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REVIEW



# Studying the right transporter at the right time: an *in vitro* strategy for assessing drug-drug interaction risk during drug discovery and development

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**ABSTRACT**

**Introduction:** Transporters are significant in dictating drug pharmacokinetics, thus inhibition of transporter function can alter drug concentrations resulting in drug-drug interactions (DDIs). Because they can impact drug toxicity, transporter DDIs are a regulatory concern for which prediction of clinical effect from *in vitro* data is critical to understanding risk.

**Area covered:** The authors propose *in vitro* strategies to assist mitigating/removing transporter DDI risk during development by frontloading specific studies, or managing patient risk in the clinic. An overview of clinically relevant drug transporters and observed DDIs is provided, alongside presentation of key considerations/recommendations for *in vitro* study design evaluating drugs as inhibitors or substrates. Guidance on identifying critical co-medications, clinically relevant disposition pathways, and using mechanistic static equations for quantitative prediction of DDI is compiled.

**Expert opinion:** The strategies provided will facilitate project teams to study the right transporter at the right time to minimize development risks associated with DDIs. To truly alleviate or manage clinical risk, the industry will benefit from moving away from current *qualitative* basic static equation approaches to transporter DDI hazard assessment towards adopting the use of mechanistic models to enable *quantitative* DDI prediction, thereby contextualizing risk to ascertain whether a transporter DDI is simply pharmacokinetic or clinically significant requiring intervention.

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## 1. Introduction

DDIs occur when one drug (termed the 'perpetrator') alters the pharmacokinetics or pharmacodynamics of a co-administered drug (the 'victim'). Pharmacokinetic DDIs are a significant patient safety concern as substantial changes in blood and tissue concentrations of a drug and/or metabolite can occur. Such fluctuating exposure levels (defined as a drug's area under the plasma concentration versus time curve [AUC], and its maximal plasma concentration at steady state [$C_{max}$]) can alter the safety and efficacy profile of a drug and/or metabolite, which is especially a concern for drugs with a narrow therapeutic index. Furthermore, the prevalence of DDI is increasing due to 1) an ageing population and the associated practice of polypharmacy as a result of co-morbidities within such a population; 50% of patients over the age of 65 years are reportedly prescribed ≥5 drugs, and 2) the use of combination therapies in diseases such as HIV and cancer [1,2]. Consequently, DDIs account for 5% of hospital admissions and 20–30% of all adverse drug reactions in oncology clinical trials [1,3]. These can lead in worst case scenarios to early termination of development of a drug, refusal of approval, or withdrawal of drug from the market, and therefore remain a major regulatory concern. DDIs might also necessitate clinical intervention either in the form of dosage adjustment, therapeutic drug monitoring, or prescribing restrictions (contraindications/exclusions). Pharmacokinetic DDIs are mediated via mechanistic changes in the processes of absorption, distribution, metabolism, and elimination of the victim drug (the substrate), brought about by the co-administered perpetrator drug's ability to inhibit (directly as an inhibitor or by competing as a substrate), or induce, drug metabolizing enzymes and/or transporters that are critical to the victim's clinical disposition. The ability to perpetrate a DDI can be limited by a drug's dose and exposure, whereas regardless of dose, all medications have the potential to be victims if they are substrates of enzymes or transporters. Changes in the exposure of a victim drug can be defined in one of two ways: 1) as a 'pharmacokinetic DDI' whereby the AUC increase falls outside of regulatory bioequivalence criteria (>1.25-fold) but without the need for clinical intervention, or 2) as a 'clinically significant DDI' whereby the magnitude of AUC increase warrants some form of clinical intervention in order to avoid adverse events. The clinical significance of any DDI mediated by a perpetrator drug is determined by the nature and severity of adverse events in relation to the increased exposure of the victim drug within the context of the victim's

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**Article highlights**

- The prevalence of DDI is increasing due to ageing populations and the associated practice of polypharmacy as a result of co-morbidities; 50% of patients over the age of 65 years are reportedly prescribed ≥5 drugs.
- From a transporter DDI risk perspective, the potential to inhibit transporters is of the greatest concern during development of investigational drugs due to the safety impact of increased exposure of some common co-medications and the adverse effect it may present to recruiting patients to clinical trials.
- Knowledge of co-medication prescription rates for a disease indication will aid the identification of critical co-medications.
- Understanding the clinically relevant disposition pathways of critical co-medications determines what transporters to focus on (and when) for evaluating DDI perpetrator potential of investigational drugs during discovery and development.
- It is vital that all *in vitro* transporter $IC_{50}$ determinations be conducted with the inclusion of a pre-incubation step with investigational drug, regardless of transporter, in order to remove any artefactual underestimation of the $IC_{50}$ ($K_i$) parameter, thereby ensuring the correct $IC_{50}$ value is obtained for accurate DDI risk assessment.
- The degree that active transport contributes to a transporter disposition pathway (defined by $f_e$ value) dictates the maximal theoretical exposure (AUCR) change possible in DDI.
- Use of transporter $f_e$ values with mechanistic static equations accurately predicts the observed AUCRs of 28 clinically significant DDIs involving six different statin victim drugs with a range of perpetrator drugs.

This box summarizes key points contained in the article.

therapeutic index (safety margin) and frequency of administration [4].

The likely extent of inter-subject variability in victim drug exposure caused by inhibition of a disposition pathway in DDI, and the subsequent potential impact on efficacy and toxicity, can be revealed separately to a degree by clinical pharmacogenetics. Indeed, clinically significant pharmacogenetic polymorphisms yielding reduced function phenotypes have been observed for various drug transporters [5] and demonstrate the important roles transporters play mechanistically in defining a drug's pharmacokinetics and its subsequent susceptibility to DDIs.

## 1.1. Drug transporters

Drug transporters are large cell membrane-spanning proteins that form channels in order to steer movement of compounds/chemicals from one side of the membrane to the other. They act as gatekeepers to facilitate either 1) the entry or 2) the exit of endobiotics or xenobiotics (e.g. drugs), into or out of cells, respectively. Two superfamilies exist, the ATP-Binding Cassette (ABC) transporter family, which are responsible for efflux out of cells, and the Solute Carrier (SLC) transporter family that primarily uptake into cells. By working either independently or in tandem, these transporters can influence the intracellular concentrations of endobiotics or drugs at their target site of action within tissues.

### 1.1.1. ATP binding cassette (ABC) transporters

ABC transporters are expressed at barrier and excretion membranes and play a protective role by pumping endobiotics/xenobiotics/drugs out of cells. These are 'primary active' energy-dependent transporters which generate the energy required for transport by hydrolyzing ATP to ADP as part of an integrated ATPase cycle [6]. They transport in a single direction only to move (efflux) a substrate against its concentration gradient. There are approximately fifty ABC transporters in seven subfamilies and of these, individual transporters from three subfamilies (ABCB, ABCC and ABCG) are often studied in relation to drug effect or response [7].

P-glycoprotein (P-gp; synonym multi drug resistance protein 1, MDR1) and bile salt export pump (BSEP; synonym sister of P-glycoprotein) are members of the 'B' subfamily (ABCB1 and ABCB11, respectively). P-gp is primarily involved in the transport of xenobiotics (e.g. drugs such as digoxin and dabigatran) and is ubiquitously expressed on the brush-border (apical) membrane of enterocytes, the canalicular (apical) membrane of hepatocytes, the brush-border (apical) membrane of renal proximal tubular cells and the apical membrane of endothelial cells forming the blood-brain barrier. Conversely, BSEP is exclusively expressed on the bile canalicular membrane of hepatocytes and secretes bile acids into bile [7–9].

Multidrug resistance-associated proteins (MRPs) 2, 3, and 4 belong to the 'C' subfamily (ABCC2, ABCC3 and ABCC4, respectively). MRP2 is ubiquitously expressed on the same membrane locations in intestine, liver and kidney as P-gp. MRP3 is expressed on the blood (basolateral) membrane of enterocytes and the sinusoidal (basolateral) membrane of hepatocytes, whereas MRP4 is located sinusoidally in hepatocytes, on the brush-border membrane of renal proximal tubular cells, and the apical membrane of brain endothelial cells. MRPs are primarily involved in the transport of conjugated endobiotics (e.g. bilirubin glucuronide, estradiol 17β-D-glucuronide) and xenobiotics (usually conjugated drug metabolites) [7,10].

Breast cancer resistance protein (BCRP), a member of the 'G' subfamily (ABCG2), is a 'half' transporter protein that dimerizes to function. BCRP is ubiquitously expressed in the same tissue barrier locations as P-gp and is primarily involved in the transport of endobiotics (e.g. estrone 3-sulfate) and xenobiotics (e.g. drugs such as topotecan and certain statins) [7,11].

### 1.1.2. Solute carrier (SLC) transporters

As a superfamily of uptake/influx proteins, SLC transporters are ubiquitously expressed at membranes of tissues and organs throughout the body where they maintain cell homeostasis and nutrient distribution by transporting endobiotics into cells [12]. Some SLC transporters are 'passive facilitative' (e.g. the organic cation transporters, OCTs) which do not use energy to transport substrates into cells, instead acting as gatekeeper channels allowing a substrate to travel by passive diffusion down its concentration gradient. Other SLC transporters are 'secondary active' and drive their transport of molecules across cell membranes through the utilization of free energy derived from voltage/ion gradients generated by a separate primary active transporter (such as a sodium/

potassium ATPase), or electrochemical gradient. This is achieved through coupling the co-transport of the ion with the transport of the intended substrate, either in opposite directions (performed by exchangers or antiporters) or in the same direction (performed by co-transporters or symporters) [12]. SLC transporters can transport bidirectionally, but only the active transporters are able to move a substrate against its concentration gradient. Substrates of SLC transporters are typically hydrophilic, charged (cationic or anionic) molecules and include endobiotics and various drugs. There are approximately 458 SLC transporters in 65 subfamilies and of these, four subfamilies (SLCO, SLC15, SLC22 and SLC47) are often studied in relation to drug effect or response.

Organic anion transporting polypeptides (OATP) 1B1 and 1B3 are members of the SLCO subfamily (SLCO1B1 or SLCO1B3, respectively). Both are uniquely expressed in liver on the basolateral membrane of hepatocytes, and are responsible for the uptake of a wide range of endogenous anionic compounds such as bile acids and sulfate and glucuronide conjugates, as well as various drug substrates (e.g. statins and sartans).

SLC15 members include peptide transporter (PEPT) 1 (SLC15A1) and 2 (SLC15A2) [13]. PEPT1 is expressed on the brush-border membrane in intestine and kidney, whereas PEPT2 is expressed primarily in kidney. Collectively PEPTs are responsible for the absorption and/or renal reabsorption of dietary peptide digestion products and peptide-like drugs (such as β-lactam antibiotics and the prodrug valacyclovir) [14].

Organic anion transporter (OAT) 1 and 3 and organic cation transporter (OCT) 1 and 2 are members of the SLC22 subfamily (SLC22A6 and SLC22A8, or SLC22A1 and SLC22A2, respectively) [12]. OCT1 is expressed on the basolateral membrane of hepatocytes and the brush-border membrane of enterocytes, where it is responsible for the uptake of endogenous and exogenous (including drugs) cations into liver and intestine, respectively [15]. OAT1, OAT3, and OCT2 are expressed on the basolateral membrane of renal proximal tubule cells and are involved in the active renal elimination of a range of endogenous anions or cations (e.g. creatinine) in addition to drugs including antibiotics (e.g. ciprofloxacin; OAT1/3), antirheumatics (e.g. methotrexate; OAT1/3), hydrophilic statins (e.g. pravastatin and rosuvastatin; OAT3), antiarrhythmics (e.g. procainamide; OCT2) and antidiabetics (e.g. metformin; OCT2).

Multidrug and toxin extrusion protein (MATE) 1 and 2-K belong to the SLC47 subfamily (SLC47A1 and SLC47A2, respectively) and function as antiporters using protons as the co-transported ion. Both MATE1 and MATE2-K are expressed on the apical membrane of renal proximal tubule cells and are responsible for the efflux of primarily cationic compounds in order to complete their active renal elimination. MATE1 is also expressed on the bile canaliculus for biliary excretion.

#### 1.1.3. *In vitro* test systems and methodologies utilized for studying transporters

Immortalized cell lines (such as human colon adenocarcinoma epithelia, Caco-2), transfected mammalian non-human and human cell lines overexpressing a human ABC or SLC transporter (e.g. MDR1 in Madin Darby canine kidney (MDCK) or Lilly Laboratories culture-pig kidney type 1 (LLC-PK1) cells; SLC transporters in human embryonic kidney 293 (HEK293) or Chinese hamster ovary (CHO) cells), or inside-out membrane vesicles prepared from insect cells (e.g. *Spodoptera frugiperda*, Sf9) or mammalian cells (e.g. HEK293) overexpressing a human ABC transporter, can all be utilized to study whether a drug is an *in vitro* substrate or inhibitor of transporters.

The industry 'gold-standard' test system for studying P-gp or BCRP transporters is the polarized cell monolayer using Caco-2 or genetically modified (e.g. MDCK-MDR1, MDCK-BCRP or LLC-PK1-MDR1) epithelial cell lines grown on semi-permeable membrane inserts (or Transwell) to form a brush-border membrane barrier separating two experimental compartments of equivalent pH (7.4). Bidirectional (apical-to-basolateral [A-B] and basolateral-to-apical [B-A] direction) apparent permeability ($P_{app}$, units: cm/s $\times$ $10^{-6}$) of substrate across the polarized cell monolayer (in triplicate wells per condition) is determined and compared (see references [16,17] for more detail). For studying SLC transporters, the 'gold-standard' is the cellular uptake assay using adherent genetically modified cell lines stably or transiently transfected to overexpress a human transporter alongside (mock) vector control cells (to correct for passive permeability and/or any non-specific binding of substrate), and grown on multiwell plates. Following incubation (in triplicate wells), uptake (accumulation) of substrate inside each cell type is determined after removal of incubation media and subsequent lysis of the cells to give a rate (pmol/mg) (see references [17,18] for more detail). Alternatively, the use of inside-out membrane vesicles overexpressing a single ABC transporter is considered the 'gold-standard' methodology for studying hepatobiliary transporters BSEP and the MRPs. This is due to the predominantly polar, poorly passively permeable properties exhibited by known substrates and inhibitors that limits significantly the cellular entry required to access the transporter in conventional polarized cell systems, rendering such test systems unsuitable due to the risk of inconclusive results and false negatives. Indeed, were an investigational drug to exhibit similar physico-chemical characteristics, then P-gp or BCRP-expressing vesicles can be used as a follow-up alternative to polarized cell monolayers. The vesicle transport assay measures the uptake (pmol/mg) of test compound into the lumen of transporter-expressing membrane vesicles in the presence of ATP (in triplicate wells) over a specified incubation time. A background incubation condition using transporter-expressing vesicles in the presence of AMP (absence of ATP), or control (non-expressing) vesicles in the presence of ATP, is typically performed in parallel to help delineate 'ATP-dependent transporter-mediated' uptake (see references [17,19] for more detail).

Assuming the use of a low non-saturating concentration of test compound, then the compound's classification as a substrate of transporters in the various *in vitro* test systems described above is determined by calculation of either an efflux ratio or uptake ratio as described in Figure 1. A test compound is considered to be a transporter substrate when the determined ratio is greater than two. This is often

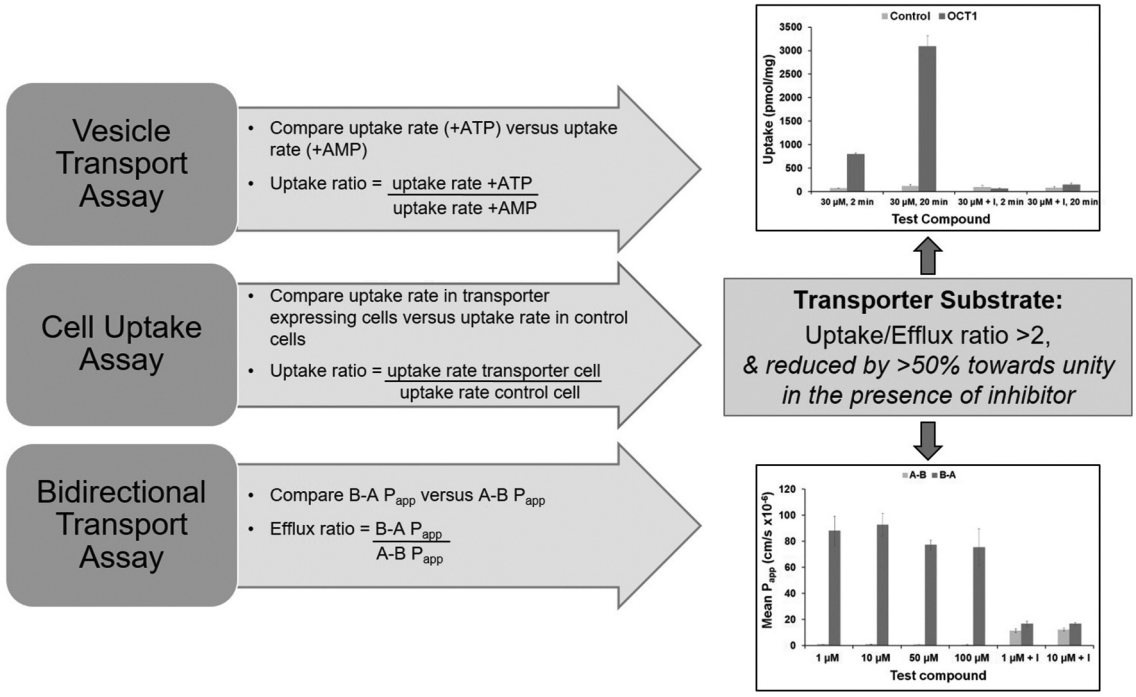


Figure 1. Summary of *in vitro* methodologies for assessing substrates of transporters. Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, The ADME Encyclopedia; Drug Transport Assessment: Transfected Cells and Membrane Vesicles by Hayley Atkinson, Robert Elsby, Philip Butler, COPYRIGHT (2021).

subsequently confirmed through use of a reference inhibitor for the transporter which would reduce the observed efflux/uptake ratio for a substrate.

The same bidirectional methodology (or the industry-recommended unidirectional (basolateral-to-apical) methodology [20–23]), cellular uptake methodology, and vesicle transport methodology described above are also employed for studying inhibition of transporters. As appropriate to the *in vitro* test system, the corrected transporter-mediated flux, or uptake rate, of probe substrate determined in the absence (solvent vehicle control) and presence of test compound is calculated and used to derive an $IC_{50}$ (concentration that decreases [inhibits] vehicle control transport activity by 50%) as described in Figure 2.

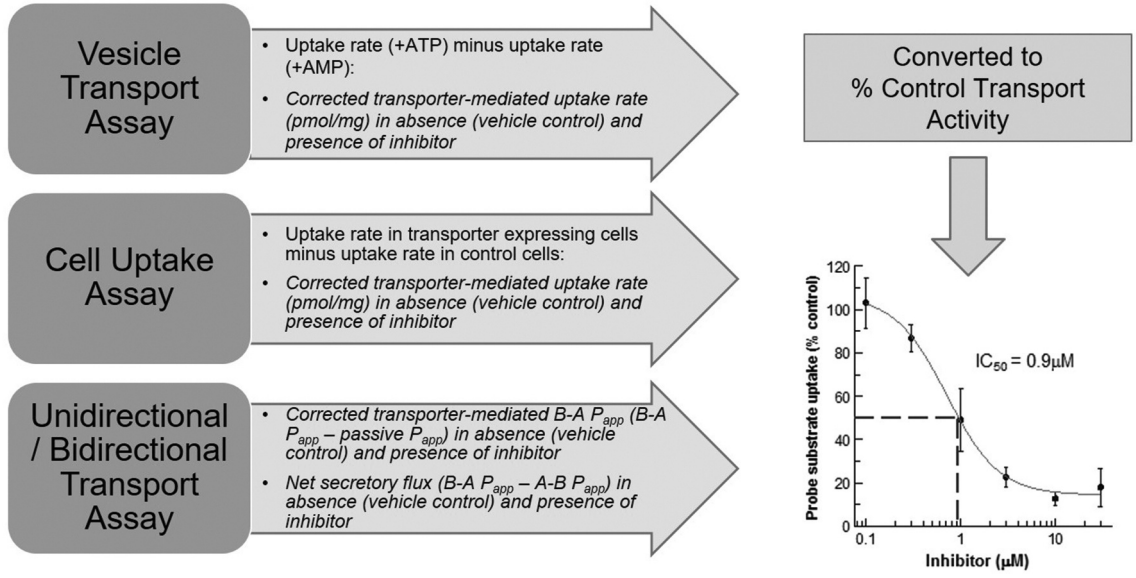


Figure 2. Summary of *in vitro* methodologies for assessing transporter inhibition. Adapted by permission from Springer nature customer service centre GmbH: Springer nature, The ADME encyclopedia; Drug transport assessment: Transfected cells and membrane vesicles by Hayley Atkinson, Robert Elsby, Philip Butler, COPYRIGHT (2021).

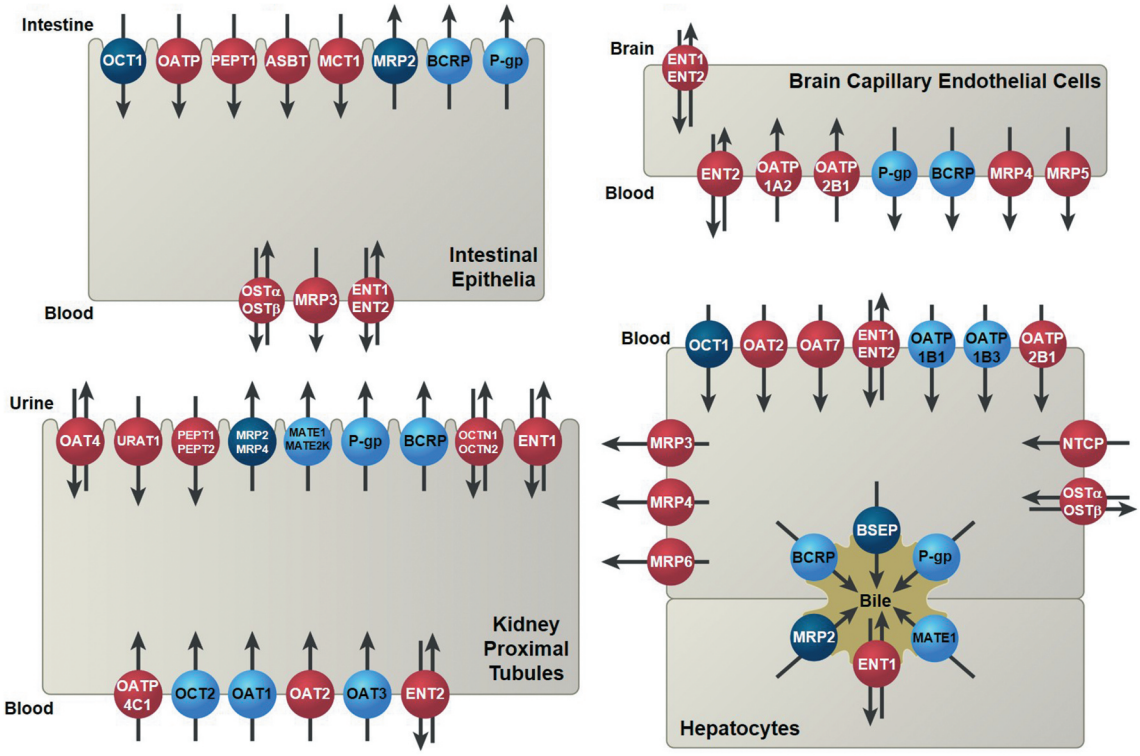


Figure 3. Transporter expression across the various ADME organs.

### 1.2. Role of transporters and their impact on drugs

Given the membrane expression of the drug-related transporters across the plethora of organs (Figure 3) involved in the absorption (intestine), distribution (blood brain barrier), metabolism (liver), and excretion/elimination (kidney) of drugs (their ADME properties), it should not come as a surprise that (depending on a drug's physicochemical properties) transporters may dictate how the body acts on a drug, and in turn, how the drug acts on the body. The impact of transporters on drugs and their development are illustrated in Figure 4 and is wide ranging. Independent of ADME considerations, transporters themselves can be targets for therapeutic intervention (e.g. URAT1 inhibition for gout and SLGT2 inhibition for Type 2 diabetes) [24], or can facilitate drug entry into target sites within tissues thereby modulating drug efficacy/pharmacodynamics (e.g. OATP1B1 or OCT1 uptake into liver is required for statin or metformin therapy, respectively). Transporters may be utilized for targeted drug delivery to improve the absorption and bioavailability of poorly absorbed oral drugs. For example, the antiviral drug valacyclovir (the amino acid conjugated prodrug of acyclovir) is readily absorbed due to it being a substrate of intestinal PEPT1, consequently enhancing the oral bioavailability of the pharmacologically active acyclovir. In contrast to the beneficial interactions above, unintentional modification of transporter function by a drug may give rise to toxicological consequences such as contributing to cholestatic drug-induced liver injury (e.g. BSEP inhibition), conjugated hyperbilirubinemia (inhibition of biliary MRP2), or cisplatin nephrotoxicity (inhibition of MATE1).

However, the biggest impact of transporters on drugs and their development is the role transporters play in dictating a substrate drug's ADME profile (its disposition) which ultimately defines its pharmacokinetics (exposure) in the body. Indeed, of the ABC efflux transporters, both P-gp and BCRP, can broadly affect the oral absorption, brain penetration, and/or biliary and renal elimination of drug substrates. In contrast, uptake by key hepatic SLC transporters such as OATP1B1 and OCT1 can often be the 'rate-determining' step in a drug's hepatic elimination/clearance prior to any subsequent metabolic pathway [25], again defining pharmacokinetic exposure.

Furthermore, due to their critical involvement in ADME, pharmacogenetic polymorphisms of specific key transporters can perturb such functional pathways thereby giving rise to inter-individual variability in drug pharmacokinetics within a population. Clinical studies have demonstrated that individuals with a single nucleotide polymorphism (c.421C.A) in the *ABCG2* gene who consequently exhibit impaired BCRP functional capability, have higher plasma levels of several BCRP substrate drugs including rosuvastatin, atorvastatin, fluvastatin and diflomotecan due to increased absorption of these drugs [5,26–29]. Additionally, the observed ethnic difference and variability between Caucasian and Asian populations with respect to exposure of the BCRP substrates rosuvastatin and atorvastatin can be explained by the higher frequency of the *ABCG2* c.421C.A polymorphism in Asian populations resulting in increased absorption of such substrates due to their impaired intestinal BCRP efflux [26,30,31]. Similarly, clinical pharmacogenetic studies have also demonstrated that individuals with a single nucleotide polymorphism (c.521 T.C) in the *SLCO1B1* gene, leading to a reduced function phenotype,

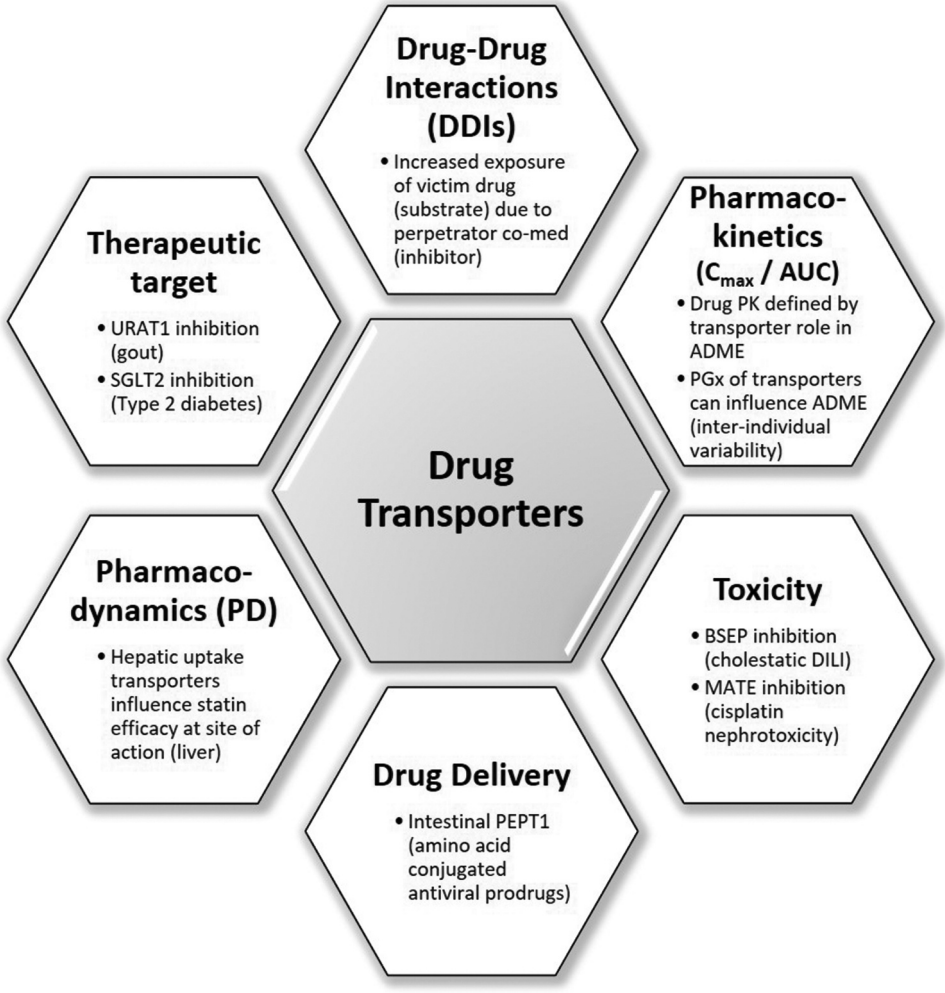


Figure 4. Impact of transporters on drug development.

exhibited higher plasma levels of several OATP1B1 substrates including simvastatin acid, pitavastatin, atorvastatin, pravastatin, rosuvastatin, repaglinide, and olmesartan due to their decreased active hepatic elimination on first pass [5,7,26,31–33]. OCT1 is another important hepatic transporter for which clinical pharmacogenetics have shown it to be the rate-determining step in the hepatic elimination of the cationic drug fenoterol. Reduced transport function arising from polymorphisms in the *SLC22A1* gene leads to elevated plasma levels of fenoterol and potential cardiovascular toxicity [34]. Collectively, these clinical pharmacogenetic observations reinforce how transporter inhibitors could alter the absorption or elimination of specific substrate drugs in clinical practice through DDIs, resulting in toxicity (e.g. myopathy in the case of statins) or altered efficacy.

### 1.3. Transporter drug-drug interactions

Examples of clinically relevant DDIs mediated through inhibition of various key ADME transporters are detailed in Table 1 and further described below based on location of the interaction.

#### 1.3.1. Intestinal transporter-mediated interactions

*1.3.1.1. P-gp.* To date, most reported clinically significant P-gp mediated DDIs concern the cardiac glycoside digoxin as the victim drug because of its narrow therapeutic index and ensuing toxicity if plasma levels are elevated above normal therapeutic range. Digoxin is susceptible to pharmacokinetic perturbations (theoretical maximum 1.43-fold increase in AUC) as a result of inhibition of P-gp by perpetrators, since P-gp efflux plays a critical role in attenuating its absorption and facilitating its active renal elimination. The majority of early clinical studies attributed decreased P-gp mediated renal clearance of digoxin (↓ approx. 30%) as the mechanism underlying observed DDIs. However, it was apparent from Pedersen *et al.* [35] along with more recent studies [22,37,39], and from scrutinizing the available reported AUC profiles with or without co-administered inhibitor in early studies, that the actual mechanism causing DDI is in fact diminished P-gp mediated intestinal efflux resulting in increased absorption of digoxin (↑ approx. 30%; from usual 70 to 100%). In pharmacokinetic DDI profiles, the increase in maximum plasma concentrations and AUC of digoxin is observed in the absence of any change in time to maximal

Table 1. Examples of clinically relevant transporter-mediated DDIs. Adapted by permission from Springer nature customer service centre GmbH: Springer nature, The ADME encyclopedia; Transporter drug interactions by Robert Elsby, Philip Butler, Robert J. Riley, COPYRIGHT (2021).

| Transporter | Victim | Perpetrator | Observed AUC increase *(AUC increase due to inhibition of specific transporter if multiple mechanisms combine for DDI*)* | Other ADME pathways contributing to observed DDI | Reference |
|---|---|---|---|---|---|
| P-gp | Digoxin | Quinidine | 1.54-fold | NA | [35] |
| | | Itraconazole | NR | NA | [36] |
| | | Talinolol | 1.23–fold | NA | [37] |
| | | Rifampin | ↓ 0.7–fold *(induction)* | NA | [38] |
| | | AZD5672 | 1.33–fold | NA | [22] |
| | | Fostamatinib | 1.37–fold | NA | [39] |
| BCRP | Topotecan | Elacridar | 2.4–fold | P-gp | [40] |
| | Rosuvastatin | Cyclosporine | 7.1–fold *(1.9–fold)* | OATP1B1/OATP1B3/NTCP | [41] |
| | | Fostamatinib | 1.96–fold | NA | [23, 42] |
| | | Eltrombopag | 1.88–fold | NA | [43] |
| | | Darunavir | 1.48–fold | NA | [44] |
| | | Lopinavir | 2.1–fold *(1.7-fold)* | OATP1B1 | [45] |
| | | Clopidogrel | 1.96–fold *(1.7-fold)* | OATP1B1 | [46] |
| | | Ezetimibe | 1.21–fold | NA | [47] |
| | | Fenofibrate | 1.07–fold | NA | [48] |
| | Fluvastatin | Cyclosporine | 1.9–fold | NA | [49] |
| OATP1B1 | Simvastatin acid | Gemfibrozil | 2.85–fold | NA | [50] |
| | | Cyclosporine | 8–fold *(4.4-fold)* | CYP3A4 | [50] |
| | Atorvastatin | Cyclosporine | 8.7–fold *(3.2-fold)* | BCRP/CYP3A4 | [51] |
| | Rosuvastatin | Cyclosporine | 7.1–fold *(1.6-fold)* | BCRP/OATP1B3/NTCP | [41] |
| | | Gemfibrozil | 1.9–fold *(1.5-fold)* | OAT3/OATP1B3 | [52] |
| | Pravastatin | Cyclosporine | 3.82–fold *(2.0-fold)* | MRP2 | [53] |
| | | Gemfibrozil | 2–fold *(1.6-fold)* | OAT3 | [54] |
| | Pitavastatin | Cyclosporine | 4.55–fold | NA | [55] |
| | | Gemfibrozil | 1.45-fold | NA | [56] |
| | | Erythromycin | 2.8-fold | NA | [57] |
| | Repaglinide | Gemfibrozil | 8.1–fold *(~2-fold based on PGx)* | CYP2C8 | [58] |
| | | Cyclosporine | 2.5–fold | CYP3A4 | [59] |
| OAT1 / OAT3 | Adefovir | Probenecid | 2.09–fold | | [59] |
| | Furosemide | Probenecid | 2.68–fold | | [60] |
| | Ciprofloxacin | Probenecid | 1.72–fold | | [61] |
| | Methotrexate | Probenecid | NR (56%↓ renal clearance) | | [62] |
| OCT2 | Metformin | Dolutegravir | 1.79–fold | | [63] |
| | Dofetilide | Cimetidine | 1.48–fold | MATE1/MATE2-K? | [64] |
| | Pindolol | Cimetidine | 1.38–1.47–fold | MATE1/MATE2-K? | [65] |
| MATE1 / MATE2-K | Metformin | Cimetidine | 1.46–1.54–fold | | [66, 67] |
| | | Trimethoprim | 1.30–1.37–fold | | [68, 69] |
| | | Pyrimethamine | 1.39–fold | | [70] |
| | Procainamide | Cimetidine | 1.35–fold | | [71] |
| | Varenicline | Cimetidine | 1.29–fold | | [72] |
| | Pilsicainide | Cimetidine | 1.33–fold | | [73] |
| OCT1 | Fenoterol | ? | ~2–fold *(based on PGx evidence)* | | [34] |

* Derived from mechanistic static equation AUCR predictions, NR = Not reported

peak concentration or change in half-life and elimination phase (representing renal clearance; which remained parallel with and without inhibitor drug). This provides the evidence for enhanced oral bioavailability as the underlying mechanism of digoxin DDI and is consistent with the ratio of unbound maximal plasma concentration of perpetrator divided by its $K_i$ being less than 0.1, indicating DDI through inhibition of renal clearance is unlikely [22]. Consequently, any reported 'apparent' decrease in digoxin renal clearance (derived from digoxin amount in urine divided by AUC) was merely artefactual reflecting an increased concentration of digoxin present in plasma due to individuals absorbing more digoxin into the body.

*1.3.1.2. BCRP.* Clinically significant DDIs attributed to inhibition of BCRP involve low permeability substrate drugs for which intestinal BCRP efflux is the rate-determining step in their absorption [6]. Topotecan [40], rosuvastatin [23,41–45], atorvastatin [74], or fluvastatin [74] are examples of such sensitive drugs which give maximum theoretical fold increases in plasma exposure (AUC) of 2.4, 2.0, 1.72 or 1.72-fold when BCRP is inhibited, due to increasing the drug's overall absorption from 40 to 97%, 50 to 100%, 40 to 69% or 58 to 100%, respectively. Conversely, in individuals who express an impaired BCRP pharmacogenetic phenotypic variant (c.421AC or c.421AA) the magnitude of exposure increase due to BCRP-mediated DDI will be significantly lower, or even negligible, due to having less functional BCRP to inhibit by a perpetrator drug. It is important to recognize that supplementary ADME pathways to intestinal BCRP may also contribute towards manifesting DDIs for atorvastatin (cytochrome P450 (CYP)3A4 and OATP1B1), fluvastatin (CYP2C9), and rosuvastatin (OATP1B1, OAT3) [74]. However, for the latter, the mechanism behind the majority of DDIs reported on the drug's label can be attributed solely to inhibition of intestinal BCRP [23].

### *1.3.2. Hepatic transporter-mediated interactions*

*1.3.2.1. OATP1B1.* Poorly permeable OATP1B1 drug substrates for which transporter-mediated uptake is the rate-determining step in their hepatic elimination, which in turn is the major clearance pathway that affects their pharmacokinetics, are susceptible to clinically significant DDIs attributed

to inhibition of OATP1B1 [1]. The commonly prescribed statins are sensitive OATP1B1 substrates for which inhibition of OATP1B1 alone (in the absence of other pathways) would result in up to a five-fold increase in plasma exposure (AUC) of the statin, with the following rank order of theoretical maxima based on the fraction excreted ($f_e$) value of the transporter for the specific statin: simvastatin acid (4.8-fold), pitavastatin (3.3), atorvastatin (3.2), pravastatin (2), and rosuvastatin (1.6) [74]. As mentioned previously, it is important to note that inhibition of other critical disposition pathways by the perpetrator drug such as CYP3A4 (simvastatin acid, atorvastatin), BCRP (rosuvastatin, atorvastatin, fluvastatin) and OAT3 (pravastatin, rosuvastatin) could exacerbate any DDI due to inhibition of OATP1B1 [74]. Unlike for the statins, for the victim drug repaglinide, inhibition of OATP1B1 contributes only in part (up to a maximum of approximately 2-fold based on clinical pharmacogenetic evidence) to the observed DDIs perpetrated by gemfibrozil (and its glucuronide metabolite) or cyclosporine due to the major underlying mechanism being attributed instead to inhibition of the critical metabolic pathways via CYP2C8 or CYP3A4, respectively [75]. By examining clinical DDI pharmacokinetic profiles, the increase in plasma concentrations of orally administered OATP1B1 victim drugs is a result of increased bioavailability due to a reduced hepatic uptake on first pass mediated by inhibition of the transporter. This is also evidenced by the absence of a change in time to maximal peak plasma concentration and in the elimination phase slope and half-life of the drug.

*1.3.2.2. OATP1B3.* Regulatory authorities and the International Transporter Consortium have considered OATP1B3 to be important for clinically relevant transporter disposition and DDIs for substrate drugs such as the sartans (telmisartan and olmesartan) and the statins (pitavastatin and rosuvastatin) [7]. Yet, despite this fact, it is difficult to discover a reported clinical DDI that can be attributed solely to inhibition of OATP1B3. This likely reflects the relatively minor contribution OATP1B3 plays towards the overall hepatic elimination of some of these listed substrates. For instance, OATP1B3 transport only accounts for a minor 10% ($f_e$ = 0.08) or 16% ($f_e$ = 0.11) of the overall active hepatic elimination of pitavastatin or rosuvastatin, respectively, with the remaining predominant fraction being mediated by OATP1B1 [74]. Consequently, it is actually inhibition of OATP1B1 (not OATP1B3) that is responsible for driving the ensuing increase in these victim drugs' exposures in DDI, with OATP1B3 simply contributing only a very small increase (≤ 1.12-fold if completely inhibited) that would be deemed within pharmacokinetic bioequivalence.

*1.3.2.3. OCT1.* The beta-agonist fenoterol is administered via inhalation for treating asthma, or intravenously for suppressing premature labour in pregnancy, and is a narrow therapeutic index drug for which increased plasma levels might result in cardiovascular side-effects in patients [34,76]. Reported clinical pharmacogenetic studies have suggested that fenoterol is a substrate of OCT1 as subjects with almost zero function OCT1 phenotype (*OCT1*3, OCT1*4*) exhibited a 1.92-fold increase in plasma concentrations (based on exposure) of intravenously administered fenoterol compared to subjects with normal transporter function [34]. When comparing the observed pharmacokinetic profiles between OCT1 phenotypes, the increase in plasma concentrations of fenoterol in the zero transporter function phenotype is a direct result of decreased hepatic elimination due to the absence of OCT1-mediated hepatic uptake [34]. Whilst to date there is no reported evidence of a clinical pharmacokinetic DDI for fenoterol with an inhibitor of OCT1, on the assumption that the OCT1 phenotype described above is almost a complete loss of transporter function, one might expect an approximately 2-fold increase in fenoterol exposure as a theoretical maximum AUC increase. It is for this reason that the International Transporter Consortium has identified inhibition of OCT1 during DDI to be a clinically relevant transporter pathway for the potential victim substrate drug fenoterol [76].

### *1.3.3. Renal transporter-mediated interactions*

*1.3.3.1. OAT1 & OAT3.* Poorly permeable, anionic substrate drugs for whom renal elimination incorporating extensive active tubular secretion (>1.5 times unbound passive filtration clearance) is a major disposition pathway are typically involved in clinically significant DDIs attributed to inhibition of OAT1 and/or OAT3-mediated transport. The antidiuretic probenecid is the main perpetrator drug causing renal DDI through inhibition of OATs. This is unsurprising given its historical use in the 1940s to purposefully decrease urinary elimination of penicillin and increase its exposure. This allowed lower doses to be used for therapeutic effect and thereby extending limited supplies of the valuable antibiotic [77]. The observed clinical DDIs perpetrated by probenecid, with any of the victim drugs adefovir, furosemide or ciprofloxacin, result in a reduction in their measured renal clearance giving approximately a two-fold increase in victim drug exposure (AUC) [59–61]. This increase is a direct result of elevated plasma concentrations within the elimination phase of the pharmacokinetic profile; evidenced by the shallowing of the elimination slope with an increase in half-life of the drug, in the absence of a significant change in its maximum plasma concentration.

*1.3.3.2. OCT2, MATE1 & MATE2-K.* Clinically significant DDIs attributed to inhibition of OCT2, MATE1, and/or MATE2-K transporters usually involve cationic, poorly permeable drugs for which active renal elimination is a major disposition pathway. The gastric acid reducer cimetidine is the most common perpetrator drug causing renal DDI through inhibition of cation transporters [64–67,71–73]. By the same token to the mechanism underpinning OAT DDIs above, inhibition of basolateral OCT2 results in a reduced renal clearance of the victim drug (e.g. dofetilide and pindolol), yielding elevated plasma concentrations and up to a 1.5-fold increase in AUC in addition to an increase in half-life of the drug.

However, depending on the victim in question, in some instances the same perpetrator cimetidine might drive a DDI through more potent inhibition of MATE1 and/or MATE2-K efflux rather than through inhibition of OCT2. This alternative mechanism is believed to underlie the DDI perpetrated by cimetidine with procainamide, varenicline or pilsicainide resulting in a similar change to each victim drug's pharmacokinetic profile (increased

plasma concentrations resulting in typically <1.5-fold increase in AUC and a longer elimination half-life) due to decreased renal clearance by cimetidine. Typically, inhibition of an apically located efflux transporter (such as MATE) involved in the urinary elimination of poorly permeable charged substrate drugs (that require basolateral uptake to enter proximal tubule cells) would not be expected to result in a change in their systemic blood concentrations. Instead, inhibition of efflux would be anticipated to increase intracellular proximal tubule concentrations of substrate without impacting blood levels. However, this turns out not to be the case for drugs that are dual substrates of OCT2 and MATE1/2-K because inhibition of MATE, in the absence of OCT2 inhibition, results in elevated blood concentrations of the victim substrate. This observation can be explained because OCT2 is a passive facilitative transporter that assists movement of a substrate (e.g. procainamide, varenicline or pilsicainide) down its passive concentration gradient. As such, when MATE is inhibited thereby preventing elimination into urine, intracellular concentrations of the substrate increase resulting in a rapid decline (towards equilibrium) of its inwardly directed concentration gradient from blood, consequently slowing the rate of uptake mediated by OCT2 into the proximal tubule cell. The effect of this indirect reduction (slowing) in OCT2 function are elevated blood concentrations since less drug is renally cleared from the systemic circulation.

The antidiabetic metformin is the most common clinically relevant victim co-medication susceptible to reduced renal clearance in DDI through inhibition of either OCT2 or MATE transporters. However, whilst dolutegravir effects its DDI with metformin through inhibition of OCT2, the predominant underlying mechanism behind the majority of clinically observed DDIs with perpetrators including cimetidine, trimethoprim and pyrimethamine is in fact inhibition of MATE1. This arises due to the significantly more potent inhibitory potential these drugs exhibit against MATE1 versus OCT2 (MATE1 $K_i$ values being 35 to 170-fold lower than corresponding OCT2 $K_i$ values, depending on the perpetrator) [78]. In contrast, for dolutegravir the reverse scenario is true with respect to its inhibitory properties hence explaining why inhibition of OCT2 drives that DDI. Irrespective of mechanism, the decrease in metformin renal clearance mediated through cation transporter inhibition results in elevated plasma concentrations and exposure (AUC) of metformin. However, due to metformin exhibiting unusual 'flip-flop' pharmacokinetic properties these elevations are not reflected as a typical AUC profile change on elimination phase, as described above for other renal DDIs, but instead appear as an effect on the 'absorptive' phase of the profile which, for metformin, actually represents its renal elimination [78].

### 1.4. Regulatory drug-drug interactions expectations for studying transporters

Only two ABC transporters (P-gp and BCRP) and eight SLC transporters (OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1 and MATE2-K) are currently deemed (from a regulatory authority and International Transporter Consortium standpoint) to have a critical role in the clinical disposition and observed clinically significant DDIs of common co-medications [7–9,76,79]. Whilst other transporters not listed above might still be important to a new drug's specific disposition, thereby warranting investigation, it is these ten transporters that are routinely required to be studied as part of regulatory submissions for all new drug applications.

#### 1.4.1. Substrate assessment

*In vitro* transporter studies required for assessing victim DDI potential and the potential for pharmacogenetic impact on pharmacokinetics for an oral drug include evaluation as a substrate of (intestinal) P-gp and BCRP and of hepatic (OATP1B1, OATP1B3 and OCT1) and/or renal transporters (OAT1, OAT3, OCT2, MATE1 and MATE2-K), depending upon

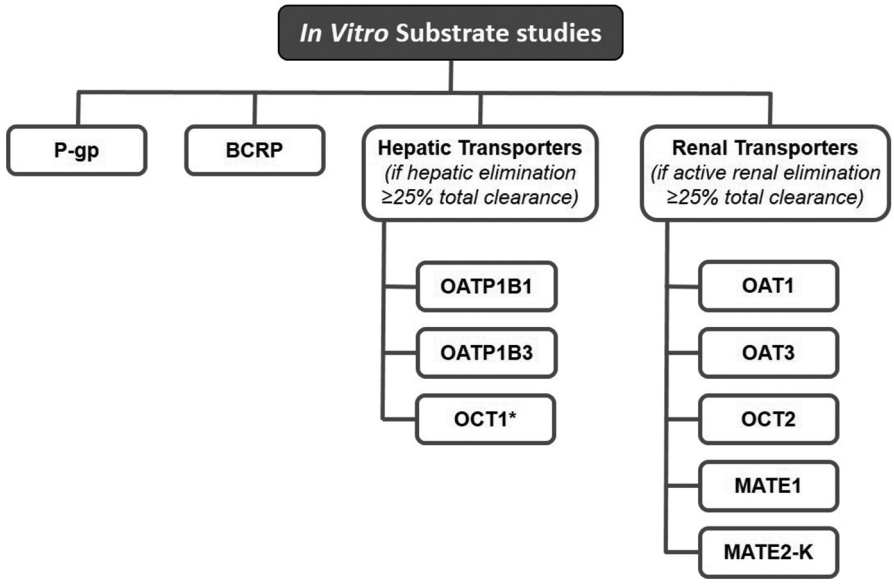


Figure 5. Regulatory expectations for studying substrates of transporters to assess 'victim' DDI potential.

whether the clearance pathway *via* each route constitutes ≥25% total plasma clearance of the drug (Figure 5). However, it is important to note that if an investigational drug has proven high intrinsic passive membrane permeability using the framework set out in the Biopharmaceutics Classification System [8,80] and is established to be BCS class I then, even if it were a substrate, transporters are unlikely to play a major role in its disposition. As such formal *in vitro* substrate assessment studies are not required by regulators and can be waived, since such a drug could not be a victim of transporter DDIs [8].

#### 1.4.2. Inhibitor assessment

In contrast to the tiered approach for substrate assessment based upon a compounds principal disposition route(s), the *in vitro* transporter studies required for assessing perpetrator DDI potential of an investigational drug towards a range of common co-medications encompass evaluation as an inhibitor ($IC_{50}$ determination) of all ten regulatory required transporters, irrespective of the drug's BCS class, transporter substrate status or principal disposition route(s) (Figure 6). This is because whilst a highly permeable drug's entry into organs will be driven by passive diffusion, it could still be a substrate of a transporter and whilst this may not impact its own disposition, it may well have the potential to inhibit (through competition) the transport of a co-administered substrate. In the same regard, circulating plasma concentrations of investigational drug have the potential to inhibit (either competitively as a substrate, or non-competitively as a non-substrate) any systemically distributed transporter even if it is located within an organ that is not involved in the drug's disposition, *e.g.* a drug only cleared by hepatic elimination can still inhibit renal transporters and potentially delay the renal clearance of victim co-medications.

#### 1.4.3. Inducer assessment

It is possible that an investigational drug might act as an inducer to increase the expression of transporter proteins and thereby increase their activity, which could result in it perpetrating a DDI with a co-administered victim substrate drug. Furthermore, since certain transporters such as P-gp share induction mechanisms with CYP enzymes such as CYP3A4, then information from CYP3A4 induction studies can be used to inform on the potential for induction of transporters by the investigational drug. However, due to the lack of well-established *in vitro* methods for studying induction of transporters at this time, it is currently not a requirement of regulatory authorities for drugs to be assessed directly as transporter inducers [8]. Consequently, the focus of this transporter drug interaction strategy is on transporter inhibition being the sole mechanism mediated by an investigational drug when perpetrating transporter DDIs.

## 2. Strategy for what/when/how to conduct *in vitro* transporter inhibition or substrate identification studies towards understanding the drug-drug interaction perpetrator or victim potential of a drug

### 2.1. Identifying critical co-medications

The key requirement before conducting any experimental work on an investigational drug is to first understand what are the likely concomitant medications (co-medications) that are prescribed to patients in the target disease indication and of these, identify those which would be considered 'critical co-medications' because they cannot easily be avoided due to having a high prescribing frequency, or be excluded due to being the 'standard of care' for treatment. An example of a standard of care medication is methotrexate for rheumatoid

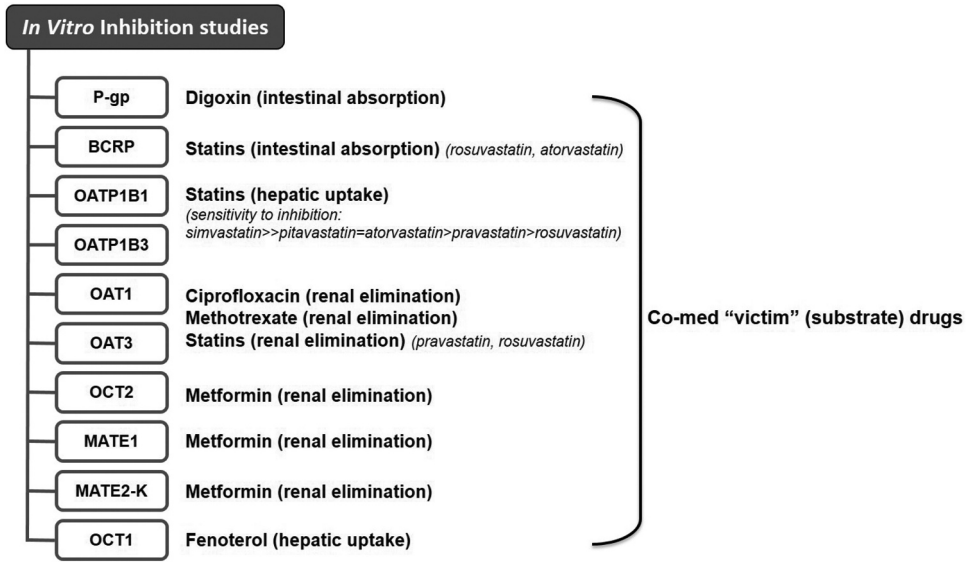


Figure 6. Regulatory expectations for studying inhibition of transporters to assess 'perpetrator' DDI potential.

arthritis; consequently all patients with this disease are on a background of methotrexate [81]. Co-medications can be classified as either 'victims' or 'perpetrators' of DDI. A consideration that contributes towards a drug's identification as a critical victim co-medication is knowledge of its therapeutic index and whether it has a wide, or narrow, safety margin, as this will define the clinical significance/prescribing implications (therefore label claims) arising from a change in exposure due to DDI. Conversely, identification of a drug as a critical perpetrator co-medication considers knowledge of whether it causes (or is predicted to cause) clinically relevant inhibition of ADME pathways at its prescribed clinical dose. Finally, it is important to note that critical co-medications could be either co-administered drugs that are used directly to treat the disease, or they could be co-administered drugs that are used to treat 'common' co-morbidities, for example statins or metformin which are used to treat cardiovascular co-morbidities in patient populations.

### 2.2. Identifying the clinically relevant pathways for critical co-medications

#### 2.2.1. Disposition pathways for victim co-medications (to prioritize the order of transporter inhibition studies for investigational drugs)

Once the critical co-medications have been identified for the chosen disease indication then the next task is to establish whether they are a substrate of transporters and whether being a substrate is important for their disposition and thus victim potential in clinical use. This is vital because whilst a victim drug might be demonstrated *in vitro* to be a substrate of multiple transporters in the literature, all apart from one transporter may be irrelevant to the clinical disposition of the drug and therefore irrelevant to any observed clinical DDI. Therefore, identifying the clinically relevant disposition pathways of the critical co-medication, *i.e.* those underpinning its absorption, distribution, metabolism, and elimination that derive its pharmacokinetics, allows determination of the crucial transporter(s) to prioritize (and potentially when) when conducting *in vitro* inhibition studies. This process prevents the generation of surplus inhibition data early on in development at other regulatory required transporters that may be irrelevant to managing clinical interaction risk for a specific victim co-medication.

Determination of the clinically relevant disposition pathways for a drug is achieved through scrutinizing evidence from a variety of sources. These include 1) the drug's regulatory authority label/summary of product characteristics text, 2) the clinical pharmacology and biopharmaceutics sections submitted to regulators in new drug applications, and 3) published peer-reviewed literature describing the drug's human mass balance, clinical pharmacogenetics, and clinically observed DDIs (and likely underlying mechanisms based on *in vitro* inhibitory profile of perpetrators). Whilst data from human mass balance is crucial to understanding whether hepatic and/or active renal elimination constitutes the major route of clearance for a drug, it is clinical pharmacogenetic data that is pivotal to understanding whether a particular transporter plays a significant role in manifesting that clearance, thereby confirming its clinical relevance to the drug's disposition. This is possible because only perturbation of those pathways that contribute significantly to *in vivo* clearance will result in a pharmacokinetic exposure change in individuals with a loss of function/reduced function transporter phenotype. Moreover, the clinically observed impact of a specific reduced function transporter polymorphism on pharmacokinetics establishes that other related transporters do not compensate for the specific loss of function. Thus highlighting how it could be misleading to consider multiple *in vitro* transporter substrate data for a victim co-medication in isolation, without the context of clinical data to aid interpretation, when attempting to identify the important disposition pathways. A further benefit of possessing clinical transporter pharmacogenetic data is that it can inform on the maximum magnitude of any exposure change that might be expected if that transporter pathway was fully inhibited by a perpetrator in DDI (if the polymorphism results in complete knockdown of function). Consequently, this knowledge also helps to delineate the underlying mechanisms contributing to clinically observed DDIs for the co-medication under consideration by establishing the existence of additional victim ADME pathways being inhibited in cases where the observed DDI exposure change exceeds that expected due to genetic polymorphism alone. Conversely, if an observed exposure change is lower than expected based on anticipating 100% transport inhibition, then this might indicate drug resistance caused by for example an anti-cancer co-medication, either directly where an efflux transporter is overexpressed, or indirectly through potential suppression of uptake transporters [82].

#### 2.2.2. Inhibition pathways of perpetrator co-medications (to prioritize the order of transporter substrate studies for investigational drugs)

Following identification of the critical co-medications it then needs to be established whether they are likely to be, or proven to be, inhibitors of transporters in the clinic. The former can be achieved by putting the co-medication's *in vitro* transporter inhibition potential ($IC_{50}$) into context with its clinical exposure data ($dose/I_{max}/f_u$) using the regulatory basic static equations described in Section 2.6 and seeing if the resultant ratio predicts the drug perpetrating a DDI through inhibition of the transporter *in vivo*. The latter can be established by scrutinizing the contraindication warning section of a drug's label to see if clinical interaction studies have already confirmed it to be a clinical inhibitor of a transporter. Considering any *in vitro* transporter inhibition data for the perpetrator co-medication in isolation without the context of clinical data could be misleading. Although the drug might be a potent inhibitor *in vitro*, the magnitude of any clinical interaction may in fact be limited if a low dose of perpetrator drug is given, or if a high dose of a highly plasma protein bound drug is given resulting in very low unbound drug concentrations at the transporter interaction site. Identifying the clinically relevant inhibition pathway(s) of the critical perpetrator co-medication allows determination of the

key transporter(s) to prioritize (and potentially when) when conducting *in vitro* substrate identification studies towards understanding an investigational drug's DDI victim potential towards that co-medication. Furthermore, this process prevents the generation of surplus substrate data early on in development at other regulatory required transporters that may not be useful to guide management of drug interaction risks with that particular critical co-medication.

### 2.3. Considerations prior to studying transporter inhibition or substrate identification

#### 2.3.1. Choice of probe substrate for inhibition assessment

When evaluating *in vitro* inhibitory potential against a particular transporter, the transport of a probe substrate is measured in one of the appropriate *in vitro* test systems described previously (Figure 2), in the absence (vehicle control) and in the presence of either a single concentration (e.g. typically when screening) or increasing concentrations (e.g. when profiling) of investigational drug. The uninhibited transport activity determined in the vehicle control condition is assigned 100% activity and all activities determined in the presence of investigational drug are converted to percentages of this control transport activity and, depending on assay format, used to either calculate percentage inhibition or derive an $IC_{50}$. The primary consideration prior to studying transporter inhibition (regardless of whether for screening or profiling) is therefore the choice of *in vitro* probe substrate for a particular transporter as observed inhibitory potential can be substrate dependent [8,83]. In line with regulatory authority recommendations, the chosen *in vitro* probe substrate needs to be one of three things in order of preference: either 1) clinically relevant to observed DDIs and capable of being used as the *in vivo* probe substrate (victim) in later clinical interaction studies, 2) a demonstrated good surrogate of a clinically relevant *in vivo* substrate, or 3) a probe substrate that usually generates more potent inhibition for known literature inhibitors in order to err on the side of caution and avoid underestimating interaction potential for investigational drugs. Clinically relevant *in vitro* probe substrates might include digoxin or dabigatran etexilate for P-gp, sulfasalazine or rosuvastatin for BCRP, pitavastatin, atorvastatin or rosuvastatin for OATP1B1, sumatriptan for OCT1, adefovir or furosemide for OAT1, benzylpenicillin or furosemide for OAT3, and metformin for OCT2, MATE1 and MATE2-K transporters [84,85]. Conversely, acceptable *in vitro* surrogate probe substrates for predicting inhibitory potential of investigational drugs towards clinically relevant substrates include estradiol 17β-glucuronide for OATP1B1 (surrogate for statins [83,86]) and estrone 3-sulfate for BCRP (surrogate for rosuvastatin [87]). Other probe substrates that are commonly used *in vitro* and for which potent inhibition by a range of inhibitors is observed, thereby fulfilling preference three for a chosen *in vitro* probe substrate, include para-aminohippuric acid (OAT1), estrone 3-sulfate (OAT3) and tetraethylammonium (OCTs and MATEs).

One further consideration regarding choice of probe substrate concerns the desired scientifically acceptable analytical endpoint for the assay, *i.e.* whether it is radiometric or liquid chromatography tandem mass spectrometric (LC-MS/MS). The use of a tritium or carbon-14 radiolabeled probe substrate has many advantages such as rapid speed of measurement (typically <1 hour) using multi-detector plate based scintillation counters, and the ability to automatically correct for any quenching effect caused by an investigational drug during counting, thereby ensuring the authenticity and accuracy of determined inhibition data. The only slight disadvantage is the current lack of availability of commercial radiolabeled sources for some of the clinically relevant *in vitro* probes, but this can easily be worked around with the use of a readily available radiolabeled surrogate probe. In contrast, provided the probe substrate is amenable to MS/MS optimization, and crucially a stable isotope labeled version of the probe exists commercially for use as an internal analytical standard, then the use of mass spectrometry as an endpoint may expand the availability of clinically relevant probes for inhibition assessment, albeit with a much longer analytical run time (multiple hours) required compared to radioactive endpoints. However, a major disadvantage of using mass spectrometry to quantify probe substrate is the vital requirement to perform a separate analyte ionization enhancement/suppression check of the highest concentration of investigational drug on the probe substrate (analyte) concentration within the incubation at the final processed concentrations anticipated to be presented to the instrument ionization chamber. Such an assessment is needed to make sure that any observed transporter inhibition is not just an artefactual result of suppression (quenching) of the probe substrate analyte response within the mass spectrometer, giving rise to a false positive. Conversely, any artefactual ionization enhancement of the probe substrate response could mask true biological inhibition resulting in false negatives. The only way to correct for such occurrences of analyte interference in order to ensure the authenticity and accuracy of determined biological inhibition data is by use of a stable isotope labeled internal standard of the probe substrate, because unlike a structurally unrelated generic 'injection-check' internal standard, the stable label responds to enhancement/suppression in an identical way to the probe substrate thereby nullifying the interference effect and removing the need to perform checks.

Finally, whilst there are many reports on their application in drug discovery, fluorescent probe substrates are not recommended in transporter inhibition evaluation for regulatory DDI risk assessment purposes during drug development as similar analytical interferences described above for mass spectrometry can occur with fluorescence measurements. Correcting for such effects is more challenging thus increasing the probability of false positives/negatives being generated in assays with such an endpoint. Furthermore, fluorescent substrates do not meet the criteria for *in vitro* probe substrates outlined above, as recommended by regulators. However, while taking on board the various caveats noted, there might be value in using fluorescent probe substrates for early high throughput transporter inhibition screens conducted in drug discovery due to speed of measurement, provided the output is simply to rank compounds rather than any potential extrapolation to clinical risk.

#### 2.3.2. Predicting whether transporters play a role in disposition for substrate assessment

Prior to undertaking any *in vitro* substrate studies it is useful practice to consider the solubility, permeability, and metabolic properties of the investigational drug using the framework of the BCS [80,88] and the modified biopharmaceutics drug disposition classification system (BDDCS) [89] in order to ascertain whether transporters are actually likely to play a role in the compounds disposition. If an investigational drug has high permeability (BCS or BDDCS class I) then, even if it were to be a substrate, it is unlikely transporters will play a role in its disposition as it will have high intrinsic membrane permeability driven by passive diffusion, enabling it to easily pass into and out of tissues and cells. As a result, any inhibition of a transporter by a perpetrator co-medication would have no impact on the disposition of the highly permeable 'victim' drug clinically and as such would not cause DDI. It is for these reasons that formal *in vitro* regulatory transporter substrate identification studies can be waived for an investigational drug if it has been proven to be a BCS class I compound [8]. The BDDCS framework can also be used to inform on the type (ABC efflux and/or SLC uptake) and location of transporters that will likely be important for the disposition of an investigational drug based on its predicted class, thereby helping to focus later substrate identification studies [89,90]. For instance, efflux transporter effects predominate in intestine and both uptake and efflux transporters can effect hepatic disposition for high permeability/low solubility BDDCS class II compounds. Whereas, uptake transporter effects generally predominate for low permeability/high solubility BDDCS class III compounds [89,90].

#### 2.3.3. Choice of *in vitro* test system for P-gp and BCRP substrate assessment

The 'gold standard' methodology for assessing an investigational drug as a substrate of P-gp or BCRP is the polarized cell monolayer. Whilst a multi-expression system like Caco-2 cell monolayers can be used to identify substrates of both P-gp and BCRP, based on experience and ease of interpretation it is recommended to utilize MDCK-MDR1 transfected cells to study P-gp and to use Caco-2 cells for studying BCRP. The advantage of this approach is that the results from the MDCK-MDR1 system can directly inform on whether a compound is a substrate of P-gp and this knowledge can be used towards interpreting the results from the Caco-2 system if efflux was still observed in the presence of a BCRP selective inhibitor, obviating the need to perform additional inhibitory confirmation in Caco-2.

The recommendation to perform BCRP substrate assessment in Caco-2 rather than transfected MDCK cells overexpressing BCRP (MDCK-BCRP) is due to the inherent weakness of MDCK-BCRP cells in correctly identifying the more polar BCRP substrates (Elsby 2013; unpublished observations) such as the clinically relevant substrate rosuvastatin and the established *in vitro* substrate estrone 3-sulfate (incidentally the same is also true for the inhibitor sulfasalazine). In both cases, the established substrates exhibit very low B-A $P_{app}$ (~$1 \times 10^{-6}$ cm/s) (and efflux ratios) associated with active transporter-mediated flux due to their presumed limited ability to cross the basolateral membrane of the MDCK cells in order to interact with, and be transported by, BCRP at the apical membrane. In contrast, in Caco-2 cells, these same BCRP substrates exhibit comparatively large B-A $P_{app}$ values (~$15 \times 10^{-6}$ cm/s or ~$50 \times 10^{-6}$ cm/s for rosuvastatin [23] or estrone 3-sulfate (Cyprotex data), respectively) yielding very high efflux ratios in the region of 50–150. The reason why Caco-2 are superior to MDCK-BCRP cells for identifying the more polar clinically relevant BCRP substrate is because of the existence of an uptake transporter OST-alpha on the basolateral membrane, facilitating the movement of rosuvastatin (or estrone 3-sulfate) across the membrane into the cell to allow its efflux by BCRP [91]. Unfortunately, MDCK-BCRP cells do not appear to functionally express this uptake transporter and so pose the risk of producing false negative results for investigational drugs that are substrates of BCRP and have low permeability. However, if the molecules being studied have reasonable permeability (*e.g.* cladribine, topotecan) then the MDCK-BCRP system should be able to correctly identify these as substrates.

With reference to the use of transporter transfected MDCK cell test systems for studying P-gp in bidirectional transport assays, the current FDA DDI guidance [8] suggests that the parental cell line (MDCK) be evaluated alongside the transfected cell line and that the efflux ratio obtained in the latter be corrected for that determined in the former (resulting from endogenous canine P-gp) in order to obtain a net efflux ratio. However, the MDCK parental cell line is not a true background control of human P-gp expressing transfected cells as it is not mock transfected with an empty vector. Furthermore, evidence suggests that endogenous canine transporter expression (P-gp and interestingly SLC transporters) is in fact reduced in the transporter transfected cell line due to the overexpression of human MDR1 when compared to parental MDCK cells [82,92]. This means that the endogenous canine P-gp efflux present in the parental cells will overestimate the endogenous contribution present in the human transporter expressing cells, thereby artefactually lowering the net efflux ratio, which may result in the occasional false negative for those compounds with low transfected cell efflux ratios. For these reasons it is recommended to conduct P-gp transporter substrate identification studies simply in MDCK-MDR1 cells and only if a drug exhibits a low efflux ratio of 2–3, then look at the evidence of efflux from the accompanying BCRP Caco-2 cell study to see whether there is involvement of P-gp. If there was no efflux in Caco-2, or efflux that was abolished with a BCRP inhibitor, then the observed efflux in MDCK-MDR1 could likely be attributed to just canine P-gp and not human P-gp. Interestingly, the down-regulation of endogenous transporters eluded to above in transfected MDCK cells diminishes with ascending cell passage as expression of the human gene wanes and thus could impact interpretation [82]. Whilst a similar phenomenon might theoretically affect endogenous transporter expression in other cell lines (e.g. HEK293) used to overexpress human SLC transporters, in all transfected cell systems any impact can be mitigated against by 1) use of a defined passage range validated to demonstrate constant functional target transport activity (representing constant expression), and 2) use of assay-ready cells frozen at a low

passage number maximizing expression of the target transporter at a constant level, thereby ensuring consistency in any endogenous expression.

As eluded to already, if an investigational drug has limited passive intrinsic membrane permeability then P-gp or BCRP bidirectional transport assays may give rise to false negative results in terms of whether a compound is a substrate. As a rough approximation, a low $P_{app}$ value of $\leq 1 \times 10^{-6}$ cm/s in both directions would typically indicate such a molecule with low intrinsic permeability properties. Therefore, in order to accurately identify whether such a molecule is indeed a transported substrate of efflux transporters then *in vitro* substrate studies must be performed using 'inside-out' membrane vesicle test systems so that the compound has direct access to the intracellular surface of the transporter. But, crucially these same vesicle test systems must not be used for more permeable compounds as the compound would not be trapped within the vesicle lumen for quantification and thus might give rise to false negatives. It is for these reasons that N-methylquinidine and not the more permeable digoxin is used as a P-gp substrate in P-gp vesicle studies [17,93].

#### 2.3.4. Anticipated clinical concentration of investigational drug

In order to make sure that the appropriate concentration level of investigational drug is utilized when performing *in vitro* transporter evaluation it is important to consider not only the location of the interaction site for each specific transporter (e.g. intestinal, hepatic inlet or renal proximal tubule), but the investigational drug's intended route of administration as this could change the dynamic of the concentration(s) chosen for investigation. For oral drug DDI risk assessment, the driving concentrations for interaction potential with P-gp and BCRP, hepatic transporters, or renal transporters are the theoretical intestinal lumen concentration ($[I]_{gut}$), maximum unbound hepatic inlet concentration ($[I]_{in,\ max\ u}$), or unbound maximum plasma concentration at steady state ($[I]_{max\ u}$), respectively (see Section 2.6 for equations).

A drug with a molecular weight of 500 given at doses between 10 and 1000 mg can have an $[I]_{gut}$ ranging from 80 μM to 8000 μM, and assuming a fraction unbound ($f_u$) = 1.0, a fraction absorbed ($F_aF_g$) = 1.0, an absorption rate constant ($k_a$) = 0.1 $min^{-1}$, with liver blood flow rate ($Q_h$) = 1617 mL/min, an $[I]_{in,\ max\ u}$ (without addition of systemic $[I]_{max,}$ *i.e.* unbound portal vein concentration) ranging from 1.24 μM to 124 μM. As such, driving concentration magnitude may significantly exceed the unbound systemic plasma levels required for efficacy. For inhaled or intra-nasal drug DDI risk assessment, the main driving concentration to consider for all transporters will be $[I]_{max\ u}$. However, if there is an ingested part of the dose, then intestinal lumen concentration, or $[I]_{in,\ max\ u}$ concentration arising from the potential absorption of the small swallowed dose, needs to be taken into consideration for intestinal P-gp and BCRP, or hepatic OATP1B1, OATP1B3, and OCT1, respectively. For intravenous administration and other parenteral routes (e.g. subcutaneous, intramuscular) the driving concentration that needs to be considered with all transporters, irrespective of their location (including intestinal P-gp/BCRP which could be impacted from drug entering the enterocyte from the blood side), is circulating free systemic concentration ($[I]_{max\ u}$).

Knowledge of the relevant anticipated concentrations above, coupled with an understanding of the regulatory authority basic static equation thresholds used to determine perpetrator DDI risk potential for a drug (see Section 2.6), allows calculation of the highest concentration level that needs to be tested *in vitro* for inhibition in order to provide adequate 'DDI cover' for an investigational drug. Consequently with oral drugs, for intestinal P-gp/BCRP inhibition, hepatic uptake transporter inhibition, or renal transporter inhibition, highest tested concentrations should be equivalent to (or greater) than 0.1x $[I]_{gut}$, 10x or 25x $[I]_{in,\ max\ u}$, or 10x or 50x $[I]_{max\ u}$, for the current FDA [8] or EMA [9] guidance, respectively. For parenteral routes of administration, the highest tested concentrations would be equivalent to (or greater) than 10x or 50x $[I]_{max\ u}$, respectively.

However, unlike inhibition studies which require a top concentration multiple times above anticipated clinical concentration to ensure perpetrator DDI cover, the concentration range chosen for victim substrate assessment studies of investigational drug simply reflects covering low (non-saturating) concentrations as well as the anticipated clinical concentration appropriate to the transporter under examination.

Once an understanding of the concentration range potentially driving (as perpetrator), or potentially being subjected to (as victim), transporter DDI has been achieved and which associated upper concentration to test, the next step is consideration of WHEN and HOW to perform transporter inhibition, or substrate identification studies, respectively, for an investigational drug under development.

### 2.4. Performing transporter inhibition and substrate screening assays during early to late drug discovery (hit-to-lead and lead optimization phases)

In early drug discovery it is common to have multiple potential drug candidates for the same disease target under evaluation since attrition of these compounds due to unfavourable efficacy (poor pharmacokinetics) or safety (not related to DDI potential) remains high. Consequently, it would not be resource or cost effective, and therefore desirable, to routinely generate *in vitro* inhibition or substrate data against all transporters towards understanding DDI perpetrator or victim potential, respectively, early in drug discovery given that the data would have limited impact for a therapeutic area.

The potential exception to this would be if a specific disease indication (or targeted patient population) had a critical co-medication as defined in Section 2.1, for example due to its use as a standard of care which cannot be excluded in the clinic, or because it would be unfavourable to exclude for drug labelling claims. In instances of a critical victim co-medication, it may be deemed necessary by project teams to conduct early *in vitro* inhibition studies specifically against the clinically relevant transporter for which being a substrate of is important to the disposition (and thus victim DDI potential) of the co-medication. Conversely, where there is a critical perpetrator co-medication, early *in vitro* substrate studies targeted against

the transporter at risk of clinical DDIs perpetrated by the co-medication might be considered.

### 2.4.1. Early *in vitro* inhibition studies

In order to discriminate between multiple candidates within a chemical series based on their transporter inhibitory liabilities with the view of taking the most favourable (least inhibiting) forward, the concentration and the number of concentrations to be tested (*e.g.* 1 or 2 giving % inhibition, or multiple to generate an $IC_{50}$) need to be optimized on a per transporter basis according to its tissue location.

For renal transporters, as the driving concentration of perpetrator for DDI is the unbound maximum plasma concentration and typically low (less than micromolar), then it is likely to be possible to rank and thus discriminate candidates based on % inhibition results using either a single concentration (*e.g.* 1 µM) or two concentration (*e.g.* 1 and 10 µM) format screening assay with concentrations of 1 µM or 1 and 10 µM; potentially fulfilling the 10 or 50x higher regulatory criteria for any future exposure prediction in human.

In contrast, for hepatic uptake transporters with oral drugs the driving unbound maximum (portal vein plus systemic $[I]_{max}$) hepatic inlet concentration can be large depending on dose and extent of human plasma protein binding. In the absence of any predicted plasma exposure, Figure 7 depicts the relationship between dose, plasma protein binding (expressed as $f_u$), minimum unbound hepatic inlet concentration (without addition of $[I]_{max}$ *i.e*, portal vein concentration) and subsequent DDI risk for inhibition of hepatic transporters such as OATP1B1/1B3 and OCT1 using FDA criteria and differing hypothetical inhibitory potencies ($IC_{50}$ values). Across all potential therapeutic doses (10–1000 mg) it is clear that an $IC_{50} \geq 10$ µM and low unbound exposure helps alleviate its DDI risk as a hepatic uptake transporter inhibitor. Furthermore, even with a reduced $IC_{50}$ of 1 µM and low unbound exposure, only doses around 100 mg or greater would be predicted to perpetrate a DDI. A drug candidate with more moderate unbound exposure would be predicted to perpetrate a hepatic transporter DDI essentially at all doses if it had a determined $IC_{50}$ of 1 µM, at doses ≥ 100 mg with an $IC_{50}$ of 10 µM, and essentially only doses above approximately 300 mg with an $IC_{50}$ of 50 µM (Figure 7). In the extreme, worst case scenario where a drug has high unbound exposure then an $IC_{50}$ of 10 µM or below would be predicted to cause DDI across all anticipated therapeutic doses and even a relatively 'weak' $IC_{50}$ of 50 µM would still cause DDI through inhibition of transporters at all doses above 30 mg (Figure 7). Consequently, it might be possible to rank and discriminate compounds based on hepatic uptake transporter (*e.g.* OATP1B1) inhibition provided a single concentration greater than 1 µM, or a larger concentration interval between two concentrations (*e.g.* 1 and 100 µM), is chosen when testing. Where a series of oral drug candidates all demonstrate high unbound exposure, then it will be more challenging to rank compounds when assessing hepatic transporter inhibition and thus an alternative 'all or nothing' approach for discriminating between them could be adopted, *i.e.* inhibition versus no inhibition at a single high test concentration.

For intestinal efflux transporters P-gp and BCRP, the driving perpetrator concentration is theoretical intestinal lumen concentration and, assuming it is soluble in intestinal fluid, this can be as high as the millimolar range depending upon dose.

**A: $IC_{50}$ = 1 µM**

$f_u$ = 1.0

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 2.06 | 1.55 | 1.24 | 1.03 |
| 30 | 6.18 | 4.64 | 3.71 | 3.09 |
| 100 | 20.6 | 15.5 | 12.4 | 10.3 |
| 300 | 61.8 | 46.4 | 37.1 | 30.9 |
| 1000 | 206 | 155 | 124 | 103 |

$f_u$ = 0.1

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 0.206 | 0.155 | 0.124 | 0.103 |
| 30 | 0.618 | 0.464 | 0.371 | 0.309 |
| 100 | 2.06 | 1.55 | 1.24 | 1.03 |
| 300 | 6.18 | 4.64 | 3.71 | 3.09 |
| 1000 | 20.6 | 15.5 | 12.4 | 10.3 |

$f_u$ = 0.01

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 0.0206 | 0.0155 | 0.0124 | 0.0103 |
| 30 | 0.0618 | 0.0464 | 0.0371 | 0.0309 |
| 100 | 0.206 | 0.155 | 0.124 | 0.103 |
| 300 | 0.618 | 0.464 | 0.371 | 0.309 |
| 1000 | 2.06 | 1.55 | 1.24 | 1.03 |

$R = 1 + [I]_{in,\ max\ u} / IC_{50} (\cong K_i) < 1.1$ No DDI risk  $R = 1 + [I]_{in,\ max\ u} / IC_{50} (\cong K_i) \geq 1.1$ DDI risk

**B: $IC_{50}$ = 10 µM**

$f_u$ = 1.0

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 2.06 | 1.55 | 1.24 | 1.03 |
| 30 | 6.18 | 4.64 | 3.71 | 3.09 |
| 100 | 20.6 | 15.5 | 12.4 | 10.3 |
| 300 | 61.8 | 46.4 | 37.1 | 30.9 |
| 1000 | 206 | 155 | 124 | 103 |

$f_u$ = 0.1

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 0.206 | 0.155 | 0.124 | 0.103 |
| 30 | 0.618 | 0.464 | 0.371 | 0.309 |
| 100 | 2.06 | 1.55 | 1.24 | 1.03 |
| 300 | 6.18 | 4.64 | 3.71 | 3.09 |
| 1000 | 20.6 | 15.5 | 12.4 | 10.3 |

$f_u$ = 0.01

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 0.0206 | 0.0155 | 0.0124 | 0.0103 |
| 30 | 0.0618 | 0.0464 | 0.0371 | 0.0309 |
| 100 | 0.206 | 0.155 | 0.124 | 0.103 |
| 300 | 0.618 | 0.464 | 0.371 | 0.309 |
| 1000 | 2.06 | 1.55 | 1.24 | 1.03 |

$R = 1 + [I]_{in,\ max\ u} / IC_{50} (\cong K_i) < 1.1$ No DDI risk  $R = 1 + [I]_{in,\ max\ u} / IC_{50} (\cong K_i) \geq 1.1$ DDI risk

**C: $IC_{50}$ = 50 µM**

$f_u$ = 1.0

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 2.06 | 1.55 | 1.24 | 1.03 |
| 30 | 6.18 | 4.64 | 3.71 | 3.09 |
| 100 | 20.6 | 15.5 | 12.4 | 10.3 |
| 300 | 61.8 | 46.4 | 37.1 | 30.9 |
| 1000 | 206 | 155 | 124 | 103 |

$f_u$ = 0.1

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 0.206 | 0.155 | 0.124 | 0.103 |
| 30 | 0.618 | 0.464 | 0.371 | 0.309 |
| 100 | 2.06 | 1.55 | 1.24 | 1.03 |
| 300 | 6.18 | 4.64 | 3.71 | 3.09 |
| 1000 | 20.6 | 15.5 | 12.4 | 10.3 |

$f_u$ = 0.01

| Dose (mg) | Minimum unbound hepatic inlet concentration (µM) (without $C_{max}$) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 0.0206 | 0.0155 | 0.0124 | 0.0103 |
| 30 | 0.0618 | 0.0464 | 0.0371 | 0.0309 |
| 100 | 0.206 | 0.155 | 0.124 | 0.103 |
| 300 | 0.618 | 0.464 | 0.371 | 0.309 |
| 1000 | 2.06 | 1.55 | 1.24 | 1.03 |

$R = 1 + [I]_{in,\ max\ u} / IC_{50} (\cong K_i) < 1.1$ No DDI risk  $R = 1 + [I]_{in,\ max\ u} / IC_{50} (\cong K_i) \geq 1.1$ DDI risk

Figure 7. Relationship between dose, plasma protein binding, unbound maximum hepatic inlet concentration $[I]_{in,\ max\ u}$ (derived assuming a fraction absorbed ($F_aF_g$) = 1.0, an absorption rate constant ($k_a$) = 0.1 $min^{-1}$, a blood-to-plasma concentration ratio = 1, with liver blood flow rate ($Q_h$) = 1617 mL/min, and without $[I]_{max}$ addition, *i.e. portal vein concentration*) and subsequent DDI risk.

As a result there is limited value in evaluating drug candidates as inhibitors of these transporters at low concentrations of 1 or 10 µM as any observed inhibition equivalent to an $IC_{50}$ value of 10 µM or below would be predicted to cause an intestinal DDI for essentially all anticipated therapeutic doses (Figure 8). In contrast, inhibitory potential equivalent to an $IC_{50}$ of 100 µM would cause DDI at doses of 100 mg and above, whilst inhibitory potential equivalent to an $IC_{50}$ of 300 µM would only cause DDI at doses of 300 mg upwards (Figure 8). Indeed, $IC_{50}$ values as high as 63 or 75 µM versus BCRP for certain perpetrator drugs have been shown to cause clinically significant DDIs with BCRP victim substrate drugs such as rosuvastatin [23]. Consequently, there is limited scope in performing single or two concentration inhibition screens to rank or discriminate drug candidates. Instead, perhaps there is more value in conducting an $IC_{50}$ profiling assay for P-gp and BCRP inhibition assessment in order to contextualize any observed inhibition with a range of possible theoretical clinical doses to understand how the DDI risk might change in relation to a future chosen dose ('managing the baggage'). Additionally, where a series of oral drug candidates are all anticipated to require high doses to achieve efficacy, then it would be prudent to test all with a maximum concentration of 300 µM and discriminate based on 'no inhibition' as testing up to this level would give DDI cover (mitigate risk) to any potential dose up to 400 mg.

The aim of early screening assay results in general is to inform on changes in the chemistry of molecules needed in order to remove specific liabilities as part of a design, make, test and analyse cycle [94]. Some progress has been made towards trying to understand structure-activity relationships for inhibition of OATP1B1 and BCRP which may provide useful guidance for early discovery teams [95,96]. However, as there are limited structure-activity relationships available in the literature for inhibition of transporters more broadly, then the majority of early transporter inhibition screening assays in drug discovery will likely be primarily focussed on simply 'managing the baggage' for candidates in terms of transporter DDI risk towards a critical co-medication. Perhaps only if the critical victim co-medication had a narrow therapeutic index would the relevant transporter inhibition data become decision making, such that molecules with no observed inhibition at high concentrations would be prioritized over other candidates even if their non-transporter properties were less favourable when compared to the other molecules in the series.

### 2.4.2. Early *in vitro* substrate studies

Studies would only be needed if the chemical series exhibits low intrinsic passive membrane permeability thereby requiring transport to enter cells. No early substrate studies would be required if the molecules have high passive permeability (BCS class I), instead such studies could be deferred to later in development. But where required, in order to discriminate between multiple candidates within a chemical series based on their transporter substrate liabilities with the view of taking the most favourable (non-transported) forward, it is important to establish what concentration and the number of concentrations that need to be tested (*e.g.* 1 or 2). These will depend on the specific transporter under consideration and its tissue location.

For identifying drugs as substrates, the chosen tested concentration needs to be sufficiently low enough to avoid saturating the transporter, when passive diffusion processes dominate, and could therefore potentially give rise to false negative results. This is usually only a concern for ABC transporters whose substrates tend to have lower $K_m$ values, as SLC transporter substrates tend to typically have sub-millimolar $K_m$ values for transport (although statin $K_m$ values for OATP1B1 are low, around 10 µM [85]). At the same time as trying to avoid saturation of the transporter, the tested concentration needs to be high enough so as not to compromise on bioanalytical method sensitivity and risk not being able to quantify

$IC_{50}$ = 1 µM

| Dose (mg) | Theoretical Intestinal Lumen concentration (µM) (dose in mol/250mL) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 133 | 100 | 80 | 67 |
| 30 | 400 | 300 | 240 | 200 |
| 100 | 1333 | 1000 | 800 | 667 |
| 300 | 4000 | 3000 | 2400 | 2000 |
| 1000 | 13333 | 10000 | 8000 | 6667 |

$[I]_{gut} / IC_{50} (\cong K_i) < 10$ No DDI risk $[I]_{gut} / IC_{50} (\cong K_i) \geq 10$ DDI risk

$IC_{50}$ = 10 µM

| Dose (mg) | Theoretical Intestinal Lumen concentration (µM) (dose in mol/250mL) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 133 | 100 | 80 | 67 |
| 30 | 400 | 300 | 240 | 200 |
| 100 | 1333 | 1000 | 800 | 667 |
| 300 | 4000 | 3000 | 2400 | 2000 |
| 1000 | 13333 | 10000 | 8000 | 6667 |

$[I]_{gut} / IC_{50} (\cong K_i) < 10$ No DDI risk $[I]_{gut} / IC_{50} (\cong K_i) \geq 10$ DDI risk

$IC_{50}$ = 100 µM

| Dose (mg) | Theoretical Intestinal Lumen concentration (µM) (dose in mol/250mL) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 133 | 100 | 80 | 67 |
| 30 | 400 | 300 | 240 | 200 |
| 100 | 1333 | 1000 | 800 | 667 |
| 300 | 4000 | 3000 | 2400 | 2000 |
| 1000 | 13333 | 10000 | 8000 | 6667 |

$[I]_{gut} / IC_{50} (\cong K_i) < 10$ No DDI risk $[I]_{gut} / IC_{50} (\cong K_i) \geq 10$ DDI risk

$IC_{50}$ = 300 µM

| Dose (mg) | Theoretical Intestinal Lumen concentration (µM) (dose in mol/250mL) | | | |
|---|---|---|---|---|
| | MW300 | MW400 | MW500 | MW600 |
| 10 | 133 | 100 | 80 | 67 |
| 30 | 400 | 300 | 240 | 200 |
| 100 | 1333 | 1000 | 800 | 667 |
| 300 | 4000 | 3000 | 2400 | 2000 |
| 1000 | 13333 | 10000 | 8000 | 6667 |

$[I]_{gut} / IC_{50} (\cong K_i) < 10$ No DDI risk $[I]_{gut} / IC_{50} (\cong K_i) \geq 10$ DDI risk

Figure 8. Relationship between dose, theoretical intestinal concentration [I]gut (derived assuming fraction unbound gut = 1.0 [1]) and subsequent DDI risk.

samples, making any substrate assessment inconclusive. It is for these reasons in early discovery high-throughput screening assays that a drug concentration of 1 or 10 µM is typically chosen when studying efflux transporters, but this might be too low for observing good uptake with some SLC transporters, so perhaps higher concentrations of 50 or 100 µM might need consideration for certain low affinity high capacity uptake transporters. If single concentration data indicate candidates in a series to be substrates of a transporter then the next question is how to discriminate between compounds to select the ones to take forward. Unfortunately, it is not possible to discriminate based on the numerical values of the determined efflux/uptake ratios of candidates as such values do not inform on whether a molecule is a 'better substrate' than another. This is because the different molecules might have different $K_m$ values for the transporter and so could be in varying states of transporter saturation, meaning that the outcome could look quite different across the candidates if they were re-tested at a lower or higher concentration. Perhaps the only way to discriminate between candidates when using single concentration data might therefore be evidence of substrate versus non-substrate classification.

Conversely, the use of two concentration levels for assessing transporter substrate status might prove beneficial in such early studies as whilst the first concentration can remain sufficiently low for the reasons given above, the second concentration level could be sufficiently high to reflect anticipated *in vivo* concentrations of investigational drug at the site of interaction. For renal transporters as the drug will interact at its unbound maximum plasma concentration, then a 10x higher second concentration might be valuable to study. For hepatic uptake transporters, or for intestinal transporters, exposed to the unbound hepatic inlet concentration, or intestinal luminal concentration, respectively, it might be prudent to cover a second concentration level that is 10–50x, or 50–100x higher than the lowest level. With this screening format, whilst all candidates might be classified as substrates based on the first concentration level, it is likely that the more clinically relevant second higher concentration level becomes discriminatory as there may be a mix of 'substrate' and 'non-substrate' activities. The latter indicating for those affected compounds that at potentially relevant *in vivo* concentrations their transport would be saturated and as such they would not be a victim of a DDI should that transporter be inhibited. Such an approach in drug discovery will help to 'manage the baggage' for candidates in terms of their transporter victim DDI risk towards a critical perpetrator co-medication. Of course, rather than managing the transporter substrate liability of candidates, another option open to project teams might be to change the chemistry of the molecules in order to increase their lipophilicity and therefore passive permeability such that they cease to be transported substrates. Obviously, this would not be an option if being an uptake transporter substrate was favourable because it enabled selective delivery to the target pharmacodynamic organ (e.g. liver in the case of statins).

## 2.5. Performing regulatory transporter inhibition profiling ($IC_{50}$) and substrate identification profiling assays during late drug discovery/early drug development through to late stage drug development and regulatory submission *(preclinical development candidate selection onwards to new drug application)*

### 2.5.1. *In vitro* inhibition ($IC_{50}$) profiling studies

Profiling concentration-dependent inhibition of transporters to obtain an $IC_{50}$ is usually performed around preclinical development candidate selection to assess the DDI perpetrator liabilities between two or three shortlisted drug candidates with a view of taking one forward into early drug development with minimal 'baggage' based on formal regulatory DDI risk assessment of the *in vitro* $IC_{50}$ value (see Section 2.6). DDI risk assessment at this stage will utilize predictions of dose and unbound plasma exposure, which will be revisited further into development when actual clinical exposure is available. Collectively, these will inform the clinical development plan around allowable co-medications and what potential follow-up clinical interaction studies will be needed during clinical development. In order to facilitate these important decisions, determined $IC_{50}$ values need to be robust and therefore generated using well validated *in vitro* test systems and methods (*e.g.* using linear conditions for incubation time and where appropriate, vesicle protein amount) [18,19,21,97]. Furthermore, $IC_{50}$ determinations, rather than single concentration assessments, are always recommended as they future-proof projects against any unexpected increases in dose (and subsequent exposure) once in the clinic and actual, rather than predicted, exposure data are available. In contrast, (percentage) inhibition data from a single concentration may become invalid if the concentration studied is no longer high enough to give DDI cover when dose increases (especially if no inhibition of transport was initially observed). The choice of which transporters (*i.e.* all regulatory required, or a select few) to study at the preclinical development candidate selection stage will depend on specific project team needs and the existence of critical co-medications for the specific disease indication.

#### 2.5.1.1. *Important assay design considerations for correct determination of $IC_{50}$*

*2.5.1.1.1. Probe substrate concentration.* For transporter inhibition $IC_{50}$ determination, in line with FDA regulatory guidance recommendations, it is industry-wide practice to use a probe substrate concentration in the incubation that is much lower (five to ten times) than its transport affinity constant (Michaelis-Menten kinetic constant, $K_m$) for the transporter such that (assuming inhibition is competitive) the determined $IC_{50}$ value is equivalent to the absolute inhibition constant $K_i$ [98]. However, why is this recommendation important? It is important because in cell-based transporter test systems it is either not possible, or extremely difficult, to determine a $K_i$ value for a transporter using the traditional experimental approach of assessing a range of inhibitor concentrations [*e.g.* 0.1x, 0.25x, 0.5x, 1x, 2.5x and 5x estimated $K_i$] over multiple probe substrate concentrations [*e.g.* 0.3x, 1x, 2x, 4x and 6x $K_m$] that would be routinely taken for drug metabolizing enzymes such as cytochrome P450s. The reason for this is, in

the absence of inhibitor, as the probe substrate concentration approaches and exceeds multiples of its $K_m$ value, then transporter-mediated processes saturate and passive diffusion of the probe substrate surpasses the active transport to become the predominant driver of movement across the cell membrane, thus making it 'appear' that the transporter has been inhibited when in fact this is not the case (contrast this to enzymes where the formation of a metabolite is constant once the enzyme is saturated and therefore can only disappear with true inhibition of formation by an inhibitor). As a result it is very difficult to evaluate the effect of inhibitor at high (saturating) probe substrate concentrations because there is effectively no active transport to inhibit, therefore making it almost impossible to experimentally determine an accurate $K_i$ value for transporters. Hence this is the reason why the industry-wide practice for transporter inhibition assessment is to use a [probe substrate] $\lll< K_m$ so that mathematically the experimentally determined $IC_{50}$ equates to the $K_i$ ensuring robust DDI risk assessment.

*2.5.1.1.2. Inclusion of inhibitor pre-incubation step for all transporters.* Early studies investigating the inhibitory potential of cyclosporine on the OATP1B1-mediated transport of probe substrate atorvastatin [99], or of the anthraquinone emodin on the OAT3-mediated transport of probe substrate Fluo [100], showed a 'time-dependent' left shift (reduction of approximately 20- or >2.4-fold, respectively) in determined $IC_{50}$ following a pre-incubation step with the inhibitor compared to just co-incubation alone. Data generated within Cyprotex in 2015 and 2016, and subsequently presented as accepted conference abstracts/posters at the AAPS Drug Transporter Workshops those same years, supported the 'pre-incubation' shift in $IC_{50}$ for cyclosporine versus OATP1B1 and collectively demonstrated that the magnitude of fold-shift was reduced when a time-matched buffer equilibration period was used for the 'no pre-incubation condition' (2-fold decrease when time-matched versus a 3.6-fold decrease when the buffer equilibration period was 10 min and the pre-incubation period with inhibitor was 30 min) [101,102]. Furthermore, the same investigations evaluated the effect of pre-incubation only (no co-incubation), co-incubation only (after time-matched buffer equilibration) and combined pre-incubation and co-incubation on the inhibitory potencies of cyclosporine, atorvastatin and clarithromycin versus OATP1B1-mediated estradiol 17β-glucuronide transport, and demonstrated that the $IC_{50}$ obtained for cyclosporine was driven by the pre-incubation mechanism only, for atorvastatin by the combination of both pre-incubation and co-incubation mechanisms and for clarithromycin that pre-incubation had no impact on the $IC_{50}$ generated. This led us to hypothesize that so-called 'time-dependent' inhibition was not a biological phenomenon related to the transporter *per se*, but rather an '*in vitro* artefact' related to the intrinsic physicochemical properties (*i.e.* passive membrane permeability) of the inhibitor and whether it needs sufficient time experimentally to be able to access intracellularly in order to exert its mechanism of inhibition. This artefactual *in vitro* phenomenon is particularly an issue when using HEK293 SLC transporter-expressing cell systems for transporter inhibition assessments as these typically utilize very short incubation times (<5 min) which may therefore impede the occurrence of any intracellular (trans-) inhibition effect. Consequently, from 2016 (ahead of draft regulatory guidance recommendations introduced in 2017) Cyprotex incorporated a 15-min pre-incubation step with investigational drug for all SLC transporters (not just OATP1B1 and OATP1B3 as suggested in current final 2020 guidance) in order to ensure that the correct value for $IC_{50}$ was obtained. This approach avoids potentially underestimating both the determined $IC_{50}$ value and subsequent DDI risk assessment which would occur if a pre-incubation step was not performed and the investigational drug needed to exert an effect at an intracellular site of the transporter as part of its mechanism of inhibition.

The recent elegant studies of Shitara and Sugiyama (2017) [103], Taguchi *et al*. (2019) [104], Panfen *et al*. (2019) [105] and Tátrai *et al*. (2019) [106] have all aided to further the understanding of the underlying mechanisms behind the artefactual *in vitro* phenomenon that arises with pre-incubation during the conduct of SLC transporter inhibition assays. For instance, Shitara and Sugiyama (2017) [103] attributed the $IC_{50}$ shift caused by cyclosporine on OATP1B1 to be a consequence of cyclosporine mediating trans-inhibition of the transporter from the intracellular compartment and that the 'pre-incubation' step allows the time needed experimentally for cyclosporine to accumulate inside the cell to a concentration level that can achieve this effect. The study by Panfen *et al*. (2019) [105] demonstrated that this trans-inhibitory effect of cyclosporine also applied to its mechanism of inhibition of OCT1-mediated probe substrate transport, for which a 50-fold decrease in $IC_{50}$ was observed against metformin following a 30 min pre-incubation with cyclosporine compared to just co-incubation alone, consistent with a measured cellular accumulation of cyclosporine during pre-incubation. Taguchi *et al*. (2019) [104] showed that when the vehicle buffer equilibration and inhibitor pre-incubation step were time-matched, then the maximum effect of pre-incubation time on the determined $IC_{50}$ for both pazopanib and cyclosporine was achieved after only a 5-min pre-incubation period and that the determined shifted (~5-fold) $IC_{50}$ values were identical after 5, 30 or 60 min pre-incubation times. This supports the in-house unpublished data generated within Cyprotex in 2018, which was presented as an accepted conference abstract/poster at the AAPS Drug Transporter Workshop (2018), demonstrating there was no difference in determined $IC_{50}$ value for cyclosporine versus OATP1B1 following either a 15-min or 30-min pre-incubation step [107]. Confirming that they were equivalent allowed use of the shorter 15-min pre-incubation time routinely, giving the added benefit that it may also lessen any impact of potential cytotoxic effects during cell incubations. Finally, the study of Tátrai *et al*. (2019) [106] looked at all regulatory SLC transporters and showed that the increased $IC_{50}$ potency achieved following pre-incubation correlated with the inhibitor reaching intracellular steady state concentrations in order to exert a (trans) inhibitory effect and that this was partly determined by the physicochemical properties (*e.g.*

lipophilicity and therefore passive permeability) of the inhibitor drug. Furthermore, the authors noted that occurrence of $IC_{50}$ shifts were related to assay duration, *i.e.* the shorter the co-incubation time with probe substrate, the larger any potential shift following pre-incubation with certain inhibitors was seen [106]. This supports the notion of 'time-dependency' being an artefactual *in vitro* phenomenon because with short co-incubations (<5 min) there simply is not enough time for inhibitor to accumulate inside the cell to exert any trans-inhibition, if this is required as part of its inhibitory mechanism at the transporter under investigation.

Consequently, for robust transporter DDI risk assessment that is relevant to the clinical situation, all *in vitro* SLC transporter inhibition assessments of an investigational drug should include a pre-incubation step of sufficient duration (*e.g.* 15 min) as standard methodology in order to ensure that the correct $IC_{50}$ value is determined right first time. This recommendation also applies to ABC efflux transporters studied in polarized cell monolayers as, despite a much longer incubation time, unpublished in-house data suggested a tendency towards a lowered $IC_{50}$ value versus P-gp following pre-incubation, the magnitude of which depended on inhibitor, so it would be prudent to err on the side of caution and include a pre-incubation as standard [107]. Additionally, for DDI risk assessment there is no routine requirement for comparison purposes to investigate inhibition potential in the absence of an inhibitor pre-incubation step in parallel. Rather, such an optional comparative study may simply be desired by a project team late on in drug development if it was perhaps deemed helpful to understand the potential mechanism of inhibition (i.e. *cis* versus *trans*) when developing a physiologically-based pharmacokinetic (PBPK) model for the drug.

*2.5.1.1.3. Whether it is necessary to assess non-specific binding/recovery or cytotoxicity of the investigational drug for $IC_{50}$ assessments.* Non-specific binding of investigational drug to plasticware and/or cells within an assay could impact on the accuracy of the determined $IC_{50}$ value were it to occur and nominal incubated concentrations are used for curve fitting. Additionally, non-specific binding might lower the top incubated concentration such that if no inhibition was observed this might no longer provide the DDI cover needed if it went below the required order of magnitude above the anticipated clinical interaction site concentration for the expected dose. Effects of non-specific binding can be reduced experimentally by either 1) pre-treating the multi-well plasticware with a bovine serum albumin buffer solution (*e.g.* 1% w/v) in order to coat surfaces and mask any non-specific binding sites prior to incubating with investigational drug in fresh assay buffer, or 2) co-incubating bovine serum albumin (*e.g.* 0.1 or 1% w/v) in the assay incubation solutions containing investigational drug. Both approaches should act to improve investigational drug recovery and retain incubation concentrations as nominal. However, the requirement to perform assessment of non-specific binding of investigational drug only applies to those studies in which transporter $IC_{50}$ assays are routinely being performed as co-incubations only without an inhibitor (investigational drug) pre-incubation step.

In contrast, for those routine ABC and SLC transporter $IC_{50}$ assays that incorporate an inhibitor pre-incubation step as standard (*i.e.* pre-incubating the cells with a range of concentrations of investigational drug and removing prior to the co-incubation with probe substrate and a fresh range of concentrations of investigational drug solution), then there is no scientific requirement to assess non-specific binding as any effect would be anticipated to be corrected for and deemed negligible, and thus would not impact on the accuracy of the determined $IC_{50}$ value. The rationale for this recommendation being that any non-specific binding sites on plasticware/cells would be saturated by investigational drug during the pre-incubation phase such that once the pre-incubation solution was removed, and immediately replaced with freshly prepared concentrations of investigational drug solution for the co-incubation, then those non-specific binding sites are masked (hidden) and so the freshly prepared concentrations of investigational drug are not subject to any binding in the incubation and thus remain as nominal.

Other factors that could influence determination of $IC_{50}$ and therefore might require consideration include potential chemical instability or cytotoxicity of the investigational drug. However, these factors are not deemed to be a concern for routine SLC transporter $IC_{50}$ assays for which the maximum overall incubation (pre-incubation plus co-incubation) time is typically less than 20 min duration and therefore too short to be of any real concern to assay output. Indeed if a molecule was completely degraded, or so toxic that it could completely kill cell test systems, within such a short time frame, then in reality the project team have more pressing critical factors to address towards that compound's development prior to conducting any transporter interaction assessments. Despite that sentiment, with respect to cytotoxicity, it is possible to detect potential cytotoxicity issues caused by investigational drug in HEK293 SLC transporter transfected cell systems during the conduct of an assay as it manifests as a concentration-dependent decrease in determined cellular protein at the higher concentration level(s). This allows assessment of whether to exclude specific impacted concentration levels from curve fitting to ensure the right data is used to derive a correct $IC_{50}$, thereby removing the need to evaluate cytotoxicity separately. In the same regard, any potential cytotoxic effects of investigational drug can be indirectly ascertained within P-gp and BCRP transporter polarized cell monolayer $IC_{50}$ assays by use of the co-incubated cell monolayer integrity marker and whether it exceeds predefined $P_{app}$ thresholds that could indicate a compromised monolayer. With regard to chemical instability, as polarized cell monolayer assays for assessing P-gp and BCRP inhibition are typically performed for longer incubation durations (*e.g.* 90 min) then it might

be possible for a compound to undergo a degree of degradation. It would therefore be a sensible approach to evaluate P-gp and BCRP substrate assessments in parallel to any corresponding $IC_{50}$ assessment as experimental determination of investigational drug mass balance (recovery) in the former can be used to inform on the likelihood of chemical instability affecting the results of the latter, and therefore any subsequent impact on interpretation of inhibition, or lack of inhibition, as appropriate.

#### 2.5.1.2. Timings for performing $IC_{50}$ studies along the value chain.

The potential timings around when to perform $IC_{50}$ studies will often depend on the disease indication and associated critical victim co-medications in accordance with the clinical development plan. They will also be influenced by the commercial development plan; for instance completing an *in vitro* DDI package to make the project attractive for partnering its clinical development, or for selling it off as an asset in order to reinvest in R&D.

One approach might be to perform inhibition studies against all regulatory required transporters during preclinical development as part of the investigational new drug (IND) application prior to human studies in order to inform the future clinical protocol around allowable co-medications and what clinical interaction studies need to be planned for. This approach will be particularly applicable for disease indications such as oncology for which 'first time in human' Phase 1 studies are actually directly in patients on a background of co-medications and therefore at risk of DDI, rather than in healthy volunteers which is the norm for the majority of therapy areas.

A second approach that could be more resource and cost-effective but more risky for those projects whose 'first time in human' Phase 1 studies are in healthy volunteers might be to defer performing transporter $IC_{50}$ studies until the read out from the Phase 1 study confirms that the investigational drug has sufficient human exposure and acceptable pharmacokinetics and will therefore progress into patient studies. The regulatory required transporter inhibition studies have to then be performed in time before 'first time in patient' studies in order to finalize the clinical protocol with respect to the list of co-medications and required clinical interaction studies. If the clinical development timelines are fast then the time interval between the end of healthy volunteer studies and the deadline for finalizing the clinical protocol prior to the start of patient studies could be tight and it is therefore possible that the *in vitro* transporter inhibition studies might end up on the critical path. Consequently, it may be useful when deciding on timings to weigh up the risk (including cost) versus the benefit of performing the inhibition studies upfront at IND prior to human studies. The resulting benefit by doing so is the ability to inform on the clinical protocol early thereby avoiding any potential future delays to progression, versus the risk that the data generated becomes surplus to requirements if the drug fails in human Phase 1 trials.

A third approach that might be undertaken as a compromise would be to mitigate transporter DDI risk versus common co-medications such as statins and the narrow therapeutic index co-medication digoxin, for which increased exposures relate to their toxicities, earlier in the drug development process in order to aid preclinical development candidate selection during Lead Optimization by understanding DDI liability 'baggage' when short-listing. The early transporter inhibition ($IC_{50}$ determination) studies that would subsequently be conducted for this purpose include P-gp (digoxin risk), BCRP (statin risk: rosuvastatin, atorvastatin, fluvastatin [74]) and OATP1B1 (statin risk). The remaining regulatory required transporter inhibition studies (OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1 and MATE2-K) for the chosen investigation drug candidate taken forward into preclinical development would then be performed at an appropriate timing as discussed above prior to clinical studies in patients.

A final approach might to defer all the regulatory required transporter inhibition studies until very late development in time for regulatory filing at NDA. Crucially however, this would only apply in the unlikely event that there were no co-medications in use in the target patient population such that a clinical risk of DDI did not exist and that the investigational drug would never be repurposed for treating other diseases in patients prescribed co-medications (otherwise this could significantly delay any repurposing).

### 2.5.2. *In vitro* substrate identification profiling studies

During the regulatory development of a drug, in order to evaluate the *in vitro* substrate potential for a particular transporter, the bidirectional transport, or uptake, of a range of concentrations of investigational drug are determined in one of the appropriate test systems described earlier (Section 1.1.3) for ABC efflux transporters (P-gp, BCRP), or uptake transporters (SLCs), respectively. The concentrations chosen need to span at least a 100-fold range (solubility permitting) and ideally be covering some therapeutically relevant concentrations such as unbound maximum plasma concentration, or 0.1x theoretical intestinal lumen concentration. For the FDA, three concentrations are required for study [108], whereas the EMA currently recommend four concentrations [9], so a default concentration range of 1, 10, 50 and 100 μM would be prudent covering both regulatory guidance. It is necessary to conduct substrate studies over a large concentration range and with a number of concentration levels in order to allow evaluation of any concentration-dependence in drug transport, which might have implications for interpretation. Typically for P-gp and BCRP polarized cell monolayer experiments, incubations are performed at a single time point that would be considered linear for transport (*e.g.* 90 min), whereas for SLC transporters, incubations are often performed over two time points (e.g. 2 and 20 min, with the former considered to be linear) to look for any evidence of time-dependence. Based on current regulatory DDI guidance, an investigational drug is classified as a substrate of a transporter if its efflux/uptake ratio determined at a low (non-saturating) test concentration is ≥2 and importantly, that this observed efflux/uptake transport activity is inhibitable by a known reference inhibitor at a concentration 10x its $K_i$ to ensure 100% inhibition of the transporter in question, thereby confirming that the investigational drug is indeed a substrate *in vitro*. For P-gp and BCRP assessment in polarized cell monolayers, the FDA guidance mentions that flux should be inhibited by the reference inhibitor by

≥50% and suggests that this is a 50% reduction in efflux ratio or a reduction in flux to unity. For SLC transporter substrate assessment, the FDA refers to a requirement of the reference inhibitor to decrease a drug's uptake to ≤50%, but doesn't explicitly confirm whether this is 'uptake ratio' or 'transporter cell uptake rate (pmol/mg)' [8]. However, it is important to remember that any 'apparent' reduction of efflux ratio or uptake ratio in the presence of reference inhibitor should not be taken in isolation as conclusive proof of substrate status. Critically, it should be evaluated alongside scrutiny of whether there is an accompanying reduction in the B-A $P_{app}$ (*i.e*. the direction of efflux transporter flux) or transporter-expressing cell derived uptake rate (pmol/mg) of investigational drug by the reference inhibitor in order to definitively confirm a compound as a substrate. This is crucial, as any experimental variability (*e.g*. causing an increase within twofold) in the more sensitive, typically low valued A-B $P_{app}$ or vector control cell uptake rate, without any observed decrease in the accompanying 'transporter-related' B-A $P_{app}$ or transporter-expressing cell uptake rate with reference inhibitor, respectively, would artefactually halve the efflux/uptake ratio. This would give the appearance that an investigational drug was a substrate based on regulatory criteria (*i.e*. reduction of 50%), whereas in fact it would be incorrect and be a false positive.

Regulatory *in vitro* transporter substrate identification studies can either be performed with radiolabeled test drug (ideally tritium, but more commonly carbon-14) with quantification by liquid scintillation counting, or with unlabeled test drug typically using liquid chromatography tandem mass spectrometry (LC-MS/MS) for quantification. With the former study type, tritium label is preferred due to having high specific activity allowing the use of low substrate concentrations without compromising on analytical sensitivity, whereas the limitation of carbon-14 labels are the low specific activity meaning that it is more difficult to achieve low substrate concentrations in assays, which could pose the risk of saturation for certain ABC-transporters. With the latter study type, for *in vitro* DMPK studies in general there is no requirement for sponsors to conduct a formal bioanalytical method validation for the test drug, such as that which would be required for formal good laboratory practice preclinical toxicology bioanalytical studies. The reason why formal method validation is surplus to requirements is because samples are generated fresh on the day of an experiment and subsequently all analysed on the day of the experiment (not in batches over multiple occasions). Therefore, there is no scientific need to evaluate inter-assay accuracy and precision, or freeze-thaw stability, nor is there a requisite for quality control samples, the sole purpose of which is to represent frozen stored samples analysed over multiple analytical occasions. For these reasons, the current FDA DDI guidance simply states that investigational drug be readily measured with no interference from the assay matrix during the conduct of transporter substrate studies [8]. In practice this can easily be achieved by use of a six to eight concentration level matrix-matched (assay buffer for transwell experiments, cell lysate for uptake experiments) calibration line to provide a robust 'fit-for-purpose' bioanalytical method. Acceptance of accuracy can be established by the back-calculated concentrations of calibration standards falling within 20% of their nominal concentrations. Bioanalytical acceptance criteria of 20% is normal industry-wide practice for supporting *in vitro* DMPK studies, reflecting the typical 20–30% coefficient of variability observed for parameters derived from *in vitro* assays.

As already discussed in detail in Section 2.5.1.1 for inhibition studies, non-specific binding, chemical instability, and potential cytotoxicity could all be present in transporter substrate identification experiments and so their impact might need consideration. In P-gp and BCRP polarized cell monolayer studies determined percentage recovery (mass balance) of investigational drug, or comparison of determined initial dosing concentrations to donor concentrations at the end of the incubation, can be used to evaluate the presence of non-specific binding or potential chemical instability, respectively. Even if recovery was low (>10-50 %), then as long as the values were equivalent for both directions of flux, there would be no impact on the outcome of a substrate assay in terms of correct classification as a transported substrate or non-substrate. Only if determined recovery was disproportionate with directional flux movement, often due to the differences in plastic type between the basolateral companion plate (*e.g*. polystyrene) and the multiwell insert plate (*e.g*. polycarbonate) giving rise to higher non-specific binding and therefore lower recovery in one direction (*i.e*. A-B) over the other, would investigators need to consider the impact this might have on substrate identification outcome. This is because the $P_{app}$ value determined in the direction of flux with the lower recovery will be an underestimate of the true value, and as such this will act to skew the calculated efflux ratio. For example if recovery of investigational drug in the A-B direction was only 20% versus 100% in the B-A direction, and the calculated efflux ratio was in the tens to hundred range in numerical value, then any increase of the underestimated A-B $P_{app}$ value that might occur in the absence of binding would be unlikely to reduce the calculated efflux ratio to below the cut-off of 2, and therefore would not change its identification as a substrate. Conversely, if the same investigational drug derived a calculated efflux ratio of only between 2–3, making it potentially borderline, then any increase of the underestimated A-B $P_{app}$ value that might occur in the absence of binding would be anticipated to reduce the calculated efflux ratio to below the threshold identifying it as a substrate, and therefore would change the classification outcome. For SLC transporter substrate identification assays whilst non-specific binding might occur, it would not impact on the classification of the investigational drug as a substrate based on determined uptake ratio, as any impact of binding would be equivalent in both the transporter transfected cells and the vector control cells since they are incubated with the same plasticware plates. As highlighted in Section 2.5.1.1 any potential cytotoxicity that might occur which could hamper correct interpretation of results can be accounted for by the use of a cell monolayer integrity marker in polarized cell

monolayer studies, or by an observed decrease in determined cellular protein with increasing investigational drug incubation concentration in SLC uptake studies. As such upfront non-specific binding, chemical instability or cytotoxicity assays need not necessarily be conducted prior to regulatory *in vitro* transporter substrate identification studies.

*2.5.2.1. Timings for performing regulatory substrate identification studies along the value chain.* Transporter substrate data is being generated to understand the investigational drug's victim DDI potential in order to inform the clinical development plan around what clinical interaction studies with known clinical transporter inhibitors might be warranted, or potentially what perpetrator co-medications to exclude in patient trials. Often such transporter substrate profiling studies are deferred until the drug is in Phase 2 clinical development since a knowledge of the principal human clearance routes (≥25% overall clearance) are required to ascertain which transporters to focus on. Such quantitative information becomes available after conducting the human radiolabelled (ADME) mass balance study. Furthermore, if the perceived DDI risk perpetrated by co-medications is considered to be low, then another option would be to consider substrate profiling studies at the later stages of drug development as required to complete the understanding of a new drug and fulfil regulatory requirements in time for filing of a new drug application (NDA).

In contrast to the approach above, regulatory transporter substrate profiling studies might need to be performed earlier prior to first time in patient studies (*e.g.* during preclinical development for oncology indications where first in humans is first in patients, or Phase 1 for other indications) if the investigational drug has a narrow therapeutic index (safety margin) and is being co-administered with critical perpetrator co-medications that cannot be excluded in order to mitigate victim DDI risk. As definitive knowledge of the principal human clearance routes will not be available at this stage, then all regulatory required transporters (P-gp, BCRP, hepatic and renal SLC) will need to be studied.

Another option available to project teams depending on the drug development plan for an oral drug might be to perform regulatory P-gp and BCRP substrate profiling studies at IND to understand whether there is a victim DDI risk around these intestinal transporters leading to increased absorption of the investigational drug, which is likely to have the biggest impact on its exposure, and then deferring the remaining SLC transporter studies until after the Phase 2 radiolabelled human ADME study readout. The same strategy could also be applied to intravenous investigational drugs for which being restricted from the central nervous system by efflux transporters plays a crucial role to maintaining their safety.

A final option might be to conduct all regulatory required transporter substrate studies (P-gp, BCRP, hepatic and renal SLC) at IND stage to complete an *in vitro* DDI package for purely commercial reasons.

### 2.5.3. Major metabolite considerations

In the current EMA DDI guideline (2013) there is no specific mention around the need to assess metabolites of investigational drugs as substrates or inhibitors of transporters, although we would anticipate some detail in the next released version [9]. In contrast, whilst it is discussed in the current FDA DDI guidance (2020), the recommendations on studying metabolites at transporters is not particularly clear [8]; in fact it was much clearer in the earlier FDA draft DDI guidance from 2012 [109]. The earlier position of the FDA was that any major metabolite whose exposure equalled or exceeded 25% of parent investigational drug AUC should also be evaluated as a substrate and inhibitor of regulatory required transporters as directed for investigational drugs [109]. The current FDA recommendation is that *in vitro* assessment of metabolites as transporter inhibitors are likely not required if the parent investigational drug has already been assessed to inhibit transporters *in vitro* such that *in vivo* DDI studies are warranted. This is because the follow-up clinical interaction study should evaluate the *in vivo* inhibition potential of the metabolite alongside that of the parent drug, obviating the need to conduct separate *in vitro* studies [8]. However, if *in vitro* assessments suggest that the parent investigational drug alone will not inhibit the major transporters, it may still be possible that *in vivo* DDIs caused by metabolites could occur. In this situation, unlike for CYPs, there is no recommendation of what the metabolite exposure threshold (relative to parent) needs to be to trigger performing *in vitro* transporter inhibition studies for a metabolite, rather that considerations should be made on a case-by-case basis. Nevertheless, there is an inference that if the metabolite is a conjugated Phase II metabolite that is more polar than the parent drug, then the *in vitro* DDI potential of this metabolite as a substrate or inhibitor of major drug transporters should be assessed since published data have shown that such metabolites might be better substrates or more potent inhibitors of transporters, thus giving a higher risk of DDI than the parent drug [8]. Our recommendation for DDI risk assessment purposes is that a metabolite is classed as major when its exposure ≥25% of parent AUC, and if this criteria is met, it be evaluated as an inhibitor of all regulatory required transporters and as a substrate based on its principal clearance routes. For the latter it is important to recognize that it may be necessary to consider other transporters not listed in regulatory guidance to understand the disposition of the major metabolite. For example MRPs might be important for the biliary or hepatic/renal elimination of certain conjugates such as glucuronides.

It is worth highlighting that in the case of oral prodrugs that are intentionally converted into their active (metabolite) form within enterocytes that both the prodrug and the active metabolite should be assessed as inhibitors of all regulatory required transporters and as substrates of intestinal transporters P-gp and BCRP. However, unlike for the active metabolite, if it is proven that there is complete conversion such that there is no detectable systemic exposure of the prodrug form, then the

prodrug itself need not be assessed as an inhibitor or substrate of hepatic and renal transporters, thereby confining its assessment to intestinal P-gp and BCRP only.

### 2.6. Qualitative perpetrator DDI risk assessment using generated $IC_{50}$ data

Using the experimentally determined definitive $IC_{50}$ (Section 2.5.1.1), for an orally administered drug at its expected therapeutic dose, transporter DDI hazard potential is assessed using basic static equations provided by regulators (Figure 9). For these, the anticipated inhibitor concentration at the specific transporter interaction site (Section 2.3.4; corresponding to $[I]_{gut}$, $[I]_{in,\ max\ u}$, or $[I]_{max\ u}$ for intestinal P-gp and BCRP, hepatic OATP1B1, OATP1B3 and OCT1, or renal OAT1, OAT3, OCT2, MATE1 and MATE2-K, respectively) is divided by the specific transporter $IC_{50}$ value (equivalent to the $K_i$ in transporter assays when [probe substrate] $\ll\ll$< $K_m$) in order to calculate a ratio. Whilst not stated in guidance, the presumed regulatory basic static equations and ratios that would apply to assessment of intravenously administered drugs are shown in Figure 10. For all assessments, if the calculated ratio value exceeds the predetermined threshold (cut-off) stipulated for the respective basic static equation, then the investigational drug 'flags' as having the potential to cause a *qualitative* DDI in the clinic through inhibition of the transporter being assessed and, as such, consideration of a follow-up clinical DDI study would be warranted to confirm whether it is a transporter inhibitor *in vivo*. The *in vitro* transporter inhibition data is included on a new drug's label, alongside any subsequent clinical DDI data, to inform physicians around what common co-medications can/cannot be co-

| Efflux transporter inhibition in the intestine | Hepatic uptake transporter inhibition in the portal vein | Renal transporter inhibition from systemic circulation |
|---|---|---|
| FDA: $I_{gut} / IC_{50} \geq 10$<br>PMDA: $I_{gut} / IC_{50} \geq 10$<br>EMA: $I_{gut} / IC_{50} \geq 10$ | FDA: $R = 1 + (I_{in,max} \times f_{u,p}) / IC_{50} \geq 1.1$<br>PMDA: $R = 1 + (I_{in,max} \times f_{u,b}) / IC_{50} \geq 1.1$<br>EMA: $(I_{in,max} \times f_{u,b}) / IC_{50} \geq 0.04$ | FDA: $(I_{max} \times f_{u,p}) / IC_{50} \geq 0.1$<br>PMDA: $1 + (I_{max} \times f_{u,p}) / IC_{50} \geq 1.1$<br>EMA: $(I_{max} \times f_{u,p}) / IC_{50} \geq 0.02$ |
| $I_{gut}$ = dose (mol) / 250 mL (or FaSSIF soluble conc.) | FDA: $I_{in,max} = I_{max} + ((F_a \times F_g \times k_a \times dose/Q_h) / R_B)$<br>PMDA / EMA: $I_{in,max} = I_{max} + (F_a \times F_g \times k_a \times dose/Q_h)$ | |
| A **positive** result for a ratio above is a flag for potential clinical DDI – consider clinical development implications | | |
| A **negative** result – **no clinical study needed** | | |

$I_{gut}$ = theoretical intestinal luminal concentration (μM)
$I_{in,max}$ (FDA) = estimated hepatic inlet plasma concentration (μM)
$I_{in,max}$ (PMDA/EMA) = estimated hepatic inlet blood concentration (μM)
$I_{max}$ = maximum plasma concentration (μM)
$f_{u,p}$ = fraction unbound in plasma
$f_{u,b}$ = fraction unbound in blood
$R_B$ = blood-to-plasma concentration ratio (default = 1.0)
$F_a$ = fraction absorbed (default = 1.0)
$F_g$ = intestinal availability (default = 1.0)
$k_a$ = absorption rate constant (default = 0.1 $min^{-1}$)
$Q_h$ = liver blood flow rate (e.g. 1617 mL/min)
$IC_{50}$ = concentration that inhibits maximal transport activity by 50% (μM); equates to $K_i$ if [probe substrate] <<< $K_m$ (assuming competitive inhibition)

Figure 9. Regulatory DDI risk (hazard) assessment basic static equations for oral drugs.

| Intestinal efflux transporter inhibition from systemic circulation | Hepatic uptake transporter inhibition from systemic circulation | Renal transporter inhibition from systemic circulation |
|---|---|---|
| FDA: $I_{max} / IC_{50} \geq 0.1$<br>PMDA: $I_{max} / IC_{50} \geq 0.1$<br>EMA: $(I_{max} \times f_{u,p}) / IC_{50} \geq 0.02$ | FDA: $(I_{max} \times f_{u,p}) / IC_{50} \geq 0.1$<br>PMDA: $1 + (I_{max} \times f_{u,p}) / IC_{50} \geq 1.1$<br>EMA: $(I_{max} \times f_{u,p}) / IC_{50} \geq 0.02$ | FDA: $(I_{max} \times f_{u,p}) / IC_{50} \geq 0.1$<br>PMDA: $1 + (I_{max} \times f_{u,p}) / IC_{50} \geq 1.1$<br>EMA: $(I_{max} \times f_{u,p}) / IC_{50} \geq 0.02$ |
| A **positive** result for a ratio above is a flag for potential clinical DDI – consider clinical development implications | | |
| A **negative** result – **no clinical study needed** | | |

$I_{max}$ = maximum plasma concentration (μM)
$f_{u,p}$ = fraction unbound in plasma
$IC_{50}$ = concentration that inhibits maximal transport activity by 50% (μM); equates to $K_i$ if [probe substrate] <<< $K_m$ (assuming competitive inhibition)

Figure 10. Presumed regulatory DDI risk (hazard) assessment basic static equations for IV drugs.

administered safely during clinical use (Figure 11). This *qualitative* DDI risk approach is illustrated by the following published case studies.

In 2011, AZD5672 was being developed for the oral treatment of rheumatoid arthritis and since patients in this disease population are of an advanced age and have co-morbidities, they are at risk of DDI because of taking multiple co-medications. One such co-medication is the cardiac glycoside digoxin. As a result of this, and to fulfil regulatory requirements, AZD5672 was subsequently assessed in a unidirectional polarized Caco-2 cell monolayer system versus digoxin as probe substrate and established to be an inhibitor of P-gp *in vitro* with an $IC_{50}$ = 32 μM [22]. Based on a predicted therapeutic dose of 100 mg and a theoretical intestinal lumen concentration of 528 μM, the basic static equation ratio ($[I]_{gut}/IC_{50} \geq 10$) was 17, indicating the potential for AZD5672 to perpetrate a DDI through inhibition of intestinal P-gp. Therefore, a subsequent follow-up clinical DDI study with digoxin was warranted and was actually performed with two doses of AZD5672 administered to steady state [22]. A lower dose of 50 mg was studied as this was not predicted to cause a DDI with P-gp based on the static equation ratio of 8.3, which was below the threshold of 10 (resulting from an $[I]_{gut}$ = 264 μM). In contrast the higher dose of 150 mg ($[I]_{gut}$ = 792 μM) was expected to cause a DDI *in vivo* (ratio = 25). The results from the clinical study confirmed that AZD5672 caused a dose-dependent DDI with digoxin through inhibition of intestinal P-gp as no change in digoxin AUC was observed with 50 mg AZD5672, but there was a clinically significant 1.3-fold increase in digoxin AUC (plus an increase in $[I]_{max}$) following co-administration with 150 mg AZD5672, which was consistent with increased digoxin absorption. In addition to confirming that AZD5672 was an inhibitor of P-gp *in vivo*, the observed DDI with digoxin was correctly predicted using the basic static equation and calculated $[I]_{gut}$ and *in vitro* P-gp $IC_{50}$ data [22].

In the second case study from 2016, another rheumatoid arthritis candidate drug fostamatinib was subsequently assessed for DDI potential versus the BCRP transporter in order to understand the DDI risk for co-administered statin medication. Using a validated membrane vesicle system versus estrone 3-sulfate as probe substrate, fostamatinib was determined to be a potent inhibitor of BCRP *in vitro* with an $IC_{50}$ = 0.050 μM [23]. Based on a therapeutic dose of 100 mg and an $[I]_{gut}$ = 691 μM, the basic static equation ratio ($[I]_{gut}/IC_{50} \geq 10$) was 13,820, indicating the potential for fostamatinib to perpetrate a DDI through inhibition of intestinal BCRP *in vivo* and that a follow-up clinical DDI study with a BCRP *in vivo* substrate was warranted. Rosuvastatin was chosen as the *in vivo* BCRP substrate since fostamatinib was not an *in vitro* inhibitor of OAT3, nor was it predicted to inhibit OATP1B1 *in vivo* (R-value <1.03) and therefore would not affect these additional rosuvastatin critical disposition pathways [23]. In the subsequent clinical interaction study, fostamatinib (100 mg) perpetrated a DDI with rosuvastatin resulting in a 1.96-fold increase in rosuvastatin AUC (plus an increase in $[I]_{max}$) due to increased absorption mediated via inhibition of intestinal BCRP [23]. The results from the clinical study confirmed that fostamatinib was an inhibitor of BCRP *in vivo* and the observed DDI was correctly predicted using the basic static equation and calculated $[I]_{gut}$ and *in vitro* BCRP $IC_{50}$ data [23].

As illustrated in Figure 11, the generated *in vitro* inhibition data and subsequent DDI hazard assessment utilizing regulatory basic static equations for transporters are used to inform the clinical development plan both in terms of potential clinical interaction studies that might be needed and around any implications for co-medications. However, the shortfall of this approach to risk assessment is that it is not very informative to physicians or the clinical development project team when putting together the clinical plan for co-medications as the perceived risk is only '*qualitative,*' *i.e.* it is an 'all or nothing' approach that simply flags DDI potential. Therefore, prior to any clinical interaction study outcome, this might lead to physicians adopting a more conservative standpoint and excluding all potential vulnerable victim co-medications from clinical trials for reasons of patient safety, regardless of whether they have narrow or wide therapeutic indices. This is because the information on risk provided is lacking regarding whether alternatives such as co-medication dose adjustment, or therapeutic drug

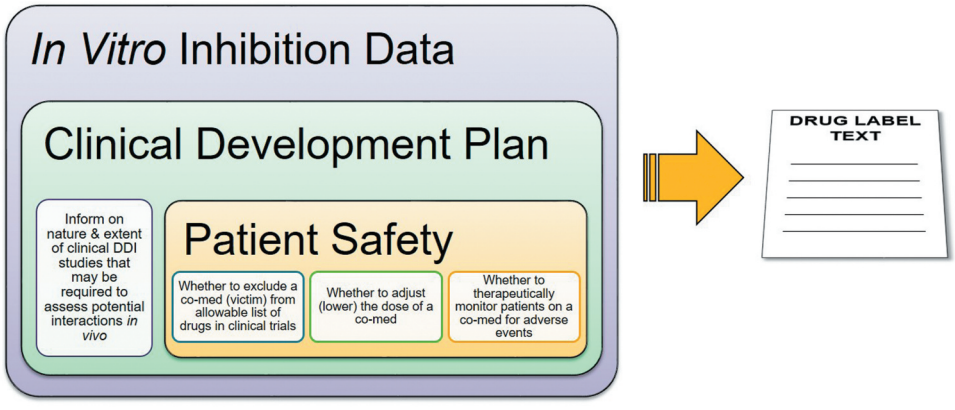


Figure 11. Transporter perpetrator DDI risk assessment in drug development.

monitoring, would help to mitigate DDI risk [4]. However, such blanket exclusions could have a major adverse impact on the recruitment of patients in clinical trials if such co-medications are deemed critical co-medications which cannot be avoided in clinical practice.

### 2.7. Qualitative victim DDI risk assessment using generated substrate data

It is quite difficult to assess 'victim' DDI risk for an investigational drug that has been shown to be a substrate of transporters *in vitro*. Factors that need to be taken into consideration include its predicted clinical concentration at the interacting site and whether the transporter could be saturated at this concentration. When a transporter becomes saturated, the passive diffusion process surpasses the active transport process for the investigational drug and as such the role of the transporter in that disposition route is diminished. This means that should a perpetrator co-medication inhibit that transport pathway clinically, it will have no effect on the exposure of the victim drug substrate and therefore would not present a DDI risk. In contrast, if the clinical concentration is at a non-saturating level where active transport processes predominate over passive processes and the transporter in question contributes significantly to a critical ADME pathway for the drug, then there could be a clinically relevant increase in the drugs exposure resulting in DDI if the transporter pathway was inhibited. In such a scenario for an identified *in vitro* substrate, consideration of a follow-up clinical DDI study may be warranted with a known transporter inhibitor to confirm whether the drug is a transporter substrate *in vivo* [8].

## 3. Quantitative prediction of transporter and complex DDIs

### 3.1. Investigational drug as perpetrator (inhibitor)

A superior and more effective approach to *qualitative* DDI risk assessment (Section 2.6) is to consider *quantitative* prediction of DDI liability using *in vitro* to *in vivo* extrapolations derived from mechanistic static or dynamic (PBPK) modelling to forecast the exposure (AUC) increase of a common victim co-medication due to inhibition of transporter pathways by a perpetrator drug [1,23,78,110]. Such quantitative DDI prediction is more effective as it immediately provides clinical context to physicians facilitating informed decisions on whether any potential interaction is simply a pharmacokinetic DDI or more importantly, using their knowledge around the therapeutic index of the victim co-medication, a clinically significant DDI requiring clinical intervention (e.g. requiring monitoring, dose adjustment or exclusion of the co-medication, or switching to a less sensitive co-medication within the same class). This enhanced understanding will also aid patient recruitment for clinical trials as the allowable co-medication list in the protocol could remain more inclusive. Furthermore, using this quantitative DDI prediction approach in early development during preclinical development candidate selection would give more visibility and context to potential DDI liabilities as a consequence of transporter inhibition when choosing between the 'baggage' of compounds. Thereby helping to reduce unexpected clinical findings and the subsequent need for risk management later in drug development [1].

As mentioned above quantitative DDI predictions can be achieved through the use of either mechanistic static equations in which the victim and perpetrator are assumed to be at the site of interaction at the same time and at maximal concentrations to give a conservative 'worst-case' prediction of maximal theoretical AUC ratio increase, or through more complex dynamic PBPK modelling that incorporates drug-specific and system (body) parameters and time. Since PBPK models are data hungry they are often useful later within clinical development when specific human pharmacokinetic parameters and other data become available to project teams. Conversely, mechanistic static models require very few input parameters making them more suited for use in early preclinical/clinical development. Mechanistic static models can also help towards validating future PBPK victim-specific profile models to ensure they are truly capturing the co-medications' derived critical disposition pathways.

#### 3.1.1. Performance of mechanistic static models in successfully predicting transporter-mediated DDIs and complex DDIs

Use of mechanistic static equations requires knowledge of the clinically relevant critical disposition pathways for a victim co-medication and subsequent determination of these pathways' fraction excreted ($f_e$), or fraction metabolized ($f_m$) value, depending on whether they are mediated *via* transporters or enzymes, respectively. The adapted Rowland-Matin mechanistic static equation below (Equation 1) is used to determine the maximum theoretical fold change in exposure (AUCR) one might expect if an individual transporter (or enzyme) pathway was inhibited in a DDI, and uses the ratio of perpetrator inhibitor concentration at the interaction site divided by $K_i$ ($IC_{50}$) for the transporter (or enzyme) within the context of the derived transporter $f_e$ value (or enzyme $f_m$ value) for the victim drug [74,111,112].

*Equation 1*:

$$\mathrm{AUCR} = \frac{1}{\frac{fe}{(1+[I]/K_i)} + (1-fe)}$$

where $K_i$ = absolute inhibition constant (equating to $IC_{50}$ for transporters if the probe [S] ⋘ $K_m$ in the transporter inhibition assay and assuming competitive inhibition, based on the Cheng-Prusoff equation [98];) and [I] = unbound maximum hepatic inlet concentration {$[I]_{in,\ max\ u} = f_u \times ([I]_{max} + (((F_aF_g \times k_a \times \text{dose (mol)})/Q_h)/R_B))$} for hepatic transporters (or enzymes), [I] = maximum enterocyte concentration {$[I]_g = (F_aF_g \times k_a \times \text{dose (mol)})/Q_{ent}$} for intestinal transporters (or enzymes) [23], or [I] = unbound maximum plasma concentration of inhibitor at steady state {$[I]_{max\ u} = f_u \times [I]_{max}$} for renal transporters. $f_u$ = unbound fraction in plasma, $F_aF_g$ = fraction of the dose absorbed after oral administration, $k_a$ = absorption rate constant ($min^{-1}$), $Q_h$ = hepatic blood flow (1617 mL/min), $R_B$ is the blood-

to-plasma concentration ratio (default = 1.0) and $Q_{ent}$ = enterocyte blood flow (300 mL/min).

Based on inhibition of a single transporter disposition pathway, the mechanistic static approach (Equation 1) has been previously evaluated to determine its performance at predicting the observed clinical DDIs with the common antidiabetic co-medication metformin (victim) by the perpetrator drugs cimetidine, trimethoprim and pyrimethamine [78]. Using unbound steady state maximum plasma concentrations as [I] and inhibition constants against the MATE1 transporter for cimetidine (7.68 μM, $K_i$ = 1.22 μM), trimethoprim (7.84 μM, $K_i$ = 2.64 μM) and pyrimethamine (0.298 μM, $K_i$ = 0.131 μM), with the MATE1 $f_e$ value (0.39) derived for metformin active renal elimination, gave predicted AUCRs of 1.51, 1.41 and 1.37, matching and therefore successfully quantifying the clinically observed AUCRs of 1.46, 1.37 and 1.39, respectively [78]. Such quantitative prediction of transporter-mediated DDI confirmed that the mechanism underpinning these clinically observed DDIs with metformin is inhibition of renal MATE1.

When inhibition of multiple transporter disposition pathways by a perpetrator may contribute to an observed DDI then the mechanistic static approach is still valid and the overall combined predicted AUCR is simply the product of the separate predicted AUCR values for inhibition of each individual pathway in isolation. Using maximum enterocyte concentration or unbound maximum hepatic inlet concentration as [I] for intestinal BCRP ($f_e$ = 0.5) or OATP1B1 ($f_e$ = 0.38), respectively, and inhibition constants against each respective transporter for fostamatinib, eltrombopag, darunavir and lopinavir, gave rosuvastatin (victim) AUCR predictions due to BCRP inhibition of 2.00, 1.87, 1.33 and 1.71, or due to OATP1B1 inhibition of only 1.01, 1.03, 1.09 and 1.25, respectively [23]. These individual transporter predictions confirmed inhibition of intestinal BCRP to be the major mechanism underpinning the clinically observed DDIs with rosuvastatin, and when combined, the overall quantified AUCR predictions (2.02, 1.93, 1.45 and 2.14) were equivalent to the observed AUC fold increases (1.96, 1.88, 1.48 and 2.10) for fostamatinib, eltrombopag, darunavir and lopinavir, respectively [23].

In the case of more complex DDIs the impact of the individual transporter pathway(s) being inhibited by a perpetrator drug can be exacerbated by that same drugs ability to clinically inhibit other critical ADME pathways belonging to the victim drug involving cytochrome P450 enzymes. In such scenarios the mechanistic static equation used for quantitative DDI prediction of reversible CYP inhibition (Equation 1) may have to be expanded to incorporate additional effects of time-dependent inhibition (Equation 2), or the additional effects of both time dependent inhibition and induction of CYPs (Equation 3), if appropriate to the perpetrator.

*Equation 2*:

$$AUCR = \frac{1}{\frac{fm}{(1+[I]/K_I)\times\left[\frac{\frac{k_{inact}\times[I]}{K_I+[I]}+k_{deg}}{k_{deg}}\right]}+(1-fm)}$$

where $k_{deg}$ = apparent first-order degradation constant of the affected enzyme (for intestinal CYP3A4 = 0.00048 $min^{-1}$ [113]; hepatic CYP3A4 = 0.00032 $min^{-1}$ [114]), $k_{inact}$ = maximal inactivation rate constant, and $K_I$ = inhibitor concentration causing half-maximal inactivation.

*Equation 3*:

$$AUCR = \frac{1}{\frac{fm}{(1+[I]/K_I)\times\left[\frac{\frac{k_{inact}\times[I]}{K_I+[I]}+k_{deg}}{k_{deg}}\right]\times\left\{\frac{1}{1+\frac{Emax\times[I]}{EC_{50}+[I]}}\right\}}+(1-fm)}$$

where $E_{max}$ = maximum induction effect determined *in vitro*, and $EC_{50}$ = inducer concentration causing half-maximal induction.

As a class, statins are examples of drugs that are susceptible to complex DDIs involving multiple clinically relevant transporter and enzyme disposition pathways [74]. This is a regulatory concern due to the frequency of statin prescriptions in many disease indications as a result of co-morbidities which makes them a common co-medication for which the potential for DDI is high [1,74]. The review of Elsby *et al*. (2012) [74] derived the clinically relevant critical disposition pathways of six statins based on a review of clinical data and of the literature, as described above in Section 2.2.1, in order to understand the mechanisms behind statin DDIs. Simvastatin acid and atorvastatin share very similar critical disposition pathways involving intestinal CYP3A4 restricting extent of absorption ($f_m$ = 0.4 and 0.31, respectively), then active hepatic uptake by OATP1B1 ($f_e$ = 0.79 and 0.69, respectively), followed by near complete metabolism in liver *via* hepatic CYP3A4 ($f_m$ = 0.83 and 0.70, respectively). One further pathway critical to atorvastatin is intestinal BCRP ($f_e$ = 0.42) whose efflux acts to restrict absorption in concert with intestinal CYP3A4. In contrast, pitavastatin has only one clinically relevant disposition pathway, namely active hepatic uptake by OATP1B1 with an $f_e$ value of 0.70. Pravastatin and rosuvastatin are more hydrophilic statins for which critical disposition pathways include active hepatic uptake by OATP1B1 ($f_e$ = 0.50 and 0.38, respectively) and active renal secretion by OAT3 ($f_e$ = 0.40 and 0.25, respectively). For pravastatin there is partial evidence indicating that intestinal MRP2 may also be important to its absorption. One further pathway critical to rosuvastatin disposition is intestinal BCRP ($f_e$ = 0.50) whose efflux acts to restrict absorption. Active hepatic uptake by NCTP ($f_e$ = 0.21) and OATP1B3 ($f_e$ = 0.11) are considered only very minor (non-critical) pathways contributing to the total active hepatic uptake (overall $f_e$ = 0.70) of rosuvastatin as their inhibition in isolation would not result in an increase in AUC outside of bioequivalence. But when combined with inhibition of the major OATP1B1 pathway they could exacerbate the effect of DDI, such as is the case with the pan-inhibitor cyclosporine. Finally, the critical disposition pathways for the sixth statin fluvastatin are intestinal BCRP ($f_e$ = 0.42) and hepatic metabolism by CYP2C9 ($f_m$ = 0.44) [74]. Using this knowledge, the inhibitory values and pharmacokinetic parameters of known perpetrator drugs listed in Table 2 have been incorporated into mechanistic static equations in order to

Table 2. Inhibitory properties and pharmacokinetic parameters of coadministered drugs that cause DDIs with statins.

| Statin | Perpetrator drug | Dose (mg)[a] | MW[a] | $[I]_g$ (μM) | $K_i$ values (μM) | | | | | CYP3A4 TDI | | $f_u$[a] | $[I]_{max}$ (μM) | $[I]_{in,\ max\ u}$ (μM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | BCRP | OATP1B1 | OAT3 | MRP2 | CYP3A4 | $K_I$ (μM) | $k_{inact}$ ($min^{-}$[1]) | | | |
| Simvastatin acid | Cyclosporine | 200 | 1202 | 55.5 | NR | 0.02[b] | NR | NR | 0.7[c] | – | – | 0.1 | 0.0009[b] | 1.029 |
| | Telithromycin | 800 | 812 | 22.5[d] | NR | 12[e] | NR | NR | 3.65[e] | 1.05[e] | 0.02772[e] | 0.35[e] | 2.76[e] | 2.426[d] |
| | Clarithromycin | 500 | 747.96 | 22.4[f] | NR | 5.3[e] | NR | NR | 57.5[e] | 13.2[e] | 0.0580[e] | 0.3[e] | 3.83[e] | 2.397[f] |
| | Gemfibrozil | 600 | 250 | 800 | NR | 2.54[g] | NR | NR | - | – | – | 0.03 | 152[b] | 9.013 |
| | Posaconazole | 200 | 700.8 | 95.1 | NR | 19.4[h] | NR | NR | 0.181[h] | – | – | 0.019 | 0.94[b] | 0.353 |
| Atorvastatin | Cyclosporine | 350 | 1202 | 97.1 | 3.7[i] | 0.02[b] | NR | NR | 0.7[c] | – | – | 0.1 | 1.1[b] | 1.911 |
| | Lopinavir | 400 | 628.8 | 42.4[j] | 8.7[i] | 0.43[i] | NR | NR | 3.65[b] | – | – | 0.02 | 14.6[i] | 0.449[j] |
| | Ritonavir[s] | 100 | 720.95 | 1.4[k] | 24[l] | 0.5[l] | NR | NR | 0.0026[m] | – | – | 0.011[l] | 0.93[b] | 0.013[k] |
| | Clarithromycin | 500 | 747.96 | 22.4[f] | – | 5.3[e] | NR | NR | 57.5[e] | 13.2[e] | 0.0580[e] | 0.3[e] | 3.83[e] | 2.397[f] |
| | Itraconazole | 200 | 705.64 | 94.5 | – | – | NR | NR | 0.0225[c] | – | – | 0.002 | 0.43[b] | 0.036 |
| Rosuvastatin | Cyclosporine | 200 | 1202 | 55.5 | 3.7[i] | 0.02[b] | – | NR | NR | NR | NR | 0.1 | 0.0009[b] | 1.029 |
| | Gemfibrozil | 600 | 250 | 800 | UNK | 2.54[g] | 1.47[n] | NR | NR | NR | NR | 0.03 | 102[b] | 7.513 |
| | Fostamatinib | 100 | 578.52 | 57.6 | 0.031[i] | >10[i] | – | NR | NR | NR | NR | 0.018 | 1.9[i] | 0.227 |
| | Eltrombopag | 75 | 442.5 | 29.4[o] | 2.1[i] | 2.7[i] | – | NR | NR | NR | NR | 0.01 | 17.7[i] | 0.232[o] |
| | Darunavir | 600 | 547.73 | 73.0[j] | 75[i] | 4.3[i] | – | NR | NR | NR | NR | 0.05 | 10.6[i] | 1.208[j] |
| | Lopinavir | 400 | 628.8 | 42.4[j] | 8.7[i] | 0.43[i] | – | NR | NR | NR | NR | 0.02 | 14.6[i] | 0.449[j] |
| | Clopidogrel | 75 | 321.9 | 77.7 | 63[i] | 1.8[i] | UNK | NR | NR | NR | NR | 0.02 | 0.0099[i] | 0.288 |
| | Clopidogrel | 300 | 321.9 | 311 | 63[i] | 1.8[i] | UNK | NR | NR | NR | NR | 0.02 | 0.13[i] | 1.155 |
| | Ezetimibe | 10 | 409.4 | 2.4[p] | 2.9[i] | 2.2[i] | UNK | NR | NR | NR | NR | 0.1 | 0.014[i] | 0.047[p] |
| | Fenofibrate | 67 | 360.83 | 61.9 | 170[i] | 20[i] | – | NR | NR | NR | NR | 0.01 | 25.8[i] | 0.373 |
| | Atazanvir | 300 | 704.9 | 142 | 69.1[b] | 1.5[b] | UNK | NR | NR | NR | NR | 0.14 | 8.68[b] | 4.900 |
| Pravastatin | Gemfibrozil | 600 | 250 | 800 | NR | 2.54[g] | 1.47[n] | – | NR | NR | NR | 0.03 | 128[b] | 8.293 |
| | Clarithromycin | 500 | 747.96 | 22.4[f] | NR | 5.3[e] | – | – | NR | NR | NR | 0.3[e] | 3.83[e] | 2.397[f] |
| | Cyclosporine | 350 | 1202 | 97.1 | NR | 0.02[b] | – | 41[q] | NR | NR | NR | 0.1 | 1.1[b] | 1.911 |
| Pitavastatin | Cyclosporine | 140 | 1202 | 38.8 | NR | 0.02[b] | NR | NR | NR | NR | NR | 0.1 | 0.0055[b] | 0.721 |
| | Gemfibrozil | 600 | 250 | 800 | NR | 2.54[g] | NR | NR | NR | NR | NR | 0.03 | 102[b] | 7.513 |
| | Erythromycin | 500 | 733.94 | 227.1 | NR | 4.88[r] | NR | NR | NR | NR | NR | 0.16 | 3.96[b] | 7.374 |
| Fluvastatin | Cyclosporine | 200 | 1202 | 55.5 | 3.7[i] | NR | NR | NR | NR | NR | NR | 0.1 | 0.0009[b] | 1.029 |

BCRP, breast cancer resistance protein

$[I]_{max}$, mean steady-state maximum plasma concentration for total (bound plus unbound) drug

CYP, cytochrome P450

$f_u$, fraction unbound (taken from the drug label accessed via Drugs@FDA database; www.accessdata.fda.gov/scripts/cder/daf)

$[I]_g$, maximum enterocyte concentration, calculated as $(F_aF_g \times k_a \times dose/Q_{ent})$, where $F_aF_g$ is the fraction absorbed (as default taken to be 1.0), $k_a$ is the absorption rate constant (as default taken to be 0.1 $min^{-}$[1]), and $Q_{ent}$ is the enterocyte blood flow (300 mL/min ≡ 18 L/h [115])

$[I]_{in,\ max\ u}$, maximum unbound liver inlet concentration, calculated as $f_u \times [(C_{max} + ((F_aF_g \times k_a \times dose/Q_h)/R_B))]$, where $F_aF_g$ and $k_a$ are as defined above, Qh is hepatic blood flow (1617 mL/min ≡ 97 L/h), and $R_B$ is the blood-to = plasma concentration ratio (as default taken to be 1.0)

$K_i$, reversible inhibition constant (assuming competitive inhibition; equates to $IC_{50}$ in transporter assays where probe substrate concentration used is ⋘ $K_m$)

$K_I$, inhibitor concentration that causes half-maximal inactivation of enzyme

$k_{inact}$, maximal rate of inactivation of enzyme

MRP, multidrug resistance associated protein

MW, molecular weight (taken from the drug label accessed via Drugs@FDA database; www.accessdata.fda.gov/scripts/cder/daf)

NR, not relevant to statin's disposition

OAT3, organic anion transporter

OATP, organic anion transporting polypeptide

[a]Value taken from either Elsby et al. (2012) [74] or Elsby et al. (2016) [23]

[b]Value taken from Elsby et al. (2012) [74]

[c]Value derived from $IC_{50}$ value reported by Obach et al. (2006) [116] divided by 2

[d]Values for $F_aF_g$ and $k_a$ taken to be 0.57 and 0.012 respectively [110]

[e]Value taken from Elsby et al. (2019) [110]

[f]Values for $F_aF_g$ and $k_a$ taken to be 0.89 and 0.0113 respectively [110]

[g]Value taken from Varma et al. (2015) [117]

[h]Value taken from unpublished Cyprotex data

[i]Value taken from Elsby et al. (2016) [23]

[j]Value for $k_a$ taken to be 0.02 [23]

[k]Value for $k_a$ taken to be 0.003 [average of the reported value from Dickinson et al. (2011) [118] and Shebley et al. (2017)[119]

[l]Value taken from Shebley et al. (2017) [119]

[m]Value derived from the mean of the $IC_{50}$ values reported by Obach et al. (2007) [114] and Shebley et al. (2017) [119], each divided by 2

[n]Value taken from Watanabe et al. (2011) [120]

[o]Value for $F_aF_g$ taken to be 0.52 [23]

[p]Value for $k_a$ taken to be 0.03 [23]

[q]Value taken from El-Sheikh et al. (2013) [121]

[r]Value taken from Izumi et al. (2013) [122]

[s]CYP3A4 $EC_{50}$ = 3.4 μM; CYP3A4 $E_{max}$ = 13.9 μM [123]

UNK, unknown; no reported evidence for or against found in the literature

quantitatively predict statin DDIs and thus decipher the individual contribution each specific ADME pathway plays towards the overall clinically observed DDI when inhibited. The overall predicted AUCRs and the contributions resulting from individual pathways are compared to the clinically observed AUCRs in Table 3. On a side note, for both

Table 3. Deciphering the mechanism(s) behind clinically observed statin DDIs using the mechanistic static equation approach.

| Statin | Perpetrator drug | Clinically observed AUCR (from label) | Predicted AUCR | Pathways inhibited (contribution to overall AUC increase) |
|---|---|---|---|---|
| Simvastatin acid | Cyclosporine (200 mg) | 8.0 | 8.2 | **$CYP3A4_{gut}$, OATP1B1** & $CYP3A4_{hepatic}$ (**1.65** × **4.44** × 1.12) |
| | Telithromycin (800 mg) | 9.4 – 10.8 | 10.7 | **$CYP3A4_{gut}$**, OATP1B1 & **$CYP3A4_{hepatic}$** (**1.66** × 1.15 × **5.61**) |
| | Telithromycin (staggered) | 4.3 | 5.4 | **$CYP3A4_{gut}$**, OATP1B1 & **$CYP3A4_{hepatic}$** (**1.49** × 1.01 × **3.61**) |
| | Clarithromycin (500 mg) | 11.6 | 11.2 | **$CYP3A4_{gut}$, OATP1B1** & **$CYP3A4_{hepatic}$** (**1.66** × **1.33** × **5.06**) |
| | Gemfibrozil (600 mg) | 2.85 | 2.61 | **OATP1B1** |
| | Posaconazole (200 mg) | 8.5 | 3.7 | **$CYP3A4_{gut}$**, OATP1B1 & **$CYP3A4_{hepatic}$** (**1.66** × 1.01 × **2.22**) |
| Atorvastatin | Cyclosporine (350 mg) | 8.7 | 7.8 | **$BCRP_{gut}$,$CYP3A4_{gut}$, OATP1B1** & $CYP3A4_{hepatic}$ (**1.68** × **1.44** × **3.15** × 1.02) |
| | Lopinavir/ritonavir (400 mg/ 100 mg) | 5.9 | 6.0 | **$BCRP_{gut}$,$CYP3A4_{gut}$, OATP1B1** & **$CYP3A4_{hepatic}$** (**1.53** × **1.44** × **1.54** × **1.78**) |
| | Clarithromycin (500 mg) | 4.4 | 4.6 | **$CYP3A4_{gut}$, OATP1B1** & **$CYP3A4_{hepatic}$** (**1.44** × **1.27** × **2.50**) |
| | Itraconazole (200 mg) | 2.5 – 3.3 | 2.6 | **$CYP3A4_{gut}$** & **$CYP3A4_{hepatic}$** (**1.45** × **1.76**) |
| Rosuvastatin | Cyclosporine (200 mg) | 7.1 | 6.0 | **$BCRP_{gut}$** & **all uptake** [OATP1B1/1B3/NTCP] (**1.88** × **3.19**) |
| | Gemfibrozil (600 mg) | 1.90 | 1.76 | **OATP1B1/3** & **OAT3** (**1.47** × **1.20**) |
| | Fostamatinib (100 mg) | 1.96 | 2.02 | **$BCRP_{gut}$** & OATP1B1 (**2.00** × 1.01) |
| | Eltrombopag (75 mg) | 1.88 | 1.94 | **$BCRP_{gut}$** & OATP1B1 (**1.88** × 1.03) |
| | Darunavir (600 mg) | 1.48 | 1.45 | **$BCRP_{gut}$** & OATP1B1 (**1.33** × 1.09) |
| | Lopinavir (400 mg) | 2.10 | 2.12 | **$BCRP_{gut}$** & OATP1B1 (**1.71** × 1.24) |
| | Clopidogrel (75 mg) | 1.40 | 1.46 | **$BCRP_{gut}$** & OATP1B1 (**1.38** × 1.06) |
| | Clopidogrel (300 mg) | 1.96 | 2.00 | **$BCRP_{gut}$** & OATP1B1 (**1.71** × 1.17) |
| | Ezetimibe (10 mg) | 1.21 | 1.30 | **$BCRP_{gut}$** & OATP1B1 (**1.29** × 1.01) |
| | Fenofibrate (67 mg) | 1.07 | 1.16 | **$BCRP_{gut}$** & OATP1B1 (**1.15** × 1.01) |
| | Atazanvir (300 mg) | 3.10 | 3.25 | **$BCRP_{gut}$** & **all uptake** [OATP1B1/1B3/NTCP] (**1.51** × **2.15**) |
| Pravastatin | Gemfibrozil (600 mg) | 2.0 | 2.28 | **OATP1B1** & **OAT3** (**1.62** × **1.41**) |
| | Clarithromycin (500 mg) | 2.1 | 1.2 | **OATP1B1** |
| | Cyclosporine (350 mg) | 3.82 | 3.70 | **$MRP2_{gut}$** & **OATP1B1 (1.87** × **1.98)** |
| Pitavastatin | Cyclosporine (140 mg) | 4.55 | 4.15 | **OATP1B1** |
| | Gemfibrozil (600 mg) | 1.45 | 2.40 | **OATP1B1** |
| | Erythromycin (500 mg) | 2.8 | 1.88 | **OATP1B1** |
| Fluvastatin | Cyclosporine (200 mg) | 1.9 | 1.7 | **$BCRP_{gut}$** |

Using liver blood flow consistent with FDA 2020 DDI guidance; 1617 mL/min (97 L/h). Using reduction in hepatic CYP3A4 $f_m$ when OATP1B1 inhibition causes % increase in AUC equivalent to 10% of inhibitable OATP1B1 activity (1.38-fold for simvastatin acid; $f_e$ = 0.79 = 376% increase (4.76-fold), 10% = 38%) (1.25-fold for atorvastatin; $f_e$ = 0.69 = 223% increase (3.23-fold), 10% = 22%; within bioequivalence). MRP2 intestinal $f_e$ = 0.66 (based on 1-FaFg = 1–0.34)

simvastatin acid and atorvastatin calculations, in order to avoid over predicting any hepatic CYP3A4-derived AUCR contribution when there is significant predicted inhibition of OATP1B1 (>10 %; corresponding to AUCR of 1.38 or 1.25 for each statin respectively), which would act to reduce the active entry of the statin into the hepatocyte thus reducing the amount available for metabolism, a decreased 'effective $f_m$' for hepatic CYP3A4 is calculated by adding together the uninhibited OATP1B1 $f_e$ value with the passive fraction value (for simvastatin acid both values also being corrected for the fraction (0.9) CYP3A4 plays to overall metabolism [74]) in order to account for the effect of OATP1B1 inhibition. The uninhibited $f_e$ value for OATP1B1 is calculated by first converting the predicted OATP1B1 AUCR to a percentage AUC increase, expressing this number as a fraction of the maximum theoretical percentage AUC increase were OATP1B1 to be completely inhibited (*i.e.* 376% for simvastatin acid, 223% for atorvastatin), then multiplying this by the OATP1B1 $f_e$ value to give the inhibited $f_e$ value, which is finally subtracted from 1.

From analysis of the different statin AUC predictions given in Table 3, it is apparent for the CYP3A4-sensitive statins simvastatin acid and atorvastatin that inhibition of hepatic OATP1B1 uptake contributes to approximately 50% of the observed increase in AUCR in DDI with cyclosporine, with the remaining exposure increases being a combined result of the reversible inhibition of intestinal and hepatic CYP3A4 (Table 3). However, the causative balance of overall DDI magnitude towards these two statins could shift entirely towards inhibition of CYP3A4 as the sole driving force of the observed DDI were the perpetrator drug predicted to be only a relatively weak inhibitor of OATP1B1 whilst instead being predicted to be a clinical time-dependent inhibitor (inactivator) of CYP3A4, alongside any potential for reversible inhibition of the enzyme. Indeed, the reported clinical DDIs on simvastatin acid by telithromycin or clarithromycin are driven solely from inhibition and inactivation of intestinal and hepatic CYP3A4 alone, with minimal impact from OATP1B1, based on predicted AUC increases of 9.31-fold (observed 9.4–10.8-fold) or 8.4-fold (observed 11.6-fold), respectively (Table 3) [110]. Separately, quantitative DDI prediction analysis of the multiple transporter pathways involved in rosuvastatin disposition revealed interestingly that 73% of the rosuvastatin DDIs listed in Table 3 are solely driven by inhibition of intestinal BCRP (and not OATP1B1 as widely assumed) as the underlying mechanism, while 18% are attributed to inhibition of both intestinal BCRP and all active hepatic uptake processes and 9% are a result of inhibition of OATP1B and OAT3.

Whilst it is clear from the data presented in Table 3 that AUCR predictions matched very closely with the clinical situation for all victim drugs, to further evaluate the performance and accuracy of the mechanistic static models for quantitative DDI prediction of statins, the observed AUCR values have been plotted against the predicted AUCR values. Subsequent correlation analysis has revealed that out of a total of 28 DDIs, 24

successfully predicted within 1.25-fold (86%) with a further 3 (11%) successfully predicting within 2-fold of the clinically observed AUCR, and only one prediction falling outside of the 2-fold margin (Figure 12). The resulting overall predictive accuracy of 97% confirms the successful performance and utility of the mechanistic static model approach for effective quantitative DDI prediction of statins. Furthermore, given the value of the approach towards delineating complex DDIs, it is conceivable that quantitative DDI predictions could be included, alongside *in vitro* transporter inhibition data, in future drug labels to inform prescribers around patient safety with respect to the DDI risk with co-medications.

#### 3.1.2. Using mechanistic static equations to predict a range of DDI risk when the disposition of the victim co-medication is unknown

It is possible that in any disease indication a newly marketed drug could be prescribed to patients as a co-medication for which knowledge of its clinically relevant disposition pathways as the victim is not yet fully established and understood. For example, whilst it might be known that a drug is a substrate of BCRP and OATP1B1 *in vitro* and that human mass balance indicates it has low absorption and its clearance is extensively by hepatic elimination, there might be insufficient quantifiable knowledge to derive the $f_e$ values for each transporter in order to accurately predict (via AUCR) DDI risk. A useful way to overcome this limitation, to at least provide some DDI risk context to physicians, could be to obtain a range of predicted exposure changes through use of generic AUCR predictions based on different hypothetical transporter $f_e$ values in order to derive a theoretical maximum AUCR of the victim co-medication that might result from inhibition of the pathway by the investigational drug across a range of anticipated clinical doses. Hypothetical transporter $f_e$ values of 0.9, 0.75, 0.5 and 0.25 could be useful to evaluate an unknown co-medication, giving maximum theoretical AUC increases due to complete inhibition of a pathway by a perpetrator drug (when $[I]/K_i > 10$) equivalent to 10-fold, 4-fold, 2-fold and 1.33-fold, respectively; with the predicted AUCR effect being dialled down with lower $[I]/K_i$ ratios.

### 3.2. Investigational drug as victim (substrate)

In order to move from *qualitative* victim DDI hazard assessment towards more *quantitative* victim DDI risk assessment an understanding of the percentage contribution of active transport versus passive processes for a drug could be used to enable a judgement to be made of its derived transporter $f_e$ value. For hepatic uptake transporters, a hepatocyte uptake assay (e.g. using the oil spin methodology) would be a useful experimental tool for achieving this goal. For renal uptake transporters, the percentage active transport can be elucidated from clinical renal clearance data by expressing the calculated observed active renal clearance (total renal clearance minus unbound passive filtration clearance) as a percentage of the total measured human renal clearance. The subsequent derivation of the transporter $f_e$ value could in turn help quantify victim DDI risk by visualizing the maximal theoretical fold increase in exposure ($AUCR = 1/(1-f_e)$) of the investigational drug were its transporter pathway to be completely inhibited by a perpetrator co-medication during DDI. Arguably it is the transporter $f_e$ value that has the most influence on the extent of any increase in victim drug exposure because even if a hepatic/renal clearance pathway accounts for a large 90% of total drug clearance, if the $f_e$ is $\leq$ 0.5 (*i.e.* 55% active; $0.5/0.9 \times 100$) then, at worst, the maximum increase in AUC due to inhibition can only ever be 2-fold as the remaining clearance (≥45%) is passively driven and not

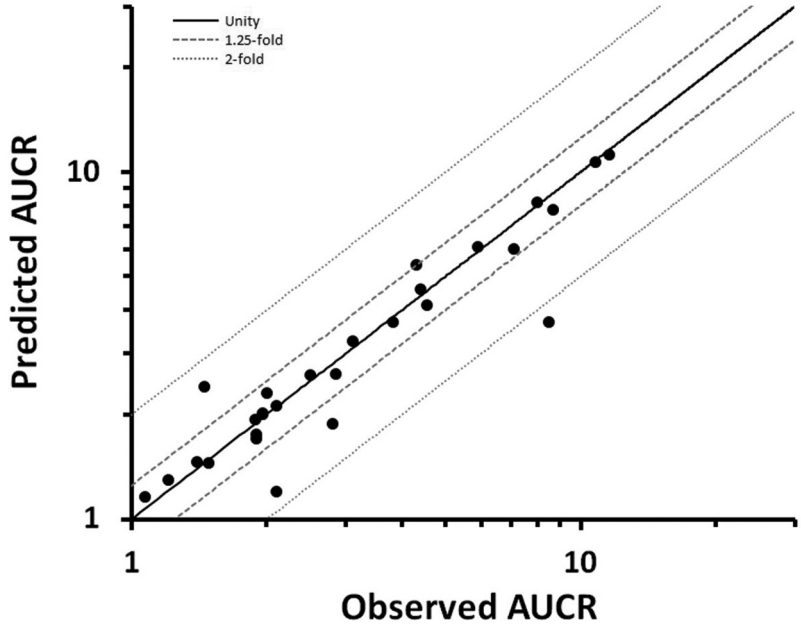


Figure 12. Predicted versus observed AUCRs for clinically relevant statin DDIs.

involved in DDI. An $f_e$ value of 0.1 (*i.e.* 11% active) would yield a maximum AUCR of only 1.11 as the majority of the clearance would be passive (89%). Collectively, all of this quantitative victim DDI knowledge then has to be contextualized with the investigational drug's anticipated therapeutic index (safety margin) to understand whether any perceived increase in its exposure through DDI poses a risk that requires clinical intervention, or will simply be a pharmacokinetic observation.

## 4. Co-medication considerations based on prescription frequency

Possessing knowledge of co-medication prescription rates within a disease indication will aid the identification of critical co-medications, for which, evidence of their clinically relevant transporter disposition (or inhibition) pathways can then be sought and subsequently used to prioritize the *in vitro* studies needed (WHAT and WHEN) to progress an investigational drug through development in order to mitigate DDI risk. Prescribing rate for medicines can be obtained via a variety of sources including from epidemiology databases, or through compiling prescription use data from large Phase 3 clinical trials conducted with thousands of patients over a reasonably long time period [34].

In the elegant review article of Bloomer *et al*. (2013) [4], across different disease indications, the prescription frequency of co-medications from the United States (US) over a three year period from 2008 to 2011 was used in conjunction with knowledge of therapeutic index (window) to categorize co-medication victim clinical drug interaction risk for a target patient population. Co-medications identified as having a wide therapeutic window and a low prescription frequency were classified as Category 1 with 'No impact of DDIs' from a victim perspective, and examples include the neuraminidase inhibitors oseltamavir and zanamavir [4]. Category 2 co-medications were classified as 'Low impact of DDIs' from a victim perspective based on having a wide therapeutic window but high prescription frequency. Statins were given as examples of drugs in Category 2 given their broad clinical use as common co-medications and 'generally excellent tolerability' [4]. However, on this latter point it may be prudent to consider the therapeutic window and subsequent susceptibility to myopathy on a statin by statin basis, as statins can have substantially different exposure increases in DDI due to the contribution ($f_e$) that OATP1B1 plays in their hepatic elimination. Simvastatin acid being the most sensitive with the largest increases in plasma concentrations, versus rosuvastatin being the least sensitive with minimal changes (<2-fold) to plasma levels [74]. Co-medications possessing a narrow therapeutic window coupled with a low prescription frequency were classified as Category 3 with 'Low/medium impact of DDIs' from a victim perspective [4]. An example of this category would be digoxin, which would only really be of concern if an investigational drug was intended for use in an elderly population or in a select patient population with high digoxin use. Finally, Category 4 co-medications were classified as 'High impact of DDIs' from a victim perspective based on having a narrow therapeutic window and high prescription frequency [4]. Oncology drugs, and in particular cytotoxic drugs, would be examples of this category. Routine categorization of the clinical victim drug interaction risk of common co-medications for the target disease indication in this way will facilitate decision-making within project teams around what *in vitro* transporter inhibition studies (in line with regulatory guidance) to perform and when along the drug development timeline to mitigate the perpetrator DDI risk of their investigational drug. For example, if a critical co-medication falls into Category 4, then comprehensive *in vitro* DDI evaluation of the investigational drug will be required early in preclinical development, or even at preclinical candidate selection stage to remove/manage risk when choosing the candidate to take forward into development. Conversely a co-medication classified as Category 1 would not likely require any dedicated *in vitro* DDI evaluation of the investigational drug until the later phases of clinical development and then only to fulfil regulatory requirements.

### 4.1. Attributes of specific co-medications

The following section describes the prescribing frequency for specific common co-medications as determined by Bloomer *et al*. [4] and highlights the transporters that are either key to their clinical disposition making them a victim of DDI, or to their potential to perpetrate DDI as inhibitors.

#### 4.1.1. Methotrexate

Methotrexate is predominantly prescribed in rheumatoid arthritis at a frequency of 28% of the patient population [4], consistent with it being the first line standard of care treatment for this disease [81]. Its clinically relevant disposition pathway is active renal elimination as a substrate of OAT1 and OAT3 [81], and as such *in vitro* OAT1 and OAT3 inhibition of investigational drugs should be prioritized.

#### 4.1.2. Ritonavir (a perpetrator)

Ritonavir is predominantly prescribed to treat HIV with a frequency of approximately 15% of the patient population [4]. It is not prescribed to any of the other disease indications mentioned in this section, presumably due to its CYP3A4 DDI perpetrator liabilities. Its clinically relevant transporter inhibition pathways include intestinal P-gp and BCRP ($[I]_{gut}/K_i$ ratios > 10) [124], and as such *in vitro* P-gp and BCRP substrate identification of investigational drugs might be prioritized.

#### 4.1.3. Metformin

The antidiabetic metformin is the major prescribed drug for treating type II diabetes (40% of the patient population). It also has a prescription frequency of 10%, or 5%, in the patient populations for hypertension, acute cerebral disease and acute myocardial infarction, or chronic obstructive pulmonary disease (COPD), asthma, human immunodeficiency virus (HIV), bronchus cancer and rheumatoid arthritis, respectively [4]. Its clinically relevant disposition pathway is active renal elimination as a substrate of OCT2 and MATE1 [78] (the expression of

MATE2-K protein has proven not to be quantifiable in human kidney [125]), and as such *in vitro* OCT2 and MATE1 inhibition of investigational drugs should be prioritized.

#### 4.1.4. Simvastatin acid

Simvastatin (acid) is the major prescription (frequency of 27%) in acute myocardial infarction patient populations. It is also prescribed to 15–20% of the patient population for hypertension, acute cerebral disease and diabetes, and to 10% of patients in COPD, bronchus cancer and rheumatoid arthritis. In asthma and HIV, its prescription frequency is approximately 5% [4]. In recent studies of age-related macular degeneration (AMD) patient populations in the US from 2007–2015 [126] or of COPD patient populations in the United Kingdom (UK) from 2007–2017 [127], simvastatin was prescribed at a rate of 42%, or 84%, respectively. Its clinically relevant transporter disposition pathway (alongside intestinal and hepatic CYP3A4) is active hepatic elimination as a substrate of OATP1B1 [74], and as such *in vitro* OATP1B1 inhibition of investigational drugs should be prioritized.

#### 4.1.5. Atorvastatin

In recent studies of AMD patient populations in the US from 2007–2015 [126] or of COPD patient populations in the UK from 2007–2017 [127], atorvastatin was prescribed at a rate of 28%, or 14%, respectively. Its clinically relevant transporter disposition pathways are active intestinal efflux as a substrate of BCRP limiting its absorption and (like simvastatin acid) active hepatic elimination as a substrate of OATP1B1 (alongside intestinal and hepatic CYP3A4) [74]. As such *in vitro* BCRP and OATP1B1 inhibition of investigational drugs should be prioritized.

#### 4.1.6. Rosuvastatin

In AMD patients in the US from 2007–2015 [126] or of COPD patients in the UK from 2007–2017 [127], rosuvastatin was prescribed at a rate of 15%, or 1%, respectively. Its clinically relevant transporter disposition pathways are active intestinal efflux as a substrate of BCRP limiting its absorption and active hepatic and renal elimination as a substrate of OATP1B1 and OAT3, respectively [23,74]. As such *in vitro* BCRP, OATP1B1 and OAT3 inhibition of investigational drugs should be prioritized.

#### 4.1.7. Clopidogrel (a perpetrator)

Clopidogrel is the major prescription (frequency of ~34%) in patient populations for acute myocardial infarction. It shares a very similar prescription frequency and disease indication profile with simvastatin [4]. Its clinically relevant transporter inhibition pathways include intestinal BCRP and hepatic OATP1B1 [23], and as such *in vitro* BCRP and OATP1B1 substrate identification of investigational drugs might be prioritized.

#### 4.1.8. Digoxin

The narrow therapeutic index cardiovascular drug digoxin is prescribed at a frequency of 5% in the patient populations for acute cerebral disease, acute myocardial infarction, bronchus cancer, diabetes mellitus and rheumatoid arthritis [4]. Its clinically relevant transporter disposition pathway is active intestinal efflux as a substrate of P-gp limiting its absorption [22,39], and as such *in vitro* P-gp inhibition of investigational drugs should be prioritized.

## 5. Conclusion

Drug transporters can play a significant role in dictating the disposition (ADME profile) and ultimately pharmacokinetics (exposure) of marketed and investigational drugs that are substrates of transport processes. Therefore perturbation of transporter function through inhibition by a co-administered drug can have a profound impact on drug levels resulting in clinical DDI. For these reasons, transporter DDIs are a key regulatory concern because they can impact on drug toxicity (and efficacy). The focus of this review article was to consider and present what different strategies could be employed by project teams for evaluating *in vitro* transporter inhibition, or substrate potential, during the drug discovery/development process towards understanding the *in vivo* risk potential of their investigational drug as a perpetrator, or victim, of DDI, respectively. As summarized in the decision tree below (Figure 13), key to judgements around the type of *in vitro* transporter studies to conduct and when during the drug discovery and development process, is the understanding of whether there are critical co-medications (victims or perpetrators) for a disease indication which cannot be avoided. These may necessitate early selective screening studies in lead optimization to remove DDI liability of candidates, rather than profiling studies during preclinical development onwards to manage risk. An overall summary of which *in vitro* transporter inhibition and substrate studies are required to fulfil regulatory expectations, and when they could be conducted along the development timeline to align with the clinical development plan, is presented in Figure 14. Conducting inhibition studies for the key transporters P-gp, BCRP and OATP1B1 earlier during preclinical candidate selection will aid to reduce unexpected clinical findings in patients on common co-medications by ensuring the right candidate with minimal liability/risk is taken forward. However, comprehensive transporter inhibition analysis to understand the DDI perpetrator potential of an investigational drug should be performed in early development prior to patient studies and at the right time to inform the clinical plan on allowable co-medications. In contrast to inhibition, as a strategy, formal regulatory transporter substrate studies to understand victim potential are typically conducted in later clinical development once knowledge of the principal human clearance routes are known. This is because the risk arising from toxicity due to any pharmacokinetic changes in investigational drug would usually be considered low due to its concentrations being closely monitored in trials and provided it has a wide safety margin. An exception to this approach would be with an investigational drug with a narrow safety margin, for which substrate studies would likely be performed prior to patient studies in order to inform on risk due to co-medications. Of course prior to such formal substrate studies it is likely for the majority of projects that their preliminary identification as efflux transporter substrates will

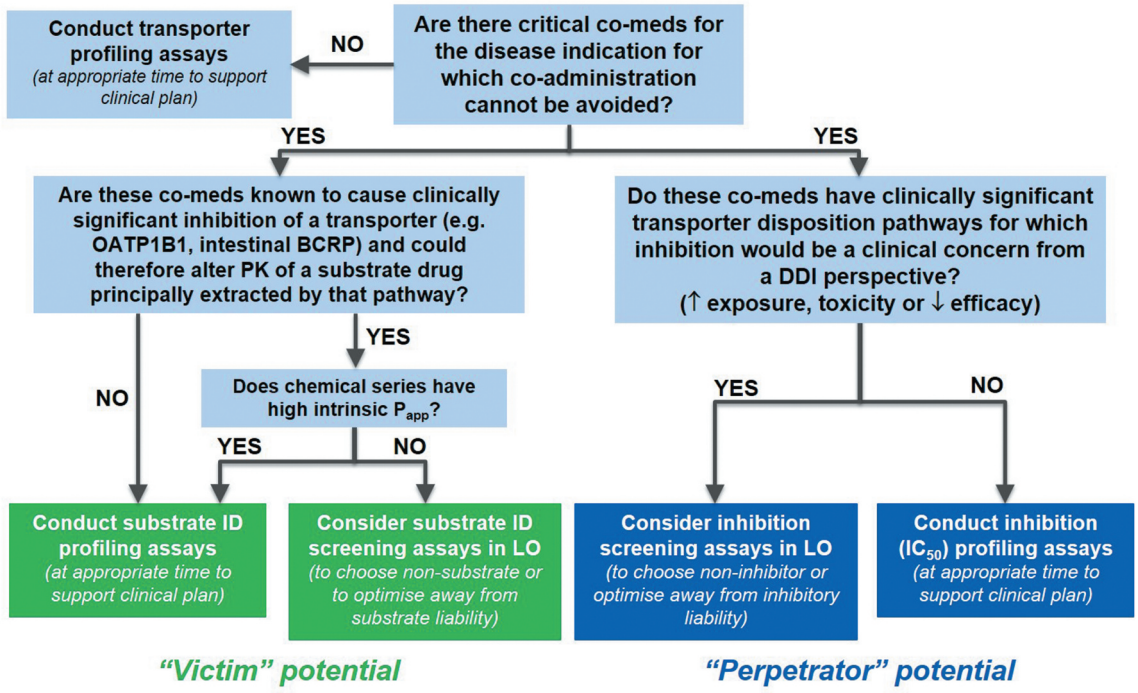


Figure 13. Transporter screening or profiling assay decision tree.

have already been ascertained from routine permeability screening assays during discovery. Furthermore, a few compounds may already have been identified as uptake transporter substrates where such knowledge was required for extrapolating *in vivo* hepatic clearance.

Whether it is the victim or perpetrator DDI potential of an investigational drug at transporters that is being considered, the use of mechanistic static equations incorporating knowledge of the clinically relevant transporter pathways for accurate *quantitative* DDI prediction and risk analysis (as demonstrated by the 97% accuracy for statin DDIs) can help to visualize the potential

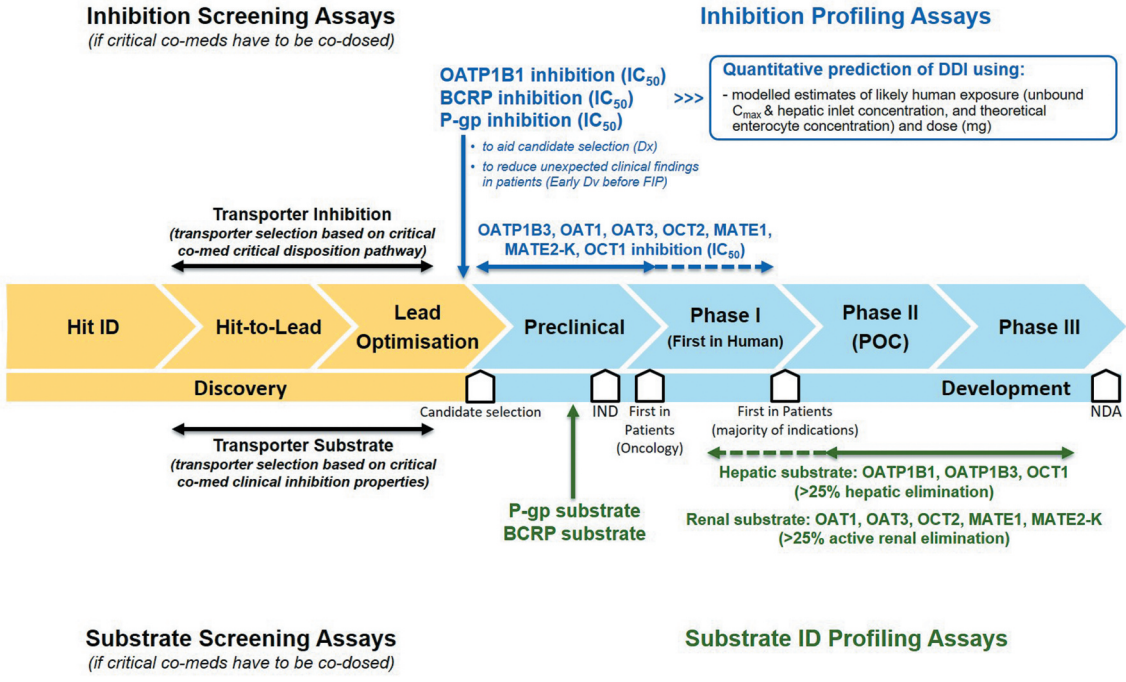


Figure 14. Overall summary of what and when transporter studies should be investigated during the drug development process.

clinical impact of DDI for project teams, rather than simply 'flagging' the hazard as is the case using the current *qualitative* approach described by regulatory guidance.

## 6. Expert opinion

As the understanding of the clinically significant role drug transporters play in dictating drug disposition and in mediating DDIs has developed, so too has the list of transporters requiring *in vitro* study by regulators grown to accommodate assessment of risk for new drugs. Currently, this encompasses ten transporters requiring routine study prior to regulatory NDA submission, which could result in a lot of *in vitro* data being generated either 1) too early in the drug discovery/development timeline and potentially becoming surplus to requirements if the investigational drug fails for reasons of poor pharmacokinetics (and efficacy) or toxicity, or 2) too late to influence finalization of the clinical development plan resulting in perhaps unnecessary co-medication exclusions that impact patient recruitment and thus delay clinical trials. In either case, there will be a cost and resource penalty, with the overall impact being considerably cheaper for the former compared with the latter. To minimize these development risks, project teams should study the right transporters at the right time for their investigational drug and the authors have tried to address this in this review by proposing *in vitro* strategies that could be employed to either mitigate/remove transporter DDI risk during development through frontloading certain studies, or to manage (contextualize) DDI risk in the clinical setting. Regardless of the approach taken it is imperative that prior assessment be made of whether there are critical co-medications (due to prescribing frequency or being a standard of care) for a disease indication and if so, an understanding gained of what their clinically relevant transporter disposition (victim) or inhibition (perpetrator) pathways are in order to determine the right transporters for priority study.

From a DDI risk perspective, for the majority of investigational drugs it is arguably their potential to inhibit transporters that is of the greatest concern during development because of the safety impact it may have on the clinical exposures of common co-medications, in addition to any adverse effects it may present to recruiting patients in trials. It is therefore vital that the inhibitory potential determined from *in vitro* transporter inhibition assays be right first time to give the 'worst case' value for accurate risk assessment purposes. To this end the authors would firstly recommend always conducting $IC_{50}$ profiling assays across a large default concentration range, rather than single concentration inhibition assessments, as the data will future-proof DDI risk against any potential future increases in dose and exposure of the investigational drug due to unexpected pharmacokinetics in humans, or if the disease indication or route of administration were to change. Secondly, all $IC_{50}$ determinations should be conducted with the inclusion of a pre-incubation step with investigational drug, regardless of transporter, in order to remove any artefactual underestimation of the $IC_{50}$ ($K_i$) parameter, thereby ensuring the correct $IC_{50}$ value is obtained for accurate risk assessment.

Regarding risk, it is clear that the industry will benefit from moving away from the current *qualitative* basic static equation approach of 'flagging' transporter DDI hazard potential, based on exceeding ratio thresholds, towards adopting the use of mechanistic static models to facilitate *quantitative* DDI prediction in order to truly mitigate or manage clinical risk. Moreover, such invaluable models are relatively straight forward and feasible to implement with minimal resource cost as the $[I]/K_i$ ratios needed are already being generated for the qualitative approach. As the authors have demonstrated for 28 clinically significant statin DDIs with six different statin drugs, mechanistic static models can accurately predict the clinically observed AUCRs mediated by inhibition of transporter (and enzyme) pathways. Consequently, the future routine use of mechanistic static models for effective quantitative prediction of transporter DDIs would enable physicians to contextualize the predicted AUCR with the therapeutic index of the victim co-medication in order to ascertain whether any DDI is simply a pharmacokinetic interaction, or a clinically significant interaction requiring intervention (co-medication dose reduction, monitoring, switching within a class, or exclusion). Furthermore, the authors can envisage a future scenario whereby once the currently emerging use of clinical endogenous biomarkers to monitor transporter inhibition *in vivo* becomes established and validated, allowing derivation of their disposition pathways and relevant transporter $f_e$ values, then the observed AUCR of the biomarker perpetrated by an investigational drug could act as a surrogate AUCR for a known victim critical co-medication that has a similar $f_e$ value for the transporter under consideration. This could be beneficial as it would not only remove the need to conduct a costly clinical interaction study with the specific co-medication later in development, but additionally the numerical AUCR and $f_e$ values for the biomarker, coupled with the *in vitro* $K_i$ value, could be incorporated into a rearranged mechanistic static equation in order to calculate [I] of the investigational drug at the interaction site (*e.g.* hepatic inlet). This value could then be utilized in place of modelled estimates of [I] for quantitative DDI predictions at other similarly located transporters which lack an established biomarker.

Unlike the current regulatory DDI risk recommendation for transporters, mechanistic static equation quantitative DDI predictive analysis, in the form of the net-effect model, is already in use by the regulators to predict the *in vivo* CYP DDI potential (via AUCR) of an investigational drug as an alternative to conducting a clinical interaction study. Based on this existing precedent, it does not feel too much of a 'leap of faith' for the transporter field to adapt and routinely use mechanistic static models for the right transporter at the right time across the breadth of the drug discovery/development value chain for effective quantitative prediction of transporter DDIs. Such a mechanistic approach can be used towards either mitigating perpetrator DDI risk early during candidate selection, or

managing clinical risk and aiding patient recruitment by informing labels and potentially providing an alternative to conducting costly clinical interaction studies with co-medications in the future.

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