scientific evidence will evolve as new information becomes available.

Section 1: Overview of transporters

A limited number of transporters, including several uptake transporters from the SLC superfamily[6] and some ATP-dependent efflux pumps from the ABC superfamily[2], have been given priority in the context of this article. Their selection was based on practical considerations and on clinical evidence that these transport proteins influence, to different degrees, drug disposition and/or side effects. FIGURE 1 and TABLES 1,2

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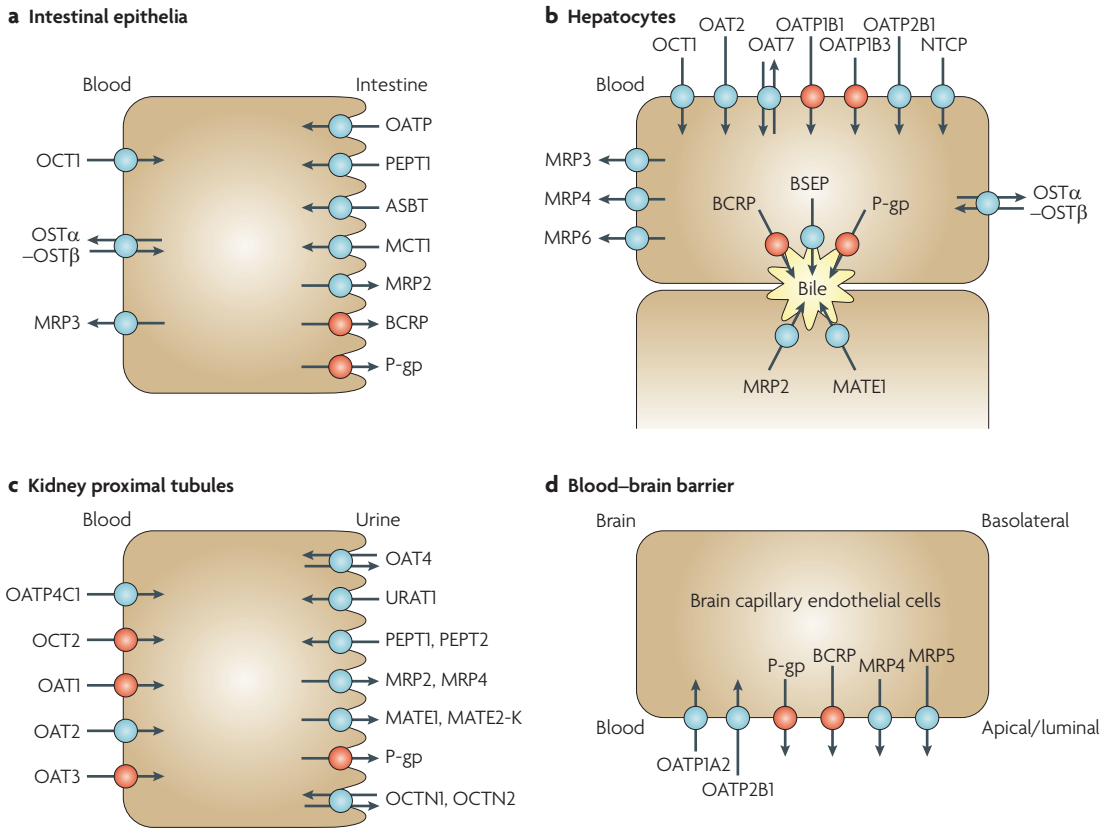

Figure 1 | **Selected human transport proteins for drugs and endogenous substances.** Transporters in plasma membrane domains of intestinal epithelia, hepatocytes, kidney proximal tubules and brain capillary endothelial cells are presented. Those coloured in red indicate that the selected transporters are described in detail in this manuscript. Those coloured in blue indicate that the transport proteins are of importance but are not described in this manuscript. **a** | Intestinal epithelia contain in their apical (luminal) membrane several uptake transporters including one or more members of the organic anion transporting polypeptide (OATP) family; peptide transporter 1 (PEPT1; SLC15A1); ileal apical sodium/bile acid co-transporter (ASBT; SLC10A2); and monocarboxylic acid transporter 1 (MCT1; SLC16A1). The apical ATP-dependent efflux pumps include multidrug resistance protein 2 (MRP2; ABCC2); breast cancer resistance protein (BCRP; ABCG2); and P-glycoprotein (P-gp; MDR1, ABCB1). The basolateral membrane of intestinal epithelia contains organic cation transporter 1 (OCT1; SLC22A1); heteromeric organic solute transporter (OSTα–OSTβ); and MRP3 (ABCC3). **b** | Human hepatocyte uptake transporters in the basolateral (sinusoidal) membrane include the sodium/taurocholate co-transporting peptide (NTCP; SLC10A1); three members of the OATP family (OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3) and OATP2B1 (SLCO2B1)); organic anion transporter 2 (OAT2; SLC22A7) and OAT7 (SLC22A9); and OCT1. Efflux pumps in the hepatocyte basolateral membrane include MRP3, MRP4 (ABCC4) and MRP6 (ABCC6). Apical (canalicular) efflux pumps of the hepatocyte comprise P-gp; bile-salt export pump (BSEP or SPGP; ABCB11); BCRP (ABCG2); and MRP2. In addition, multidrug and toxin extrusion protein 1 (MATE1; SLC47A1) is located in the apical hepatocyte membrane. **c** | Kidney proximal tubules contain in the apical (luminal) membrane OAT4 (SLC22A11); urate transporter 1 (URAT1; SCL22A12); PEPT1 and PEPT2 (SLC15A2); MRP2 and MRP4; MATE1 and MATE2-K (SLC47A2); P-gp; organic cation/ergothioneine transporter (OCTN1; SLC22A4); and organic cation/carnitine transporter (OCTN2; SLC22A5). Basolateral uptake transporters in proximal tubule epithelia include OATP4C1 (SLCO4C1); OCT2; and OAT1, OAT2 and OAT3 (SLC22A8). **d** | Apical (luminal) transport proteins of brain capillary endothelial cells contributing to the function of the blood–brain barrier include the uptake transporters OATP1A2 and OATP2B1; and the efflux pumps P-gp, BCRP, MRP4 and MRP5 (ABCC5). Note that localization of transporters to particular membranes and tissues is sometimes controversial; therefore, the International Transporter Consortium erred on the conservative side in only showing the localization of transporters for which good evidence exists.

Membrane transporters
Membrane-associated proteins that govern the transport of solutes (for example, drugs and other xenobiotics) into and out of cells. Transporters can play a vital role in determining drug concentrations in the systemic circulation and in cells. The two major superfamilies of membrane transporters are the ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies.

Blood–brain barrier
The blood–brain barrier consists of endothelial cells connected by tight junctions. The endothelial cells, which are surrounded by astrocytes, separate the circulating blood and the brain interstitial space. The role of the blood–brain barrier is to protect the central nervous system by restricting and preventing the entry of toxic substances including drug molecules and bacteria into the brain. Included in this protective barrier are transporters such as P-glycoprotein.

Drug–drug interaction
(DDI). Concomitant administration of multiple drugs can result in altered levels of the drugs compared with administration of the drugs alone. DDI can result in higher (inhibition) or lower (induction) levels of the drug. For example, if drug A ('perpetrator' drug) inhibits a membrane transporter that drug B ('victim' drug) uses to enter into the cell, administration of drug A can lower the level of drug B in the cell and also potentially increase the level of drug B in the systemic circulation.

Drug-metabolizing enzymes
(DMEs). These are enzymes responsible for chemical modification of drugs usually to increase rate of elimination. The activity of these enzymes can be altered through inhibition or induction, thus affecting the rate of metabolism.

describe the selected transporters as well as other important transporters. Note that clinical data documenting the importance of these transport proteins with respect to drug disposition and/or toxicity continues to emerge. TABLES 1,2 include clinically relevant information with respect to DDIs and genetic polymorphisms. Examples of clinically relevant DDIs that can be explained, in part or in full, by modulation of transporter activity are compiled in TABLE 3. Below, we present an overview of P-gp, breast cancer resistance protein (BCRP; also known as ABCG2); organic cation transporters (OCTs) and organic anion transporters (OATs); and organic anion transporting polypeptides (OATPs). For ease of reading we refer to MDR1/P-gp

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Table 1 | **SLC transporters of emerging clinical importance in the absorption and disposition of drugs**

Transporter/alias (Gene)	Selected substrates	Selected inhibitors	Organs/cells	Comments
OATP1B1/OATP-C, OATP2, LST-1 (*SLCO1B1*)	Bromosulphophthalein, oestrone-3-sulphate, oestradiol-17β-glucuronide, statins*, repaglinide*, valsartan, olmesartan*, bilirubin glucuronide, bilirubin, bile acids	Saquinavir, ritonavir*, lopinavir*, rifampicin*, cyclosporine*	Hepatocytes (sinusoidal)	• Has a role in disposition and excretion • Has clinically relevant polymorphisms • Has a role in clinical drug–drug interactions
OATP1B3/OATP-8 (*SLCO1B3*)	Bromosulphophthalein, cholecystokinin 8, statins*, digoxin, fexofenadine, telmisartan glucuronide, telmisartan*, valsartan, olmesartan, oestradiol-17-β-glucuronide, bile acids	Rifampicin*, cyclosporine*, ritonavir, lopinavir*	Hepatocytes (sinusoidal)	• Has a role in disposition and excretion
OAT1 (*SLC22A6*)	Para-aminohippurate, adefovir, cidofovir, zidovudine*, lamivudine*, zalcitabine*, acyclovir*, tenofovir*, ciprofloxacin*, methotrexate*	Probenecid*, novobiocin	Kidney proximal tubule, placenta	• Has a role in disposition and excretion • Has a role in clinical drug–drug interactions
OAT3 (*SLC22A8*)	Oestrone-3-sulphate, non-steroidal anti-inflammatory drugs, cefaclor, ceftizoxime, furosemide*, bumetanide*	Probenecid*, novobiocin	Kidney proximal tubule, choroid plexus, blood–brain barrier	• Has a role in disposition and excretion • Has a role in clinical drug–drug interactions
OCT2 (*SLC22A2*)	*N*-Methylpyridinium, tetraethylammonium, metformin*, pindolol, procainamide, ranitidine amantadine, amiloride, oxaliplatin, varenicline*	Cimetidine*, pilsicainide, cetirizine*, testosterone, quinidine	Kidney proximal tubule, neurons	• Has a role in disposition and excretion • Has clinically relevant genetic polymorphisms • Has a role in clinical drug–drug interactions
OATP1A2/OATP-A (*SLCO1A2*)	Oestrone-3-sulphate, dehydroepiandrosterone sulphate, fexofenadine*, bile salts, methotrexate, bromosulphophthalein, ouabain, digoxin, levofloxacin, statins*	Naringin, ritonavir, lopinavir, saquinavir, rifampicin*	Brain capillaries endothelia, cholangiocytes, distal nephron	• Has role in disposition and excretion
OATP2B1/OATP-B (*SLCO2B1*)	Oestrone-3-sulphate, bromosulphophthalein, taurocholate, *statins, fexofenadine, glyburide, taurocholate	Rifampicin, cyclosporine*	Hepatocytes (sinusoidal), endothelia	• Has a role in disposition and excretion • Has a role in clinical drug–drug interactions
OCT1 (*SLC22A1*)	Tetraethylammonium, *N*-methylpyridinium, metformin*, oxaliplatin	Quinine, quinidine, disopyramide	Hepatocytes (sinusoidal), intestinal enterocytes	• Has a role in disposition and excretion • Has clinically relevant genetic polymorphisms • Has a role in clinical drug–drug interactions
PEPT1 (*SLC15A1*)	Glycylsarcosine, cephalexin, cefadroxil, bestatin, valacyclovir, enalapril, aminolevulinic acid, captopril, dipeptides, tripeptides	Glycyl-proline	Intestinal enterocytes, kidney proximal tubule	• Has a role in absorption, disposition and excretion • Has a role in clinical drug–drug interactions
PEPT2 (*SLC15A2*)	Glycylsarcosine, cephalexin, cefadroxil, bestatin, valacyclovir, enalapril, aminolevulinic acid, captopril, dipeptides, tripeptides	Zofenopril, fosinopril	Kidney proximal tubule, choroid plexus, lung	• Has a role in excretion
MATE1 (*SLC47A1*)	Metformin, *N*-methylpyridinium, tetraethylammonium	Quinidine, cimetidine, procainamide	Kidney proximal tubule, liver (canalicular membrane), skeletal muscle	• Has a role in disposition and excretion • Has a role in clinical drug–drug interactions
MATE2-K (*SLC47A2*)	Metformin, *N*-methylpyridinium, tetraethylammonium	Cimetidine, quinidine, pramipexole	Kidney proximal tubule	• Has a role in disposition and excretion

*Can potentially be used for *in vivo* (clinical) studies.

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Table 2 | **ABC transporters of emerging clinical importance in the absorption and disposition of drugs**

Transporter/ alias (Gene)	Selected substrates	Selected inhibitors	Organs/cells	Comments
MDR1/P-gp, ABCB1 (*ABCB1*)	Digoxin*, loperamide*, berberine, irinotecan, doxorubicin, vinblastine, paclitaxel, fexofenadine	Cyclosporine*, quinidine*, tariquidar, verapamil	Intestinal enterocytes, kidney proximal tubule, hepatocytes (canalicular), brain endothelia	• Has a role in absorption, disposition and excretion • Has a role in clinical drug–drug interactions
BCRP/MXR (*ABCG2*)	Mitoxantrone, methotrexate, topotecan, imatinib, irinotecan, statins*, sulphate conjugates, porphyrins	Oestrone, 17β-oestradiol, fumitre-morgin C	Intestinal enterocytes, hepatocytes (canalicular), kidney proximal tubule, brain endothelia, placenta, stem cells, mammary glands (lactating)	• Has a role in absorption, disposition and excretion • Has clinically relevant genetic polymorphisms • Has a role in clinical drug–drug interactions
BSEP/SPGP, cBAT, ABCB11 (*ABCB11*)	Taurocholic acid, pravastatin, bile acids	Cyclosporin A, rifampicin, glibenclamide	Hepatocytes (canalicular)	• Has a role in excretion • Has clinically relevant genetic polymorphisms • Has a role in clinical drug–drug interactions
MRP2/ABCC2, cMOAT (*ABCC2*)	Glutathione and glucuronide conjugates, methotrexate, etoposide, mitoxantrone, valsartan, olmesartan, glucuronidated SN-38	Cyclosporine, delaviridine, efavirenz, emtricitabine	Hepatocytes (canalicular), kidney (proximal tubule, luminal), enterocytes (luminal)	• Has a role in absorption, disposition and excretion • Has clinically relevant genetic polymorphisms • Has a role in clinical drug–drug interactions
MRP3/ABCC3 (*ABCC3*)	Oestradiol-17β-glucuronide, methotrexate, fexofenadine, glucuronate conjugates	Delaviridine, efavirenz, emtricitabine	Hepatocytes (sinusoidal), intestinal enterocytes (basolateral)	• Has a role in disposition
MRP4/ABCC4 (*ABCC4*)	Adefovir, tenofovir, cyclic AMP, dehydroepian-drosterone sulphate, methotrexate, topotecan, furosemide, cyclic GMP, bile acids plus glutathione	Celecoxib, diclofenac	Kidney proximal tubule (luminal), choroid plexus, hepatocytes (sinusoidal), platelets	• Has a role in disposition and excretion
MDR3/ABCB4 (*ABCB4*)	Phosphatidylcholine, paclitaxel, digoxin, vinblastine.	Verapamil, cyclosporine	Hepatocytes (canalicular)	• Has a role in disposition • Has a role in clinical drug–drug interactions

ABC, ATP-binding cassette. *Can potentially be used for *in vivo* (clinical) studies.

Pharmacokinetics
Pharmacokinetics describe what the body does to a drug. Pharmacokinetic parameters result from the absorption, distribution, metabolism and excretion properties of drugs, and provide information about the rates of elimination and systemic concentrations of drugs. The functional activity and level of a drug transporter can determine the amount of drug molecule transported into or out of specific tissues, which will determine the level of drugs in that tissue and also the systemic circulation.

Substrate or inhibitor
A substrate of a drug transporter is translocated across a membrane by the transporter. An inhibitor of a drug transporter can impair the uptake and/or efflux of another drug. Many inhibitors are not substrates, that is, they are not transported by the transporter. Substrates can competitively inhibit other substrates that interact at the same site(s) on the transporter.

as P-gp throughout this report. Although evidence is available demonstrating that various members of the multidrug resistance protein (MRP) and the multidrug and toxin extrusion transporter (MATE) families are involved in drug disposition, as mentioned in various sections, because of the need to limit the scope of this manuscript, they are not discussed in detail here. Because of the extensive body of literature obtained over many years on the interaction of drugs with P-gp, as well as the vast experience in studying this transporter in the industry, P-gp will be described in more detail than other transporters. Many issues discussed for P-gp are relevant for other transporters. In this article capitalized letters are used for human genes and proteins (for example, SLCO and OATP), whereas only the initial letter is capitalized for rodent genes and proteins (for example, Abcg2 and Bcrp). The standard human gene nomenclature is listed in TABLES 1,2, but, in general, the transport proteins or gene products responsible for transport function are referred to by the names commonly used in the field.

P-glycoprotein

General description. P-gp mediates the ATP-dependent export of drugs from cells. It is expressed in the luminal membrane of the small intestine and blood–brain barrier, and in the apical membranes of excretory cells such as hepatocytes and kidney proximal tubule epithelia. P-gp has an important role in limiting entry of various drugs into the central nervous system. In addition, it also plays a part in the intestinal absorption and in the biliary and urinary excretion of drugs. The level of expression and functionality of P-gp can be modulated by inhibition and induction, which can affect the pharmacokinetics, efficacy, safety or tissue levels of P-gp substrates[5,17–21].

Substrate and inhibitor selectivity. Initially discovered as a result of its interaction with multiple anticancer drugs, P-gp is responsible for the efflux across biological membranes of a broad range of therapeutic drugs. Recently, a high-resolution structure of the mouse P-gp has been described, which revealed distinct binding sites for drugs[22]. A select number of substrates and inhibitors of

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Table 3 | **Selected transporter-mediated clinical drug–drug interactions**

Implicated transporter*	Interacting drug	Affected drug	Clinical pharmacokinetic impact on affected drug‡
Organic anion transporting polypeptides	Cyclosporine	Pravastatin	AUC ↑890% and C_{max} ↑678%[102,204]
	Cyclosporine	Rosuvastatin	AUC ↑610%[205]
	Cyclosporine	Pitavastatin	AUC ↑ 360% and C_{max} ↑560%[206]
	Rifampicin (single dose)	Glyburide	AUC ↑125%[207]
	Rifampicin (single dose)	Bosentan	C_{trough} ↑ 500%[208]
	Lopinavir/ritonavir	Bosentan	Day 4: C_{trough} ↑ 4,700%[208]; day 10: C_{trough} ↑ 400%[208]
	Lopinavir/ritonavir	Rosuvastatin	AUC ↑107% and C_{max} ↑365%[209]
Organic anion transporters	Probenecid	Cidofovir	CL_r ↓32%[210,211]
	Probenecid	Furosemide	CL_r ↓66%[210]
	Probenecid	Acyclovir	CL_r ↓32% and AUC ↑40%[210,212]
Organic cation transporters	Cimetidine	Metformin	AUC ↑50% and CL_r ↓ 27%[213,214]
	Cimetidine	Pindolol	CL_r ↓~34%[215]
	Cimetidine	Varenicline	AUC ↑29%[216]
	Cimetidine	Pilsicainide	AUC ↑33%, CL_r ↓28%[217]
	Cetirizine	Pilsicainide	CL_r ↓41%[218]
	Cimetidine	Dofetilide	CL_r ↓33%[219]
P-glycoprotein	Quinidine	Digoxin	CL_r ↓34–48%[220,221]
	Ritonavir	Digoxin	AUC ↑86%[222]
	Dronedarone	Digoxin	AUC ↑157% and C_{max} ↑75%[223]
	Ranolazine	Digoxin	AUC ↑60% and C_{max} ↑46%[224]
Breast cancer resistance protein	GF120918	Topotecan	AUC ↑143%[225]

*Implicated transporter refers to the likely transporter; however, because the studies are carried out *in vivo* it is not possible to assign specific transporters to the drug–drug interaction. ‡Percent change refers to the difference between the area under the curve (AUC), or C_{max}, in the presence and the absence of the inhibitor (interacting drug) normalized to the AUC in the absence of the inhibitor. For clearance values (CL_r), the values are normalized for the absence of the inhibitor. C_{trough} is the minimum drug concentration observed after administration of a dose of the drug and the concentration prior to the administration of a subsequent dose.

human P-gp are shown in TABLE 2. P-gp substrates are generally hydrophobic molecules, of which many are cationic. Multiple binding sites for substrates and inhibitors on P-gp have been identified using site-directed mutagenesis[23–25].

Methodology for evaluating function. Cell lines that express P-gp and inside-out membrane vesicles prepared from these cell lines can be used to determine whether a drug is a P-gp substrate or inhibitor. As P-gp is localized in the apical plasma membrane in polarized cell monolayers, a high efflux ratio of basal-to-apical to apical-to-basal (the so called B-A/A-B ratio) indicates a potentially significant role for P-gp in transporting drugs across cell monolayers. A high efflux ratio *in vitro* has been shown to correlate well with studies that have demonstrated a role for P-gp in drug penetration to the central nervous system in mice[26,27]. By contrast, a high efflux ratio does not always translate into poor oral absorption. The involvement of P-gp in absorption of a drug is more pronounced if the drug has a poor apparent permeability coefficient (Papp), or in cases in which there is interplay between metabolism and efflux.

Mice deficient in *Mdr1a* or *Mdr1a/b* are widely used as powerful tools for assessing the role of P-gp *in vivo*[9]. Knockout mice can be used as a reference for complete inhibition of P-gp, and represent the 'worst case' scenario. Sasongko *et al.*[28] demonstrated that the AUC_{brain}/AUC_{blood} ratio of ^{11}C-verapamil in humans increased by less than twofold (88 ± 20%) in the presence of a high intravenous dose of the P-gp inhibitor cyclosporine. By contrast, an almost eightfold (770%) increase was observed in the *Mdr1a/b* knockout mice compared with control mice. These data can be interpreted in multiple ways, but illustrate the challenge in predicting human transporter-mediated DDIs based on preclinical animal data.

Clinical significance. The clinical significance of a P-gp inhibitor can be investigated in humans by assessing the P-gp-mediated clearance or exposure of a probe substrate in the presence of the inhibitor. Digoxin is transported by P-gp *in vitro* and should be considered in DDI studies with new molecular entities (NMEs) that are inhibitors of P-gp (TABLE 3, BOX 2). Current clinical

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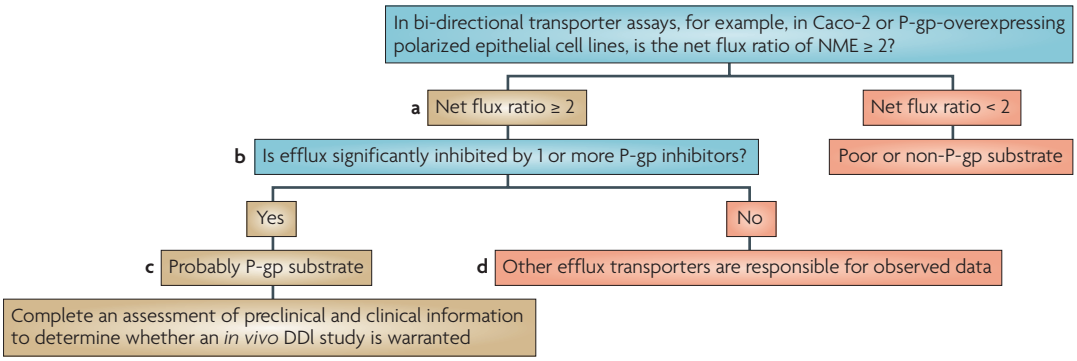
Box 2 | **Decision trees for P-glycoprotein or BCRP substrate interactions**

Although the current US Food and Drug Administration draft Drug Interaction Guidance provides a decision tree specifically for P-glycoprotein (P-gp; also known as MDR1, ABCB1)[14,15,183], we broadened the decision tree here and in BOX 3 to include both P-gp and breast cancer resistance protein (BCRP; also known as ABCG2). This is because similar *in vitro* methodologies and criteria are used to suggest clinical drug–drug interaction (DDI) studies for these two transporters. Generally, most P-gp substrates are organic cations or neutral molecules, relatively hydrophobic, and have a range of molecular masses (200 daltons to greater than 1,000 daltons). BCRP substrates tend to overlap with P-gp but also include acids or drug conjugates that are not good substrates for P-gp. A new molecular entity (NME) is considered to be a potential P-gp or BCRP substrate if the efflux ratio — basal to apical (B-A) to apical to basal (A-B) — is ≥ 2 in an epithelial cell system that expresses one or both transporters (see footnote and (**a**) in the figure). A net flux ratio cut-off higher than 2 or a relative ratio to positive controls may be used to avoid false positives if a ratio of 2 is deemed non-discriminative as supported by prior experience with the cell system used. Additional corroboration that an NME may be a P-gp or BCRP substrate can be achieved with the use of inhibitors; information especially valuable if non-transfected cells are not included as controls for endogenous transport activity. Reduction of the flux ratio by the P-gp (or BCRP) inhibitors should be greater than 50% (see (**b**) in the figure). If the flux ratio is not reduced by P-gp (or BCRP) inhibitors, then other efflux transporters may be responsible for the observed net flux (see (**d**) in the figure).

The conclusions from *in vitro* efflux studies are dependent on the cell line used. For example, for cells heterologously expressing P-gp or BCRP (for example, MDCK or LLC-PK1), in which expression of the transporters can be high, the efflux ratio would be expected to be much higher than 2 for strong substrates. A classification of relevant substrates versus non-substrates will depend on the assay system. Validation studies of *in vitro* assay systems using known weak and strong substrates and, where possible, correlations with *in vivo* model systems, should be established. Meaningful efflux ratios are dependent on the transporter expression; thus, results should be related to a reference compound. In cell lines such as Caco-2, which express multiple uptake and efflux transporters, reduction of efflux in the presence of an inhibitor may support efflux by P-gp and/or BCRP, depending on the selectivity of the inhibitor used (for example, cyclosporine inhibits multiple transporters).

If *in vitro* experiments suggest that the NME is a P-gp and/or BCRP substrate (see (**c**) in the figure), preclinical and clinical information should be assessed to determine whether a clinical *in vivo* DDI study is warranted. In particular, the relative contribution of the transporter-mediated pathway to the overall clearance of the drug is the primary determinant of whether an inhibitor will have a major effect on the disposition of the NME. For example, the pharmacokinetics of an NME that has high solubility, high permeability and/or is highly metabolized are less likely to be affected by a co-administered drug that is a P-gp inhibitor[15,173,174]. Therefore, an *in vivo* interaction study may not be needed. By contrast, an NME that has poor solubility, limited permeability and is metabolically stable (eliminated primarily as the parent compound) is more likely to demonstrate a pharmacokinetic change in the presence of an inhibitor. The possible clinical consequence of such an interaction needs to be assessed based on careful evaluation of available exposure–response data[186,187]. The recommended clinical study with a known P-gp inhibitor should consider the therapeutic use of the NME; however, cyclosporine is a reasonable choice, as it is known to inhibit P-gp *in vivo*. As noted previously, because cyclosporine inhibits multiple transporters, care must be taken in the design and interpretation of DDI studies in which it is used as an inhibitor.



This figure shows a decision tree for P-gp and a similar tree could be used for BCRP. Although flux systems have traditionally been used to determine whether an NME is a substrate of P-gp or BCRP, inside-out vesicles expressing P-gp or BCRP, or transfected cell monolayers grown on solid support with appropriate controls (for example, inhibitors and positive controls) as described in Section 2, also can be used.

Pharmacodynamics
Pharmacodynamics describe the extent and time course of the effects of drugs on physiological and pathophysiological processes. The relationship between drug concentration and drug effect is important in determining pharmacodynamics.

data indicate that there are no consistent examples in which inhibition of P-gp in the blood–brain barrier resulted in adverse effects[29–31]. It is therefore difficult to extrapolate the data obtained for P-gp inhibitors in knockout mice to humans to indicate the potential for a clinically significant DDI at the human blood–brain barrier[31].

Findings from many studies on the effect of *ABCB1* polymorphisms[20] on P-gp substrates have not been consistently reproduced; therefore, routine application of *ABCB1* polymorphism analysis to clinical studies is not warranted at this time. Studies with larger numbers of samples may be needed to clarify the role of *ABCB1* polymorphisms in pharmacokinetics and pharmacodynamics[32].

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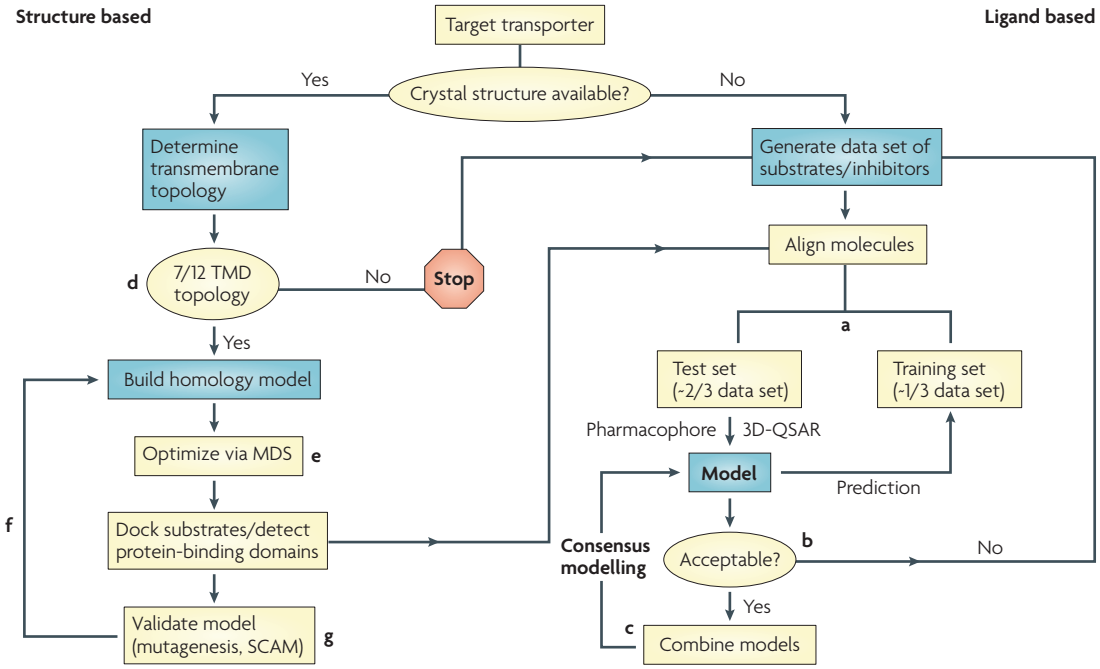

Figure 2 | **Decision tree for computer modelling of transporter proteins.** Ligand-based methods such as pharmacophore and 3D-quantitative structure–activity relationship modelling (3D-QSAR) will be required when crystal structure templates are not available or can be used to complement existing homology models. Generally, a high-throughput *in vitro* assay is used to generate a data set of transporter ligands (substrates or inhibitors) and their corresponding activity values (K_m/J_{max}, K_i, or percentage inhibition). **a** | Data sets are split into a training set used to construct a model and a test set to validate the model. **b** | Criteria for model acceptance depend on its ability to successfully predict biological activity of test set molecules. **c** | Acceptable models may be combined to generate synergistic consensus models. **d** | To generate homology models with high confidence, the membrane topology of both the target and scaffold proteins must match. Modelling of transporters with divergent topology should be attempted with great caution as it is currently unclear how the arbitrary deletion or insertion of a membrane helix may distort the overall helical packing of membrane proteins. **e** | To further increase fidelity, models should be optimized using molecular dynamics simulations (MDS), preferably embedded in a lipid bilayer surrounded by an aqueous environment. **f** | When all criteria are satisfied, homology models may be used to determine regions of substrate binding or interaction and mapping of possible permeation pathways. **g** | Biochemical validation — for example, substituted cysteine accessibility method (SCAM) — will therefore be needed. TMD, transmembrane domain.

BCRP

General description. BCRP is a 'half ABC transporter' consisting of 655 amino acids and six transmembrane domains[33]. BCRP was identified originally as a determinant of multidrug resistance in cancer cell lines *in vitro*[34,35]. BCRP is expressed in the gastrointestinal tract, liver, kidney, brain endothelium, mammary tissue, testis and placenta. It has a role in limiting oral bioavailability and transport across the blood–brain barrier, blood–testis barrier and the maternal–fetal barrier of some selected substrates[36,37]. The physiological functions of BCRP include the extrusion of porphyrins from haematopoietic cells and hepatocytes, as well as the secretion of vitamin B_2 (riboflavin) and possibly other vitamins (such as biotin and vitamin K) into breast milk[36].

Substrate and inhibitor selectivity. BCRP actively extrudes a broad range of endogenous and exogenous substrates across biological membranes[37]. TABLE 2 lists selected substrates and inhibitors of human BCRP. High-speed screening and quantitative structure–activity relationship (QSAR) analysis methods suggest that one amine bonded to one carbon of a heterocyclic ring is an important component for drug interactions with BCRP. In addition, fused heterocyclic ring(s) and two substituents on a carbocyclic ring of the fused heterocyclic ring(s) are also important chemical moieties for the interaction with BCRP[38–40]. Many protein kinase inhibitors such as imatinib (Gleevec; Novartis) carry such structural components. Substrates of BCRP include pitavastatin (Livalo; Kowa Pharmaceuticals America)[41] and phytoestrogens, such as genistein, daidzein and coumestrol[42].

Methodology for evaluating function. Polarized and non-polarized cell lines expressing BCRP are used to assay BCRP-mediated transport and inhibition[43]. In addition, membrane vesicles containing BCRP that constitutively express the transporter can also be used[43]. Prazosin and cimetidine can be used as a positive control in cell lines, whereas several relatively polar, hydrophilic substrates (for example, methotrexate, oestrone 3-sulphate and sulphasalazine) can be used as controls in vesicular

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transport assays. Since the initial publications of the *Bcrp*$^{-/-}$ knockout mouse[36,37], drug disposition studies in *Abcg2*$^{-/-}$ knockout mice have delineated an important role for intestinal Bcrp as a rate-determining barrier for the oral bioavailability of several drugs. These include topotecan (Hycamtin; GlaxoSmithKline)[44] and sulphasalazine (Salazopyrin; Pharmacia), which had 10-fold to 110-fold increase in relative AUC[45]. BCRP is also a moderate determinant of the bioavailability of nitrofurantoin[46], some fluoroquinolones[47] and imatinib[48].

Clinical significance. Recent clinical studies have demonstrated that subjects with reduced BCRP expression levels, correlating with the Q141K variant, are at increased risk for gefitinib (Iressa; AstraZeneca)-induced diarrhoea and altered pharmacokinetics of 9-aminocamptothecin, diflomotecan, irinotecan (Camptosar; Pfizer), rosuvastatin (Crestor; AstraZeneca), sulphasalazine and topotecan[49–55]. Inter-individual differences in BCRP function probably contribute to variable bioavailability, exposure (AUC and C_{max}), and pharmacological response of drugs that are BCRP substrates. The most significant clinical effects are likely to be for drugs that have a low bioavailability and have a narrow therapeutic index.

OCTs and OATs

General description. A distinct family of proteins within the SLC superfamily is encoded by 22 genes of the human SLC22A family, and includes the electrogenic OCTs (isoforms 1–3) and the OATs (significant isoforms in humans include OAT1–4 and 7, and URAT1)[6,7,56,57]. The genes encoding OCT1–3 and OATs encode proteins that are 542–556 amino acids long with 12 predicted transmembrane-spanning domains[57]. The tissue distribution and localization of OCTs and OATs are summarized in TABLE 1 and FIG. 1. There are several published reviews of the molecular characteristics, expression and function of OCTs[7,57–60] and OATs[57,61–63].

Substrate and inhibitor selectivity. TABLE 1 summarizes various compounds that interact with human OCTs and OATs. OCTs transport relatively hydrophilic, low molecular mass organic cations. Properties of inhibitors of OCT1 and OCT2 have been identified and include a net positive charge and high lipophilicity[59,64]. OAT1, OAT3 and OAT4 support exchange of intracellular 2-oxoglutarate for extracellular substrate[62]. OAT1 and OAT3 mediate the basolateral entry step in renal secretion of many organic anions, and have distinct selectivities for different structural classes of type I organic anions. That is, monovalent (or selected divalent) anions that are less than 500 daltons[58]; although OAT3 can also transport some positively charged drugs such as cimetidine.

Methodology for evaluating function. *In vitro* assays that are commonly used to characterize OCT function include *Xenopus laevis* oocytes, membrane vesicles and cell lines derived from various tissues including proximal tubule (Caki-1), placenta (BeWo) and colon carcinoma (Caco-2) cells[65–69]. These are in addition to various human embryonic kidney (HEK293) cell lines expressing recombinant OCTs[68,73,83]. Functional assessment of OATs generally uses heterologous expression in cultured cells or *X. laevis* oocytes, and such systems have been used to develop structure–activity relationships for the interaction of OATs[70,71]. Studies performed in *Oct*$^{-/-}$ knockout mice have demonstrated the pharmacokinetic, pharmacological and physiological relevance of OCTs in organic cation disposition and activity[8,72–74]. Studies using *Oat1*$^{-/-}$ and *Oat3*$^{-/-}$ knockout mice have also allowed an initial assessment of the influence of each transporter on the renal handling of selected compounds, and have led to the identification of endogenous OAT substrates[75,76].

Clinical significance. Recent studies suggest that genetic variation in OCT1 may be a significant determinant of inter-individual variability in the disposition and response to cationic drug substrates, particularly metformin[73,77]. However, such studies have been controversial[78]. Recent studies suggest that the activity of OCT1 is positively associated with the degree to which patients with chronic myeloid leukaemia respond to imatinib[79,80]. In Chinese and Korean populations, a common OCT2 variant (A270S) has been associated with a significant reduction in renal clearance of metformin[81,82], but the effect of this variant may differ depending on the ethnic group[83]. By contrast, genetic variants in the OATs have not been associated with changes in drug disposition. DDIs with the OATs and the OCTs can occur, and may result in reduced renal clearance (TABLE 3). Creatinine, which is often used to assess filtration clearance, undergoes renal tubular secretion, which could be inhibited by inhibitors of OCT2. Therefore, other methods should be used to assess filtration clearance of NMEs in DDI studies involving OCT2 inhibitors. DDIs involving the OATs and the OCTs are discussed in more detail in Section 3.

OATPs

General description. The OATPs (SLCOs) represent a superfamily of important membrane transport proteins that mediate the sodium-independent transport of a diverse range of amphiphilic organic compounds. These include bile acids, steroid conjugates, thyroid hormones, anionic peptides, numerous drugs and other xenobiotic substances[84] (TABLE 1). The general predicted OATP structure consists of proteins with 12 transmembrane domains[85]. The mechanism of transport appears to consist of anion exchange by coupling the cellular uptake of substrate with the efflux of endogenous intracellular substances such as bicarbonate in a process that seems to be electroneutral[85–87]. OATP1B1 was cloned by several groups[88–91] and its localization along with other OATPs is shown in FIG. 1. Interestingly, OATP family members are poorly conserved evolutionarily and orthologues for human OATPs may not exist in rodents.

Substrate and inhibitor selectivity. OATP1B1 transports a broad range of compounds such as bile acids; sulphate and glucuronate conjugates; thyroid hormones; peptides; and drugs such as methotrexate and HMG-CoA reductase inhibitors (TABLE 1). Similar to OATP1B1, OATP1B3 also transports bile acids; monoglucuronosyl

IC_{50}
IC_{50} value, as used in this paper, is the concentration of an inhibitor needed to inhibit one-half of the transport rate of the substrate measured in the absence of the inhibitor. The IC_{50} of a drug is determined by a concentration–transport rate curve. To determine the IC_{50} value of an inhibitor of a drug transporter, the effect of increasing concentrations of the inhibitor on the transport rate of a substrate is determined.

Fraction unbound
(fu). This fraction represents the ratio of unbound drug concentration to total drug concentration in the plasma. The fu is determined by the affinity of drugs to the binding proteins (for example, albumin and α1-acid glycoprotein) in plasma and the total number of binding sites on the proteins.

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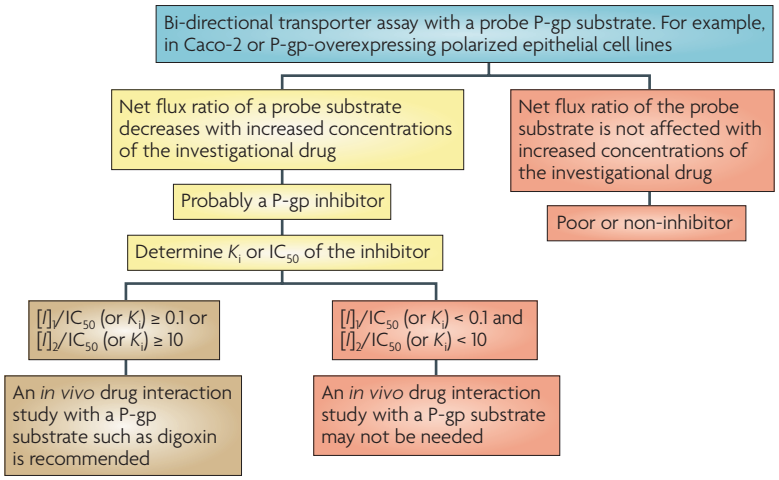
Box 3 | **Decision trees for P-glycoprotein or BCRP inhibitor interactions**

A new molecular entity (NME) is considered to be a potential P-glycoprotein (P-gp; also known as MDR1, ABCB1) and/or breast cancer resistance protein (BCRP; also known as ABCG2) inhibitor if the net flux ratio of a P-gp (or BCRP) probe substrate is decreased in the presence of the NME using a bi-directional transport assay (see footnote 1 below). Generally, the potency of inhibition is reported only as an IC_{50} value, which is dependent on the test system used. There are several practical challenges in evaluating whether NMEs that inhibit efflux transporters *in vitro* are also inhibitors *in vivo*. Orally administered drugs that are classified as *in vitro* efflux inhibitors provide the dual challenge of considering both intestinal efflux inhibition, in which intestinal drug concentrations will be the highest, and tissue efflux inhibition, in which circulating concentrations will be more relevant. Zhang *et al.*[15] proposed that drugs exhibiting an $[I]_1/IC_{50} \geq 0.1$ or $[I]_2/IC_{50} \geq 10$ should be evaluated to determine whether inhibition occurs *in vivo*. In that publication, $[I]_1$ is the mean NME steady-state total C_{max} at the highest clinical dose, and $[I]_2$ is the theoretical maximal gastrointestinal NME concentration after oral administration calculated as the highest clinical dose (mg) in a volume of 250 ml[15]. The $[I]_1/IC_{50} \geq 0.1$ calculation is familiar to those working on metabolic inhibition, as this approach has been used for many years.

In this paper, to be consistent with decision trees for other transporters (for example, organic cation transporter 2 (OCT2) and organic anion transporting polypeptide (OATP)) and the notion that unbound drug is the pharmacologically active species, mean steady-state C_{max} values for unbound drug following administration of the highest proposed clinical dose is used for $[I]_1$ in the decision tree (see footnote 2 for further discussion). Using this approach, a drug (molecular mass of 500 daltons) with an *in vitro* IC_{50} value of 10 μM will exceed an $[I]_1/IC_{50}$ value > 0.1 at an unbound C_{max} > 1 μM (assumes fraction unbound (fu) = 0.1 and C_{max} = 10 μM). This suggests that there is limited drug interaction potential with P-gp or BCRP. By contrast, for the same drug, an $[I]_2/IC_{50}$ value > 10 will be exceeded at a dose of approximately 12 mg or greater. Thus, a drug with an inhibition potency of approximately 10 μM or less *in vitro* is likely to exceed $[I]_2/IC_{50}$ value > 10 as many drugs require oral doses greater than 12 mg.

If an NME meets either $[I]_1/IC_{50} \geq 0.1$ (for example, IC_{50} is ≤ 10-fold the unbound C_{max} value) or $[I]_2/IC_{50} \geq 10$, an *in vivo* drug interaction study with digoxin is recommended. Digoxin is a unique drug (see footnote 3), and may not be the best reference for decisions regarding interaction studies with other P-gp substrate drugs. Fenner *et al.*[188] analysed drug interactions with digoxin and suggested that interactions of digoxin with P-gp inhibitors were limited (for example, most inhibitors caused < 2-fold changes in plasma concentrations of digoxin). However, owing to its narrow therapeutic index, interactions that alter the digoxin AUC > 1.25-fold are clinically important for digoxin. Therefore, the interpretation of the significance of P-gp for drug–drug interactions should not be over-extrapolated based on the importance for digoxin safety, as most other drugs have a much larger therapeutic index. In addition, if the NME inhibits a cytochrome P450 enzyme as well as P-gp, selection of a drug that is a dual substrate of cytochrome P450and P-gp may be deemed appropriate. If the NME is a pure P-gp inhibitor, other than for clarifying a digoxin dose adjustment requirement, no drug–drug interaction studies may be indicated depending on the therapeutic area of the NME and its drug labelling considerations.

For an NME that is a BCRP inhibitor, possible candidate probe substrates include sulphasalazine[189], rosuvastatin[190], pitavastatin[41], ciprofloxacin[47] and dipyridamole[191]. However, many of these remain to be tested as selective BCRP probe substrates in clinical studies.



Note 1: A unidirectional assay based on the probe substrate can also be considered[188]. Inside-out vesicles expressing P-gp or BCRP, or transfected cell monolayers grown on solid support with appropriate controls (for example, inhibitors and positive controls, as described in Section 2), can also be used for inhibition assays. Note 2: If fu cannot be accurately determined owing to high protein binding, then assume fu = 0.01, to err on the conservative side. Alternatively, unbound portal vein concentrations may be used and might be more applicable to liver uptake[192,193]. Note 3: Digoxin has an absolute bioavailability of approximately 70% from immediate release tablets. By contrast, the absolute bioavailability from a liquid-filled formulation (for example, Lanoxicaps) is approximately 90%. Therefore, consideration should also be given to the dosage form of digoxin used in an interaction study. In addition, digoxin may be a substrate for other transporters[15,92,188,194]. Hence, caution is advised when extrapolating results from digoxin to other P-gp substrates.

bilirubin; bromosulphophthalein; steroid conjugates; the thyroid hormones T3 and T4; leukotriene C_4; and drugs such as methotrexate and rifampicin[86–91]. However, OATP1B3 also exhibits unique transport properties in that it is able to mediate the cellular uptake of the opioid peptide deltorphin II[92]; the hepatotoxic cyclic peptide amanitin; and the cardiac glycosides digoxin and ouabain[92]. It is also involved in the uptake of the angiotensin II receptor antagonist telmisartan and its glucuronide conjugate[91], and the selective uptake of the intestinal

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peptide cholecystokinin 8 (REFS 93,94). TABLE 1 lists additional details on the substrate specificity of OATP1B3, and the specificities of OATP1A2 and OATP2B1 are also noted.

Methodology for evaluating function. *In vitro* assessment of OATP transporter function has relied on a number of transient and stable heterologous expression systems[84]. These include *X. laevis* oocytes, recombinant virus, or stable cell lines expressing an individual or multiple transporters[95]. In addition, stable expression of OATP transporters in polarized cells such as MDCK-II has been reported, typically in combination with efflux transporters such as MRP2 (REFS 96,97). Isolated hepatocytes with OATP inhibitors can also be used to study OATP transport[98,99]. More recently, *in vivo* drug disposition profiles from *Oatp1b2*$^{-/-}$ knockout mouse models have been reported[100,101] and may reflect, in part, the activity of both human OATP1B1 and OATP1B3. Additional studies are needed to more fully validate the utility of rodent models to predict human OATP-mediated drug disposition.

Clinical significance. The clinical relevance and DDIs related to OATPs (TABLE 3) have been noted only for certain OATPs; for example, OATP1B1 and OATP1B3 expressed primarily in the liver. DDIs involving the OATPs have focused primarily on OATP1B1. Inhibition of OATP1B1-mediated hepatic uptake appears to contribute to the significant increase in statin (for example, rosuvastatin) concentrations in blood after cyclosporine administration[102,103] (TABLE 3). Because cyclosporine is an inhibitor of multiple transporters, the specific transporter involved in the clinical DDI cannot be ascertained; although OATP1B1 is a likely candidate. In addition, a series of functional polymorphisms of OATP1B1 have been characterized[88]. The frequencies of the *SLCO1B1* 388G allele in Caucasians, African Americans, and Asians is approximately 40%, 75% and 60%, respectively[88,104–106]. Another common single nucleotide polymorphism is 521T>C in codon 174, which has frequencies of approximately 15%, 2% and 15% in Caucasians, African Americans, and Asians, respectively[88,104–106]. The c.388G allele often occurs in a haplotype with the c.521C allele. These haplotypes — c.388G-c521T (**1B*), c.388A-c.521C (**5*) and c.388G-c.521C (**15*) — have different activities and also occur at different frequencies in various ethnic groups. In addition, the promoter variants (-11187G>A and -10499A>C) may be in linkage disequilibrium with common coding region single nucleotide polymorphisms[104].

Pharmacokinetic studies indicate that individuals with the *SLCO1B1*5* or **15* haplotypes have increased exposure to statin drugs such as pravastatin[106], pitavastatin[107], simvastatin acid[108], atorvastatin[109] and rosuvastatin[110]. They also have increased exposure to other drugs such as repaglinide[111], atrasentan[112], irinotecan[113] and ezetimibe (Zetia; Merck/Schering–Plough)[114]. The **5* haplotype is rare (with a frequency of 2% in Caucasians and is absent in individuals of other ancestries), whereas the **15* haplotype is more common (16% in Caucasians, 2% in sub-Saharan Africans and 9–12% in Asians)[104]. However, because of the large number of patients on statins, even less common variants may have an effect in many individuals. Compelling clinical evidence supporting an important role for *SLCO1B1* polymorphisms has come from a genome-wide association study of simvastatin-induced myopathy[115].

Section 2: Methods for studying transporters

The sections below provide, in order of increasing complexity, assay systems that are currently available for measuring transporter activity. Although application of these systems is less labour intensive when radiolabelled or fluorescent substrates are available, the use of sensitive liquid chromatography–mass spectrometry methodology has provided alternative high-throughput detection methods[116].

Membrane-based assay systems

ATPase assay. Substrate-dependent ATP hydrolysis has been used to evaluate the interactions of substrates and inhibitors with some ABC transporters by colorimetric analysis of inorganic phosphate release during the transport process. The simplicity of this assay makes it a practical technique, which can be used in high-throughput assays to screen for compounds that interact with some ABC transporters[117]. Drawbacks of this technique include inconsistency between ATPase activity and the transport rate of some substrates and inhibitors; a high incidence of false positives and negatives; and the requirement of high substrate concentrations.

Membrane vesicle transport assay. Inverted plasma membrane vesicles have been used primarily to study efflux transporter activity, in particular for ABC transporters[118]. A wide variety of cell lines have been used to prepare membrane vesicles, such as drug-selected cells, transfected cells and baculovirus-infected insect cells. Assay mixtures should contain an ATP-regenerating system to maintain constant ATP concentrations during the assay period; a blank in which ATP is replaced by 5′-AMP is recommended. A major advantage of this methodology is that drugs are directly applied to the cytoplasmic compartment and influx, rather than efflux, is measured. This enables detailed kinetic and QSAR analyses for substrate or inhibitor interaction with the target transporter[40]. Determining the uptake or efflux of the hydrophobic compounds may be problematic because of the high degree of background binding to cell membranes in vesicle or cellular systems. Vesicle transport assays with hydrophobic compounds may require different size-exclusion techniques[116–118].

Cell-based assay systems

Cell-based assay systems can be used in drug discovery to identify substrates and inhibitors for individual transporters and for developing QSAR models. In addition, these systems can be used for mechanistic studies to assess transport mechanisms, the rate-limiting step in trans-epithelial transport, and transporter-based DDIs.

Unbound inhibitor concentration
[*I*]. The unbound drug is the pharmacologically active species and is available for inhibition of transporters. Typically, mean unbound steady-state C_{max} values following administration of the highest proposed clinical dose is used for $[I]_1$ in the decision tree. Note that the definition of $[I]_1$ used in this report differs from that described by Zhang *et al.* (Ref. 15) in the original published P-glycoprotein decision tree. Defining [*I*] as the unbound steady-state maximum concentration was intentionally done to provide consistency across the all the decision trees included in this report.

K_i
K_i is the inhibition constant of the drug inhibitor, and for competitive inhibition is determined as, $K_i = IC_{50}/[1 + ([S]/K_m)]$, in which [S] is the concentration of the drug substrate and K_m is the affinity of the drug substrate for the drug transporter.

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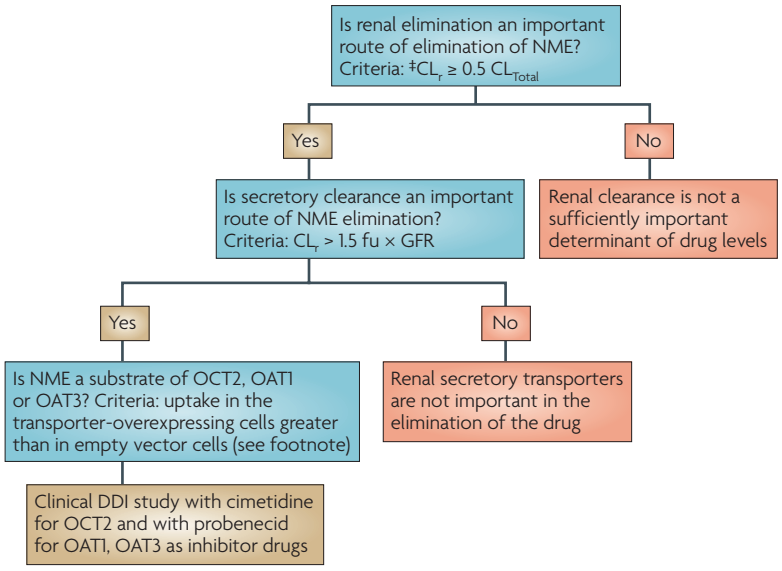
Box 4 | **Decision trees for OCT or OAT substrate interactions**

Generally, most organic cation transporter (OCT) and organic anion transporter (OAT) substrates are small organic ions (less than 400 daltons) that are relatively hydrophilic. OCT substrates are typically cations and OAT substrates anions. Of note, OAT3 can also transport weakly basic compounds such as cimetidine and sitagliptin (Januvia; Merck). The key clinical renal transporters in the basolateral membrane of the proximal tubule are OCT2, OAT1 and OAT3. Transporters in the apical membrane of the proximal tubule that are important, but not a focus of this manuscript, include the multidrug and toxin extrusion transporters (MATE1 and MATE2-K) and the organic anion transporter (OAT4), as well as some ATP-binding cassette transporters (for example, MRP2 and MRP4).

In evaluating whether a new molecular entity (NME) is an OCT or OAT substrate, the first step to consider is whether the renal clearance (CL_r) of the NME is an important determinant of the total clearance (CL_{Total}). That is, if $CL_r \geq 0.5 \times CL_{Total}$ (0.5 was selected because 50% was considered to represent a substantial and clinically important percentage of the CL_{Total}). When CL_r is a substantial component of CL_{Total} (or when the fraction of CL_r to CL_{Total} cannot be estimated because oral bioavailability cannot be determined), the second condition to establish is whether renal clearance involves secretion. That is, if CL_r > 1.5 fraction unbound (fu) × glomerular filtration rate (GFR). If fu cannot be accurately determined, for example, plasma protein binding is greater than 99.9%, or is not known, then assume fu = 0.01, to err on the conservative side. Greater than 1.5 implies that a substantial fraction of the CL_r is due to renal tubular secretion.

If both conditions are met, that is, $CL_r \geq 0.5\ CL_{Total}$ and CL_r > 1.5 fu × GFR, then *in vitro* uptake into a recombinant cell line expressing renal transporters should be conducted. These assays should include control cells without recombinant transporters, or transfected cells with known OCT or OAT inhibitors, or both. To determine whether an NME is a substrate of an OCT or OAT (or OATP as discussed in BOX 6), the ratio of NME uptake in the transporter-expressing cells versus the control (or empty vector) cells should be statistically greater than 1 (see footnote). Michaelis–Menten studies can be used in the transfected cells to determine the kinetic parameters of the NME.

For confirmed *in vitro* substrates of OCT2 or OAT1/3, a clinical drug–drug interaction (DDI) study could be performed with cimetidine as an inhibitor of organic cation transport, or probenecid as a broad non-specific OAT inhibitor. Cimetidine and probenecid are model inhibitors; however, these drugs may not be prescribed commonly. Therefore, sponsors should consider other more clinically relevant renal transporter inhibitors, if available, for use in DDI studies. Examples of labels that describe DDIs mediated by OCTs or OATs include metformin, pramipexole and varenicline.



The ratio of NME uptake in the cells expressing the transporter versus the control (or empty vector) cells should be statistically greater than 1. No agreement was reached by the International Transporter Consortium regarding the magnitude of the ratio. However, it is important that uptake into the transfected cells be significantly greater than background in a control cell line and be inhibited by a known inhibitor of the transporter. A positive control should be included.
‡If the CL_r/CL_{Total} is unknown, go to 'Yes'.

Data from multiple experimental systems are often useful in confirming the involvement of a transporter with a potential substrate. Various cell-based assay systems are described below.

Polarized cell lines without recombinant transporters. Vectorial transport systems in which flux can be measured in two directions (namely, apical-to-basolateral and vice versa) are standard methods for evaluating drug transport across the small intestine and the blood–brain barrier. However these systems have some limitations. For example, Caco-2 cells can vary in their transporter expression profiles compared with the small intestine; although some data support strong correlation with human jejunum[119]. Although endogenous expression levels of transporters may be low in MDCK and LLC-PK1 cell lines, these have similar limitations. Immortalized human kidney cells (for example, Caki, IHKE-1 and NRK52E) can be used to understand regulation of key drug transport processes; although limitations include their inability to replicate transport processes present in the intact kidney[120]. Recently, chemically synthesized RNA interference molecules have been used to silence expression of various genes in mammalian cell lines. By selectively knocking down endogenous transporters, the new cell lines could be used to study the role of a particular transporter in the transport of drugs[121–123].

Single- and double-transfected cell lines. Recombinant transporters that are stably or transiently expressed in various cell lines can be used to characterize drug transporter interactions. Cell-based assay systems for quantitative drug transport studies include cell lines expressing uptake or efflux transporters, or both[123,124]. Cultured cell lines used to study drug transporter interactions include cell monolayers such as MDCK, LLC-PK1, HEK293 or CHO cells grown on solid support. Such monolayers can be used to assess uptake and efflux by single recombinant transporters, or by multiple recombinant transporters, and kinetic measurements can be obtained. Various ABC transporters have been analysed in monolayers of polarized cells, such as MDR1-transfected MDCK II cells, by measuring B-A/A-B flux ratios of compounds. Apart from single-transfected cell lines, polarized cells that stably express multiple transporters — for example, a recombinant uptake transporter in their basolateral membrane and an efflux transporter in their apical membrane — have been developed[97,124–127]. These cell lines may overcome the limitation of certain single-transfected cells that often lack the endogenous uptake or efflux transporters to provide a complete mechanism for trans-cellular transport of a molecular entity. It is noted that compounds may undergo uptake or efflux by multiple transporters in intact organs, and therefore even double-transfected cell lines may not predict the true *in vivo* situation[128].

Primary cells. Primary-cell-based assays are derived from intact tissue, which, at the time of isolation, express the full complement of drug transporters present in a

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given tissue or cell. Thus, human primary cells may be used to study drug disposition and clinically relevant drug interactions. However, these cells can adapt to culture conditions over time, requiring strict definition of culture methods to ensure proper polarization, expression and localization of drug transporters. For example, brain microvessel endothelial cells require monitoring of tight junction integrity, and renal proximal tubule cell usage is limited by tubule collapse in culture. In addition, investigation of intestinal drug transport in isolated intestinal cells or shed enterocytes is limited by their inability to form well-defined monolayers in culture.

Sandwich-cultured primary hepatocytes. Suspended primary hepatocytes have been shown to reproduce hepatic uptake function[129]. Unfortunately, the *in vivo* polarity of hepatocytes is rapidly lost upon isolation, leading to the inability to assess the potential for canalicular efflux. This polarity can be regenerated when hepatocytes are cultured in a sandwich configuration between two layers of gelled collagen[130,131]. The drug-metabolizing capabilities and the regulatory machinery of hepatocytes are retained, as well as expression, localization and function of various hepatic transport proteins in the sinusoidal and canalicular membranes[132]. Utilizing sandwich-cultured rat and human hepatocytes, good correlations have been demonstrated between *in vitro* and *in vivo* biliary clearance for some compounds[133]. This system can provide an estimate of intracellular drug and/or metabolite concentrations. A caveat of the sandwich-cultured hepatocyte model is that it is not as simple to establish as primary cells, and the nature of the culture system does not allow for considerations of other factors that would influence uptake and efflux of compounds (for example, loss of sinusoidal flow or lack of exteriorized bile flow).

Considerations for use of in vitro systems in evaluating transporter interactions. *In vitro* technologies for determining the interaction of drugs with transporter proteins are described here and in the literature (see sections above). These techniques and tools continually evolve, and so prescriptive approaches in an article such as this are at risk of being out of date in the near future. The ideal experimental approach considers many factors such as stage of development (discovery versus clinical phases); physicochemical properties of the NME; and the required outputs from an investigation (for example, efflux ratio, kinetic parameters K_m and V_{max}, and uptake clearance). Whatever the selected experimental approach, it is essential that the system be well characterized with known substrates or inhibitors, and that appropriate controls are incorporated to verify test results. Due consideration should be given to the choice of concentrations; time points (for example, within linear ranges, giving appropriate signal-to-noise ratio); calculation methods appropriate to the test system; and justification that the probe substrates or inhibitors allow tangible extrapolation to the clinical situation.

Absorption, distribution, metabolism and excretion (ADME). The acronym ADME describes the factors that determine the overall exposure to an administered drug. Absorption describes uptake from the site of administration. Although usually referring to oral route, other routes can apply, for example, subcutaneous or buccal. As drug levels are typically measured systemically, oral absorption can be a function of movement across the mucosal surface of the intestinal tract, through the liver and into the systemic circulation via the vena cava. Distribution describes the extent of partitioning into tissues. Metabolism refers to a chemical change usually catalysed by enzymes such as the cytochrome P450s. The drug and its metabolites are excreted or eliminated usually by the kidney (in urine) or from the liver (via bile) or intestines into the faeces.

Intact organ/*in vivo* models

Numerous transporter-gene knockout and naturally-occurring transporter-deficient animal models have been characterized in recent years, and their commercial availability has been increasing steadily[134]. Knockout models have illustrated the role of transporters in physiology, protection of major blood–tissue barriers, and the absorption and excretion of xenobiotics and endogenous compounds. For instance, although the localization of P-gp in the blood–brain barrier has been known, its profound role in limiting brain exposure of some xenobiotics became apparent only after demonstrating that *Mdr1a*$^{-/-}$ mice displayed a 100-fold higher sensitivity towards ivermectin[135]. And although a key role for BCRP in protecting the blood–brain barrier remains to be demonstrated, recent evidence obtained in *Bcrp*$^{-/-}$/*Mdr1a*$^{-/-}$/*Mdr1b*$^{-/-}$ triple-knockout mice treated with lapatinib (Tykerb/Tyverb; GlaxoSmithKline) suggested that Bcrp and P-gp may have a combined effect at the blood–brain barrier[136].

Numerous transporters have been implicated in the clearance of various drugs and metabolites. The importance of a transporter-based elimination pathway is determined by the fraction of total clearance mediated by the transporter. Appreciable alterations (greater than twofold) in exposure occur only when a major elimination transport pathway is inactivated or extensively inhibited (that is, unbound inhibitor concentration $[I]/K_i >> 1$, in which $[I]/K_i$ is the concentration of inhibitor relative to its inhibition constant)[137]. Transporter knockout models are useful in establishing the extent of the contribution of the ablated pathway to overall clearance in that species.

Species, strain, sex, diet and housing condition differences, as well as compensatory mechanisms, are a limitation of knockout and mutant models. All these variables should be considered carefully in the interpretation of data, and in attempts to extrapolate findings across species. For example, hepatic *Mrp2* expression in rats is approximately tenfold higher than in humans[138], and species differences in substrate specificities have been observed[139]. Therefore, the relevance of biliary excretion via Mrp2 in rats compared to other species should be confirmed further with, for example, knockout mice and *in vitro* systems[138,140,141]. Gender differences in the expression of certain transporters may exist; higher hepatic BCRP expression in males has been established in both mice and humans[142]. Strain differences have also been reported. For example, hepatic Mrp4 is upregulated only in certain *Mrp2*$^{-/-}$ mouse strains[143,144]. Direct translation of preclinical transport findings to the clinic is challenging because of species differences in transporter expression, substrate affinity, physiological function and interplay between transporters and enzymes. Humanized transporter animal models[145] and stem cells are promising tools that may prove to be useful in the near future to study the absorption, distribution, metabolism and excretion (ADME) properties of compounds.

Contribution of transporters *in vivo*

Estimating the contribution of transporters to total tissue uptake and excretion is necessary for understanding their importance in drug disposition. This estimate allows

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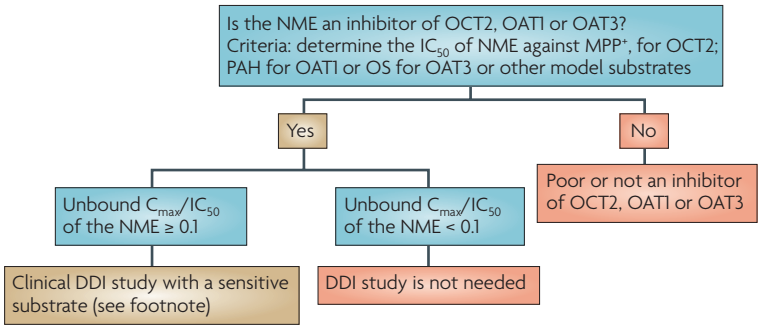
Box 5 | **Decision trees for OCT or OAT inhibitor interactions**

Evaluation of organic cation transporter (OCT) or organic anion transporter (OAT) inhibitors requires determination of an IC_{50} value in an *in vitro* study. If the IC_{50} value is ≤ 10-fold the unbound C_{max} value in cells expressing OCT2 (or OAT1/3), a clinical drug–drug interaction (DDI) study is recommended. Ten-fold was selected to err on the conservative side and to be consistent with the P-glycoprotein (P-gp; also known as MDR1, ABCB1) and breast cancer resistance protein (BCRP; also known as ABCG2) decision trees. For inhibitors of OCT2, the new molecular entity (NME) can be clinically evaluated for its potential to inhibit the renal clearance of metformin, a commonly used anti-diabetic drug. However, OCT2 inhibitors may also be multidrug and toxin extrusion transporter (MATE) inhibitors, and clinical inhibition interactions with metformin may involve MATE1 or MATE2-K in addition to, or even instead of, OCT2. Therefore, some caution in the interpretation of *in vivo* inhibition studies for OCT2 should be exercised. For OAT1 and OAT3, several potential substrates may be used as 'victim' drugs (see footnote). Some inhibitors of OAT1/3 may also inhibit apical transporters (for example, ATP-binding cassette transporters); therefore, clinical inhibition data should be interpreted cautiously.

Selection of an appropriate probe substrate victim drug for the clinical DDI study should be based upon therapeutic considerations; for example, a victim drug should be selected that is likely to be co-administered with the NME in a therapeutic setting. Other considerations should include the therapeutic window of the victim drug and the maximum effect that would be expected if the secretory clearance of the victim drug was totally inhibited.



For NMEs that are OAT inhibitors, multiple candidate probe substrates could be used in clinical DDI studies, including zidovudine, lamivudine, zalcitabine, acyclovir, ciprofloxacin, tenofovir and methotrexate. Some of these substrates may be transported by multiple transporters. For example, some data suggest that methotrexate renal elimination may be affected by co-administration of non-steroidal anti-inflammatory drugs; however, the mechanisms may include other transporters in addition to OATs[195,196]. MPP^+, 1-methyl-4-phenylpyridinium; OS, oestrone-3-sulphate; PAH, para-aminohippuric acid.

Drug clearance
The clearance of a drug is defined as the proportionality constant between rate of elimination and plasma concentration. The total clearance (CL_{Total}) reflects the rate of removal from the systemic circulation (the site of monitoring concentrations). Clearance by an organ can also be defined (for example, renal clearance (CL_r) via excretion, and hepatic clearance (CL_h) via metabolism and/or biliary excretion). Renal clearance of a drug is determined by glomerular filtration, and tubular secretion and reabsorption, both of which may be mediated by transporters.

the prediction of the extent to which inhibition of, or a genetic polymorphism in, a particular transporter, will affect drug concentrations in plasma and tissues. Unfortunately, unlike cytochrome P450 (CYP) enzymes, selective inhibitors or antibodies for most drug transporters have not been identified; although some progress has been made. For instance, potent inhibitors for BCRP and P-gp have been described[2]. The relative contribution of OATP1B1 and OATP1B3 can be estimated in human hepatocytes by measuring the proportion of uptake of test compounds that is inhibited by oestrone-3-sulphate, which is an OATP1B1 inhibitor[146]. A method using reference compounds (substrates) for specific liver uptake transporters also has been proposed (for example, cholecystokinin 8 for OATP1B3)[147,148]. The reference compound should be a specific substrate for a particular transporter. The contribution of a specific transporter to the uptake of the test compound in human hepatocytes can be calculated by taking the ratio of the uptake of the reference compound in human hepatocytes to the uptake in cells transfected with the specific transporter (relative activity factor) and multiplying that ratio by the uptake of the test compound in the transporter-expressing cells. Unfortunately, for most transporters, reference compounds are not yet available. Instead of relative activity factor values, the ratio of the expression level of a specific transporter in human hepatocytes to that in transporter-expressing cells estimated by the band density of Western blot analysis of cell-surface protein can also be used[148].

The relative importance of uptake and efflux transporters in the pharmacokinetics of drugs *in vivo* in humans can be quantitatively estimated by using physiologically-based pharmacokinetic (PBPK) modelling. For example, the effects of changes in OATP1B1 and MRP2 activity on systemic and hepatic exposure of pravastatin were simulated using a PBPK model incorporating blood, liver (the clearance and pharmacological target organ), and peripheral organs[149]. Application of this modelling approach to other drugs has demonstrated its broader validity[150,151].

Imaging techniques can qualitatively and quantitatively elucidate the role of transport proteins in drug disposition (for example, whole body autoradiography and positron emission tomography (PET)). PET is a non-invasive imaging method that is useful for accurately measuring pharmacodynamic end points in humans and preclinical species. Examples of end points include the level of dopamine D_2 receptor occupancy of antipsychotics in the brain[152], or the amount of tracer accumulation (for example, ^{11}C, ^{13}N or ^{18}F) in an organ over time to quantify transporter activity. [^{11}C]-*N*-Acetyl-leukotriene E_4 has been used to study hepatobiliary elimination in normal animals, including monkeys, and in *Mrp2*-deficient mutant rats[153]. [^{11}C]-Verapamil was used to measure the effect of inhibition of P-gp activity in the blood–brain barrier[28,152,154]. PET probes are currently being developed for defined transporters; however, to use these probes a cyclotron must be in close proximity owing to the short half-lives of some PET tracers, such as [^{11}C]-labelled compounds.

Gamma scintigraphy is a non-invasive imaging method utilizing short-lived gamma-emitting radioisotopes (for example, ^{99m}Tc) that can be used to quantify transporter activity and modulation. However, available probes have not been characterized completely for specific transporters. ^{99m}Tc-sestamibi has been used to assess MDR1 P-gp activity[155,156], while ^{99m}Tc-mebrofenin and analogues have been used to measure MRP2 function[157–159]. Gamma scintigraphy has been used to directly quantify biliary and intestinal drug clearance in humans[160,161]. Use of ^{99m}Tc probes to assess hepatic transport function in patients receiving irinotecan[162] and vinorelbine[163] provides interesting translational applications for gamma scintigraphy and the possibility to individualize drug dosage regimens. But, practical reasons may limit the application of imaging methods in the later phases of drug development.

Interplay of efflux transporters and enzymes

The considerable overlap in the substrate specificity and tissue localization of CYP3A and P-gp has led to the hypothesis that this enzyme and transporter pair act as a

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coordinated absorption barrier against xenobiotics[164,165]. Clinical studies investigating the importance of intestinal CYP3A and P-gp through inhibition or induction of these proteins have demonstrated that the role of P-gp in the intestine extends beyond simply limiting absorption of the parent drug[164,166,167]. P-gp also increases the access of drug for metabolism by CYP3A through repeated cycles of absorption and efflux. That is, after penetration into enterocytes, molecules that escape metabolism are eliminated from the cells via P-gp or other apical ABC transporters but then re-enter the enterocytes[168,169]. It has been shown that the residence time of the drug in the intestine is prolonged with the aid of P-gp, thereby increasing the chance of local metabolic conversion by CYP3A4 (REFS 168,169).

Transporters and enzymes in drug clearance

Drug uptake followed by metabolism and excretion in hepatocytes and kidney proximal tubule epithelia is a major determinant of the systemic clearance and exposure of many drugs. Vectorial transport of anionic drugs (for example, statins and angiotensin-converting enzyme inhibitors) from the blood to the bile or urine via an uptake and efflux transporter is important for determining drug exposure in the circulating blood and in organs. Sugiyama and co-workers[170,171] have characterized the interplay of enzymes and transporters to understand the importance of parameters that determine the intrinsic unbound drug clearance in the intestine, liver and kidney. For highly permeable drugs[172–174] in which neither active uptake nor efflux is rate-limiting, the traditional organ clearance models incorporating blood flow, extent of protein binding, and intrinsic metabolic and excretion clearance may hold. However, most anionic drugs and some hydrophilic organic cationic and zwitterionic drugs exhibit poor membrane permeability[172–174]. These types of drugs require transporters for their efficient penetration into or out of the liver or kidney. In such cases, the intrinsic organ clearance is a hybrid of three parameters: the intrinsic clearances for cellular uptake and efflux into the systemic circulation; the traditional organ intrinsic clearance, representing metabolism; and biliary and renal excretion. Each of these uptake, metabolic or efflux clearances can be rate-determining for certain drugs[175]. Therefore, the effects of changes in metabolism and transport activity resulting from interactions with co-administered drugs, or polymorphisms, should be considered in predicting potential changes in the systemic exposure of drugs. Modelling systemic exposure changes for some drugs can be done with PBPK modelling as described in the section above, thus providing initial predictions of drug interaction potential. The role of transporters, including transporter–enzyme interplay, in the pharmacokinetics of orally administered drugs has been reviewed recently[176].

Computational models

The application of computational modelling algorithms to gain insight into transporter–substrate interactions has met with increasing success by the availability of high-quality data sets and atomic resolution structures of several major facilitator proteins. This burgeoning field can be divided broadly into indirect ligand-based techniques, such as pharmacophore and 3D-QSAR modelling, and direct structure-based approaches, such as homology or comparative modelling based on available crystallographic data[177] (FIG. 2). However, synergistic models fusing both techniques are becoming increasingly important[178]. Ligand-based models describe a protein's structural requirements for substrate or inhibitor interaction by correlating the molecular features of validated substrates or inhibitors with their biological activity[38–40,64,178–180]. Most models are limited to predicting putative inhibitors; although some models predict substrates. However, such models generally do not robustly predict the rate of substrate transport[177,178,181]. Pharmacophore models can be used successfully to screen large databases and identify novel transporter ligands. Structure-based approaches generate three-dimensional models of a target transporter based on the structure of an appropriate scaffold protein; currently available structures include bacterial solute transporters and an ABC transporter (Sav1866). A unique challenge in modelling membrane transporters concerns the generation of robust models using low levels of sequence identity and divergent membrane topologies. Ultimately, the docking of ligands may guide ligand-based and rational drug design as well as allow for screening of large databases.

Section 3: Drug development issues

The identification of membrane transporters that influence the disposition and safety of drugs is a new challenge for drug development programmes, as well as for regulatory agencies worldwide. Drug transporter information is becoming common in drug labels, and provides important mechanistic ADME information that is useful for patients, physicians, regulatory agencies and research scientists. For instance, the label for mycophenolate mofetil highlights the role of MRP2 in the hepatic disposition of mycophenolic acid; the atorvastatin label indicates the role of OATP1B1; and the varenicline label discusses the involvement of OCT2 in its interaction with other drugs. Other examples include the sitagliptin (Januvia; Merck) label, which has information about the role of P-gp and OAT3 in the drug's clearance; the cidofovir label, which includes information on the potential modulation of renal toxicity by co-administration of an OAT inhibitor, probenecid; and the lapatinib label, which includes information on P-gp, BCRP and OATPs[182]. Transporter substrate or inhibition studies should be undertaken specifically to clarify the impact transporters have on a drug's disposition, efficacy and safety, including their importance in DDIs. Thus, the ability to move a test compound rapidly into the clinic and to plan effectively for its clinical development will, in part, depend on preclinical assessments of the interaction of drug candidates with transporters.

The ITC discussed the characteristics of an NME that would trigger an *in vitro* interaction study with a specific transporter. An understanding of the physicochemical properties of the drug (for example, molecular mass, charge and lipophilicity) and the organ(s) involved in

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Box 6 | **Decision trees for OATP interactions**

Organic anion transporting polypeptide (OATP) substrates

Generally, investigations involving OATPs are focused on the OATP1B family in the liver. Most OATP1B substrates are large (350–1,000 daltons) organic anions that are relatively hydrophilic. In addition to parent drugs, a number of Phase II drug conjugates are OATP1B substrates. For the identification of substrates, heterologous expression systems including OATP1B1, OATP1B3 and possibly other hepatic OATPs such as OATP2B1, should be considered. Criteria for establishing whether an NME is an *in vitro* substrate of an OATP are discussed in the footnote of BOX 4. If a new molecular entity (NME) is a substrate of an OATP, then a detailed assessment of non-clinical (for instance, permeability, metabolism, tissue-to-plasma ratio in liver and other organs) and clinical data (for instance, dose linearity) should be completed to determine whether a human pharmacokinetic study is warranted (**a**).

If a clinical study is necessary, the NME could be given together with a single dose of an OATP inhibitor such as rifampicin or cyclosporine, as both are broad OATP inhibitors. However, some care should be taken in the interpretation of the clinical data with respect to the interaction because these agents can affect many other transporters as well as absorption, distribution, metabolism and excretion processes. In addition, the pharmacokinetics of the NME could be studied in individuals with OATP1B1 single nucleotide polymorphisms (SNPs) associated with lower OATP activity (as outlined in Section 1). Functional SNPs in OATP1B3 have been described[197], and appear to be associated with alterations in bilirubin levels[198]; however, to date, no phenotypic consequence related to pharmacokinetics has been observed. Therefore, at this time, clinical studies in individuals with OATP1B3 SNPs are not recommended.

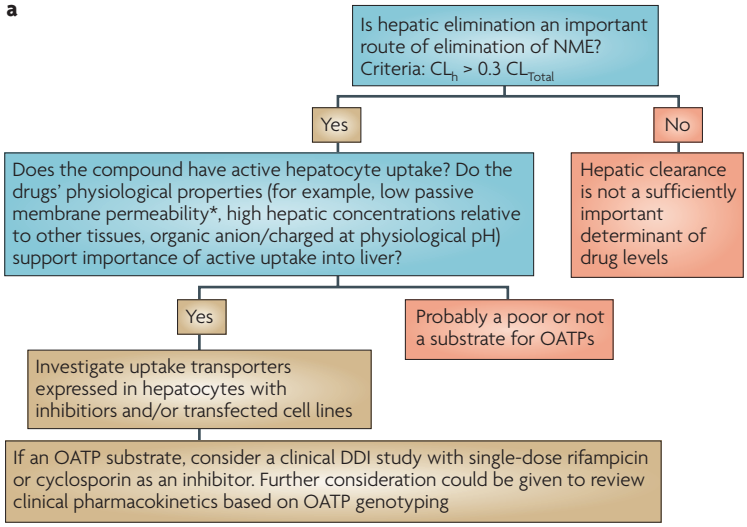
OATP inhibitors

To assess the potential of an NME to inhibit OATPs, the uptake of a prototypical substrate such as oestradiol-17-β-glucuronide, a statin or bromosulphophthalein in a heterologous expression system for OATP1B1 or OAT1B3, should be measured in the presence of the NME, and the IC_{50} value of the NME should be determined (**b**). A known inhibitor such as rifampicin or cyclosporine should be included as a positive control[127,199]. The predicted or actual plasma concentration *in vivo* should be considered as a starting point for interpreting the observed IC_{50} value. If the IC_{50} value is ≤ 10 times the unbound C_{max} (for example, unbound $C_{max}/IC_{50} \geq 0.1$ or $[I]/IC_{50} \geq 0.1$), then the NME may be an *in vivo* OATP inhibitor. If this criterion is met, it is recommended that an *in vitro–in vivo* extrapolation approach (R value) be completed[192,193], which takes the maximal unbound drug concentration at the inlet of the liver ($I_{in,max}$) into account (see figure (**b**) and BOX 7).

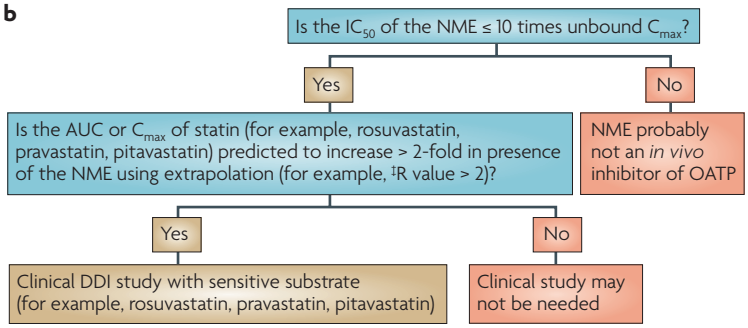
If the extrapolation suggests a > 2-fold change in exposure, consider proceeding to a clinical drug–drug interaction (DDI) study using atorvastatin, pravastatin, pitavastatin or rosuvastatin as OATP1B1 and OATP1B3 non-selective *in vivo* probe substrates[192,193]. Note that calculation of an R value is only one method of many that are available for extrapolation. *In vitro* confirmation that the NME inhibits the uptake of the statin, which is proposed as an *in vivo* probe substrate, should be obtained before proceeding to the clinical DDI study.

To estimate the magnitude of the expected clinical effect of the NME on the statin, calibration of the R value should be performed using OATP inhibitors such as rifampicin or cyclosporine as positive controls[127,199]. It has been noted that telmisartan may prove to be useful as an OATP1B3 selective substrate; thus telmisartan can be considered if a potential drug interaction specific to OATP1B3 needs to be assessed *in vivo*[98]. Examples of how the R value is calculated and used are described in BOX 7.

a



b



*Low permeability needs to be defined by each laboratory based on standards, such as atenolol (a Biopharmaceutics Classification System reference drug). A general guide would be 1×10^{-6} cm per sec (10 nm per sec) or lower is classed as low permeability. ‡R value is defined as $1+(fu \times I_{in,max}/IC_{50})$, in which $I_{in,max}$ is the estimated maximum inhibitor concentration at the inlet to the liver and is equal to: $I_{max} + (F_a \times Dose \times k_a/Q_h)$. I_{max} is the maximum systemic plasma concentration of the inhibitor; F_a is the fraction of the dose of the inhibitor, Dose, which is absorbed; k_a is the absorption rate constant of the inhibitor; and Q_h is the hepatic blood flow (for example, 1,500 ml per min).

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Box 7 | **OATP1B1 decision analysis: case studies**

Current information suggests that HIV protease inhibitors are organic anion transporting polypeptide (OATP) inhibitors. Lopinavir is administered at high doses (400 mg) yielding total C_{max} values of approximately 15–20 μM, representing approximately 0.23–0.3 μM free drug (fraction unbound (fu) = 0.015)[200]. *In vitro* inhibition assays demonstrated that lopinavir inhibited OATP1B1 with an IC_{50} value of 0.1 μM. As the IC_{50} value is less than ten times the unbound C_{max}, completion of an extrapolation is recommended for OATP1B1 (see BOX 6 for OATP inhibition decision tree). An R value for lopinavir of 9.2 is calculated[201] using the following equation and parameters: $R = 1+(fu \times I_{in,max}/IC_{50})$; $I_{in,max}$ = 54.8 μM[200] (see BOX 6 footnote for further explanation of calculation of the R value and of definition of $I_{in,max}$); the fraction of drug absorbed, F_a = 1.0; the absorption rate constant, k_a = 0.03 per min[202]; hepatic blood flow, Q_h = 1,500 ml per min. These estimates assume that the fraction of the substrate transported by OATP1B1 is 100%. However, the magnitude of clinical interaction will be attenuated if the fraction transported by a single pathway is less than 100% (for example, a second route of transport). For rosuvastatin, the contribution of OATP1B1 to the hepatic uptake is estimated to be 54%. As shown in the drug label, the clinical drug–drug interaction (DDI) with lopinavir and ritonavir combination (400 mg and 100 mg) twice a day for 10 days yields a twofold change in AUC and fivefold change in C_{max} of rosuvastatin. This is probably due to lopinavir inhibiting OATP1B1 (ritonavir's effect on OATP1B1 is discussed below).

A similar analysis can be completed for two other HIV protease inhibitors: for ritonavir — 100 mg dose; fu = 0.02[198]; C_{max} = 1.73 μM[203]; $I_{in,max}$ = 4.51 μM, (using F_a = 1.0, Q_h = 1,500 ml per min and k_a = 0.03 per min[202]) — the unbound concentration is < 1 μM, when used as a boosting agent only. However, the IC_{50} value (0.3 μM) is less than ten times the unbound ritonavir concentration (0.05 μM). The extrapolation yields an R value of 1.3, suggesting that the drug interaction potential of ritonavir with OATP substrates is minimal based solely on plasma concentrations, and that a DDI study to evaluate the clinical significance of OATP inhibition is not needed.

For amprenavir (600 mg dose; fu = 0.1; C_{max} = 12.0 μM; $I_{in,max}$ = 35.8 μM)[203], unbound concentrations are > 1 μM and the IC_{50} value (5.5 μM) for OATP1B1 is fivefold higher than the unbound concentration (1.2 μM). Extrapolation provides an R value of 1.7, suggesting that a DDI study may not be required. Indeed, the lack of an interaction between amprenavir (when administered as fosamprenavir) and rosuvastatin was confirmed in a clinical study[201]. Furthermore, in this study, ritonavir was present as a boosting agent, suggesting that this low-dose ritonavir does not inhibit OATP *in vivo*.

clearance of the NME will help determine which transporter–NME interactions should be studied *in vitro*. For example, an NME that is cleared exclusively by the kidneys should not (initially) be evaluated as a substrate for the hepatic transporter OATP1B1. Furthermore, consideration of the physicochemical and pharmacokinetic properties of an NME is also important in assessing the likelihood of a clinical DDI. For example, the solubility, permeability and degree of metabolism of an NME will affect its likelihood for a DDI with a P-gp or BCRP inhibitor (BOX 2).

Of the numerous drug transporters identified so far, P-gp, BCRP, OATPs, OCTs and OATs have been implicated (and studied) most frequently in clinical practice. As outlined in Section 2, various *in silico, in vitro* and *in vivo* tools are available to evaluate the role of transporters in the disposition of a drug. Examples of potential decision trees for these clinically relevant transporters are included here (BOXES 2–6), and are intended to provide guidance to sponsors and regulatory agencies as to when to conduct clinical studies to investigate the importance of transporters in a drug's disposition. The recommendations proposed herein are generally intended to support clinical development and filing of a new drug application. Case studies of decision analyses are provided in BOX 7. The timing of transporter investigations should be driven by efficacy; safety; clinical trial enrolment questions (for example, exclusion and inclusion criteria); the need to further understand the ADME properties of the drug molecule; and transporter information needed for drug labelling. The proposed decision trees represent starting points for discussion. The US Food and Drug Adminstration draft Drug Interaction Guidance[14,15,183] is recommended for use in conjunction with this report as a starting point to develop a strategy for addressing transporter issues during drug development.

The lack of specific inhibitors and substrates for transporters is well recognized, and represents an area of research that warrants further research. However, this should not be seen as a reason not to move forward with a clinical (or preclinical) drug interaction study, as the primary reason for conducting a drug interaction study is to provide information for dose adjustments. Even though it is desirable to understand the mechanisms responsible for DDIs, elucidating the mechanisms is not the primary objective of clinical DDI studies; understanding the required dose adjustments for patients is the single most important objective. The supporting preclinical and *in vitro* transporter data are essential in the design of the clinical DDI study.

Summary and conclusions

The focus of the ITC has been to provide, where possible, consensus on the current knowledge of transporters in drug development. The ITC identified areas in which there was agreement on the current status of the field, existing challenges and future directions. Importantly, the ITC has provided some guidelines that industry and regulatory agencies can apply to assist in the development of safe and effective medications (see decision trees in Section 3). Although this topic has been addressed to some extent previously[184], the field of drug transporters is rapidly advancing. Consideration must be given to the role of transporters in the absorption (for example, intestinal P-gp and BCRP); distribution (for example, P-gp at the blood–brain barrier, and OATP1B1, OATP1B3 and OATP2B1 for hepatocyte uptake); and excretion (for example, OATs and OCTs for renal elimination) of NMEs in development. One could also argue that metabolism should be included to complete the ADME paradigm, as intracellular drug concentrations can be affected by an interplay between transporters and DMEs, as summarized in Section 2 (REFS 166,169).

There are significant challenges that result from these rapid developments. Our applied knowledge of DMEs progressed through the 1990s and was successfully practiced to address specific issues in the pharmaceutical industry. A comparison between the experiences with DMEs and the current challenges with transporters is inevitable, and can also be informative. Will successes in the transporter field parallel the successes in the field of DMEs? And will the fields be integrated to provide a more holistic approach to clinical trials of NMEs? Overall, from a mechanistic perspective, transporters offer complexities that are distinct from those of DMEs. Typically, tissue-specific drug concentrations are determined

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by both uptake and efflux transporters within a tissue. Drug concentrations measured in plasma, therefore, may not reflect levels in organs such as liver and brain; this important concept has been illustrated in studies with transporter-knockout mice. Furthermore, in contrast to DMEs, which are largely concentrated in the liver and intestine, transporters are present in varying abundance in all tissues in the body, and have important roles in drug distribution and tissue-specific drug targeting, as well as in drug absorption and elimination. Another complexity of drug transporters, which is also seen for DMEs, is the issue of redundant specificities among transporters within a particular tissue. For example, in a given tissue, there may be redundant influx and efflux transporters such that multiple transporters with overlapping substrate specificities will determine tissue-specific drug concentrations. Standardized, well-characterized, generally accessible *in vitro* assay systems are needed to define the roles of the different transporters in drug absorption, distribution and elimination. These are in addition to clinically relevant, selective probe substrates, inhibitors and tracers suitable for imaging studies. The Critical Path Initiative has highlighted the enormous need for modern tools for use in drug development[185].

In this manuscript, we have suggested models for decision trees that can be used in the development of drugs. Much time was spent by the ITC in discussing whether to include such decision trees here. Many expressed concerns that decision trees could and would be rigidly interpreted as the sole path for *in vitro* and *in vivo* testing of transporter–drug interactions. Importantly, there was concern that the field was not sufficiently advanced, and that there are gaps in our knowledge of the *in vivo* role that transporters play in drug absorption and disposition in humans. Moreover, tools such as selective probes and inhibitors were not available at this time. However, others felt that the field was evolving and that decision trees, interpreted with flexibility, might be useful in the development of drugs and the collection of data to refine the decision trees. All felt that considerable caution must be exercised in the use and interpretation of the decision trees presented here, and all acknowledged that the decision trees will need to be modified regularly as the field of transporter pharmacology grows. At some point in the future, integrated transporter and enzyme decision trees should be considered. The proposed decision trees attempt to define what can be extrapolated from current knowledge to aid in guiding the need, or lack thereof, for clinical studies. The decision trees that were constructed included conservative criteria for each decision so as to reduce false negatives. However, concern was expressed that an abundance of false positives will have a detrimental effect on the development of new drugs. Thus, a balance in the decision trees is clearly needed. The evolution and appropriate application of these decision trees will require constant monitoring. How can this be achieved with an assured and encompassing measure of success? Constant questioning of current practices with ongoing dialogue between academic institutions, regulatory agencies and industry will ensure the development of safe drugs.

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Competing interests statement

The authors declare competing financial interests: see web version for details.

DATABASES

UniProtKB: http://www.uniprot.org
BCRP | MRP2 | OCT1 | OCT2 | OAT1 | OAT3 | OAT4 | OATP1A2 | OATP1A2 | OATP1B1 | OATP1B3 | P-gp

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