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Predicting transporter-mediated drug-drug interactions based on *in vitro* cell permeability assays

Drugs may fail in clinical development or post-marketing for a variety of reasons: they may simply not be as effective as anticipated; poor pharmacokinetic properties may prevent them from reaching their intended target in therapeutic doses; or they are proven unsafe. Regardless the reason, late-stage drug failures are costly – wasting time, resources and manpower – and have contributed significantly to the declining overall productivity in the pharmaceutical industry in recent years.

By Dr Chris Bode

To improve drug efficacy and safety, two over-arching strategies can have a major impact on advancing safer, better characterised drugs through preclinical and clinical development and failing unsafe compounds earlier. First, build into the design of new drug compounds improved pharmacodynamic and pharmacokinetic properties either at the outset or during the lead optimisation process. And second, develop robust tools and assay protocols to characterise the Absorption, Distribution, Metabolism and Excretion (ADME) properties of drug candidates, and use these to generate accurate and reliable predictions before advancing experimental compounds into clinical trials (**Figure 1**).

A compound's ADME profile provides a window into its potential efficacy and toxicity. *In vitro* models that closely mimic conditions in the human body yield ADME data that can help guide selection of compounds to take forward into clinical development. In some instances, the data are sufficiently predictive to support regulatory filing of a new drug application (NDA). Historically, *in vitro* model systems have primarily been used to evaluate drug metabolism, particularly the breakdown of compounds by liver enzymes that comprise the cytochrome P450 (CYP) metabolic pathways. Preclinical testing routinely includes *in vitro* assays to screen drug candidates in model systems based on human liver microsomes. These tests became mandatory in 1997, when the US Food and Drug Administration (FDA) required the industry to institute preclinical studies to test for potential drug-drug interactions (DDIs) that can result when two or more drugs compete for access to particular CYP enzymes.

The highly publicised withdrawal of the once-successful allergy drug terfenadine (Seldane®) underscores the importance of screening for DDIs in early drug development. When taken together with ketoconazole, an antifungal agent and a CYP3A4 enzyme inhibitor, terfenadine levels in the circulation may rise to dangerously high levels. This can cause a disorder called Torsades de Pointes, a cardiac arrhythmia that can be fatal. When given on its own, terfenadine was a safe drug. But its risk for DDIs when co-prescribed with other commonly used drugs necessitated its withdrawal from the market and is testament to the risks posed by DDIs.

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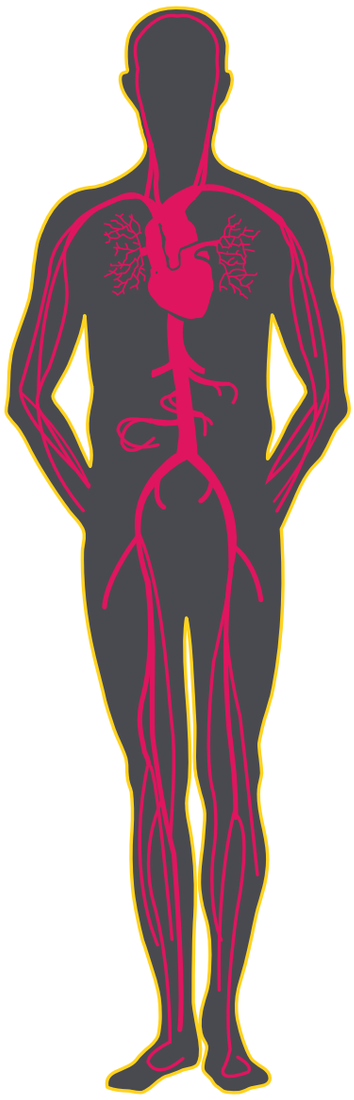

Figure 1
Pharmacokinetics is the discipline that describes the concentration to a drug over time in the body, most commonly the blood plasma compartment

A new mechanism for drug interactions

The demise of Seldane and the resulting market and industry repercussions were a wake-up call, and the industry began to look more closely at DDIs as an important and preventable cause of drug failures in late-stage testing in humans or after a drug is already on the market. For several years, the focus remained almost exclusively on the role of metabolic pathways, mainly the CYP enzymes, in terms of DDI risk. The FDA signalled the value and acceptance of predictive *in vitro* assays for DDIs in ADME model systems when it formally advised the industry that a negative finding *in vitro* was sufficient for a regulatory filing, whereas a positive result would necessitate a clinical drug interaction study[1].

Increasingly, another mechanism of drug interactions that was distinct from, yet could also overlap with and complement CYP-mediated DDIs, was emerging as an independent and significant risk factor for serious clinical outcomes. This mechanism involves a large group of transporter proteins that mediate the passage of drugs into and out of tissues and cells. In particular, interest has converged on efflux transporters, which, as their name suggests, transport compounds out of cells or prevent drugs and other molecules perceived as foreign from gaining entry in the first place. This class of transporters is believed to serve a protective function, but it is easy to see how problems could arise if they were to prevent the flow of drugs into target tissues where they are intended to exert a therapeutic effect, or into cells where they can be converted to active metabolites or metabolised for clearance from the body. In cancer, for example, over-expression of certain transporter proteins can prevent cytotoxic doses of chemotherapeutic drugs from accumulating in tumour cells, a common mechanism of resistance to chemotherapy.

As interest in transporters and understanding of the role they can play in transporter-mediated DDIs increased, the FDA formally recognised this risk in its 2006 guidance update[2]: "Various reported interactions attributed earlier to other mechanisms of interaction, such as protein-displacement or enzyme inhibition/induction, may be due in part to the inhibition or induction of transport proteins." Highlighting several well-characterised transport proteins, including P-glycoprotein (P-gp), organic anion transporters (OATs), organic anion transporting polypeptides (OATPs), organic cation transporters (OCTs), multidrug resistance-associated proteins (MRPs), and breast cancer resistant protein (BCRP), the FDA advised: "Of the various transporters, P-gp is the most well understood and may be appropriate to evaluate during drug development."

An unmet need identified

In urging the pharmaceutical industry to develop a standard methodology to study the activity of drug efflux transporters – in a manner akin to the *in vitro* tests available for evaluating CYP enzyme interactions – the FDA identified a critical unmet need. Techniques and pharmacologic tools did not exist to identify or inhibit the activity of specific transporter proteins, to test for transporter-mediated drug interactions, or to predict potentially dangerous DDIs that could result when one compound inhibits or induces the same transporter protein for which another compound is a substrate.

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Summarising the perspective of the Pharmaceutical Research and Manufacturers of America (PhRMA) on drug transporters, Bjornsson et al wrote in 2003[3] "at present, no accurate prediction of transporter involvement in humans is possible from nonclinical studies". As an example of a transporter-mediated DDI, the authors described the increased bioavailability of talinolol in the presence of verapamil, a P-gp inhibitor, concluding: "Some drug interactions, previously believed to be P450-mediated, are now considered at least in part due to inhibition of transport proteins." Although the industry was increasingly incorporating functional cell-based assays into the preclinical testing paradigm, in the early 2000s this was not yet the case for permeability studies, as "most if not all cell-based systems presently available contain multiple transporter proteins, which can lead to confounding results"[3].

The scientific literature clearly documents that human transport proteins alone or in combination with metabolic enzymes can affect the pharmacokinetics and pharmacodynamics of a drug through drug-drug interactions[4]. Established examples of transporter-based DDIs include digoxin and quinidine, fexofenadine and ketoconazole or erythromycin, penicillin and probenecid, and dofetilide and cimetidine.

The most extensively studied human transporter is P-gp, a surface glycoprotein that functions as an ATP-dependent efflux transporter[5]. P-gp is expressed on the surface of cells in the intestine, liver, kidney, brain, placenta, adrenal glands and testes. It is recognised for its ability to confer multidrug resistance to various types of tumour cells. Known substrates, inhibitors, and inducers of P-gp are listed in **Table 1**.

This collection of commonly prescribed drugs and a herbal remedy documents the broad structural variability and spectrum of therapeutic classes of drugs that are subject to efflux by P-gp. Some of these compounds will block P-gp-mediated transport of co-administered drugs, causing harmful DDIs. A variety of herbal constituents, which may be taken by patients for their medicinal value, as well as some phytochemicals found in foods, can also affect P-gp-mediated drug transport, altering the pharmacokinetics of a drug and causing adverse drug reactions or reduced drug effectiveness[6].

Another efflux transporter is the breast cancer resistance protein (BCRP), which is present in the intestine, liver, breast and placenta. Like P-gp, it has been linked to chemotherapeutic drug resistance in tumour cells when present at elevated levels. BCRP has several known drug substrates (**Table 1**).

The complexity of drug interactions

Transporter-mediated DDIs can be relatively straightforward. For example, two co-administered drugs that interact with the same transporter protein present on the cells lining the small intestine may compete for binding to the transporter, altering the intestinal absorption of one or both drugs and, as a result, their systemic bioavailability. Co-administration of drugs that are substrates for the same efflux transporter can enhance the bioavailability of one of the drugs, which can be a problem if it has a narrow therapeutic range (eg, digoxin, a P-gp substrate). At the level of the blood-brain barrier (BBB), drug interactions involving efflux transporters may allow compounds to pass into the brain that would not normally be able to cross the BBB, causing unwanted side effects. A drug that interferes with transporter activity in the liver or kidney may alter biliary or renal excretion, respectively, of a co-administered compound, and drugs that interact with placental efflux transporters could alter passage across the

TRANSPORTER	SUBSTRATES	INHIBITORS	INDUCERS
P-gp	Colchicine, digoxin, fexofenadine, indinavir, paclitaxel, topotecan, vincristine	Cyclosporine A, elacridar, erythromycin, itraconazole, ketoconazole, quinidine, ritonavir, valspodar, verapamil, zosuquidar	Rifampicin, St John's Wort
BCRP	Daunorubicin, doxorubicin, rosuvastatin, sulfasalazine, topotecan	Elacridar, gefitinib	

Table 1: Compounds interacting with the efflux transporters P-gp and BCRP

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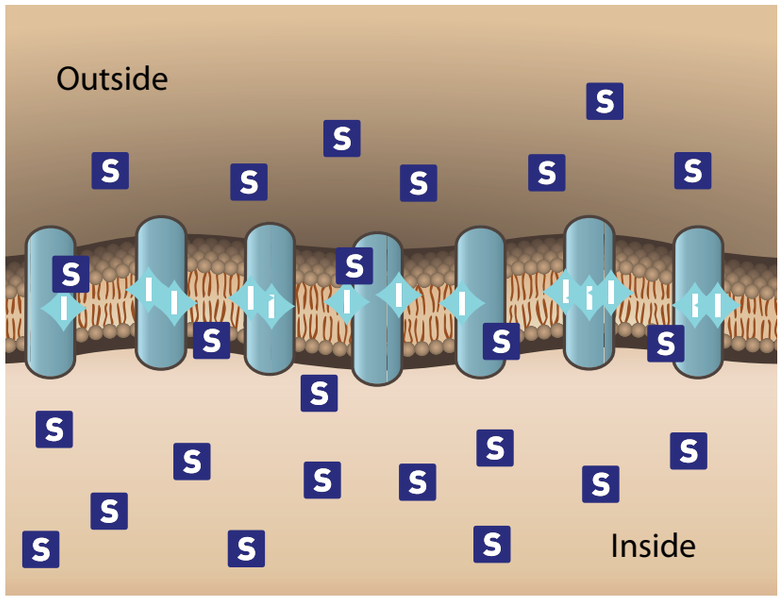

Figure 2
In the presence of an inhibitor ('I') of an efflux transporter, a drug that is a substrate ('S') of the same transporter readily crosses the cell membrane, whereas it would be excluded in the absence of the inhibitor

placental barrier, which protects the foetus from exposure to toxic agents (**Figures 2** and **3**).

This picture becomes more complicated with the realisation that many drugs may inhibit one or more metabolic enzymes as well as one or multiple transporter proteins. The challenge then is how to distinguish between permeability and metabolic mechanisms that underlie a drug interaction. Does one, the other, or both cause a particular DDI? If both are involved, are the effects competing, complementary, or synergistic? Does one mechanism modulate the effect of the other, either magnifying or diminishing the outcome of the interaction? The realisation that inhibition of a transporter protein can magnify drug interactions involving CYPs adds an additional level of complexity to emerging *in vitro* models of DDIs.

A real-world example of an especially strong DDI illustrates the difficulties in dissecting the mechanisms at play and the results of combination effects. Gemfibrozil, a fibrate drug used to treat elevated plasma lipid levels, increases blood levels of repaglinide, a drug prescribed to treat insulin resistance and elevated blood glucose levels in patients with Type II diabetes. As Type II diabetes is typically associated with the spectrum of disorders collectively called metabolic syndrome – which includes overweight/obesity, hypertension, and hypercholesterolemia – repaglinide and gemfibrozil are often co-prescribed.

Studies have demonstrated that gemfibrozil not only elevates the peak plasma concentration of repaglinide approximately 2-fold, but also prolongs its half-life in the circulation 2- to 3-fold[7,9]. As a result, the area under the repaglinide plasma concentration vs time curve (AUC) is elevated 8-fold. As a point of reference, note that the FDA considers a 'sensitive' CYP3A4 substrate to be a drug for which the AUC is elevated by at least 5-fold in the presence of a CYP3A4 inhibitor, while a 2-fold increase in AUC is considered significant for a substrate of any other CYP[2].

Repaglinide is metabolised by CYP2C8 and CYP3A4. Gemfibrozil is an inhibitor of CYP2C8; it has no effect on CYP3A4[8]. Thus, the logical conclusion is that the drug interaction is likely the result of inhibition of CYP2C8 by gemfibrozil, which prevents the metabolism of repaglinide, causing elevated drug levels in the bloodstream. However, gemfibrozil is only a moderate inhibitor of CYP2C8, and the strength of this interaction is not sufficient to account for its effect on repaglanide levels[8]. Subsequent studies led to the conclusion that the gemfibrozil-replaglinide DDI is likely caused by a metabolite, the glucuronide conjugate of gemfibrozil, which is an irreversible inhibitor of CYP2C8 and also appears to block the uptake of repaglinide into liver cells[7,9].

Itraconazole, an antifungal agent and a potent inhibitor of the CYP3A4 enzyme, which by itself increases the AUC of repaglinide less than 2-fold, magnifies the effect of gemfibrozil on repaglinide: the combination of gemfibrozil and itraconazole raised the repaglinide AUC 19-fold[10]. The combination of gemfibrozil and itraconazole results in a dramatic and potentially dangerous increase in exposure to repaglinide. As a result of this finding, an advisory was issued warning of the increased risk of hypoglycemia for patients taking both gemfibrozil and repaglinide, and the added risk for patients also taking itraconazole. The 'precautions' section of the repaglinide label was revised to recommend that patients on repaglanide should not start taking gemfibrozil, and vice versa. Furthermore, patients already taking both drugs should not be given itraconazole and should have regular monitoring of blood glucose levels and possible adjustment of the dose of replaglinide.

Notably, even with the range of *in vitro* assays and screening tools available to test for this drug interaction, the magnitude of the DDI effect caused by the combination of itraconazole, gemfibrozil and repaglinide could not have been predicted. Improved assay systems are needed to enable quantification of multi-drug interactions. For transporter-mediated DDIs, refined transporter models are needed that are able to represent the fraction of drug transported by a particular

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transporter[11]. And robust *in vitro* methods are needed to identify and characterise the mechanisms and effects of DDIs caused by multiple enzyme/transporter combinations.

A new model system for transporter-mediated DDIs

The unmet need for predictive *in vitro* model systems encompasses a demand for assays to identify and characterise transporter-based mechanisms that may have a role in DDIs, and for tools to screen for DDI risk that involves the interplay of metabolism- and transporter-mediated protein families. Innovative systems have been developed for *in vitro* testing and predictive modelling of transporter-mediated DDIs. This approach relies on RNA interference (RNAi) to silence the activity of a single transporter in a cell-based assay system[12]. *In vitro* RNAi is typically a transient phenomenon, but by utilising a lentiviral vector coding for short hairpin RNA (shRNA), it is possible to create a stable *in vitro* model system that mimics how a drug would be absorbed in the human body, while eliminating the function of one efflux transporter of interest at a time. A series of transporter assays in parallel, each including a cell line modified to silence a different individual transporter, would offer a powerful tool for preclinical screening of drug permeability and possible DDIs. The ability to evaluate a compound either alone or together with one or more probe substrates in transporter assays can yield data to help define and quantify a compound's absorption properties and reveal potential transporter-mediated drug interactions.

Compounds that are subject to P-gp-mediated efflux have an increased risk for a drug interaction if they are co-administered with a drug that is a P-gp inhibitor. *In vitro* assays can be used to predict the risk for DDIs. The FDA has identified the bidirectional cell monolayer transport assay as the definitive *in vitro* assay format to predict DDIs involving transporters. As far as the FDA is concerned, a negative result *in vitro* would be sufficient proof of minimal DDI risk. Whereas a positive result would require clinical, *in vivo* testing to assess the magnitude of a potential DDI. And the consequences of a clinical DDI can include dose adjustment, labelling changes (including the dreaded 'black box' warning) or, rarely, withdrawal from the market.

Human Caco-2 cells represent an industry standard *in vitro* model system to predict intestinal drug absorption, as first documented by Hidalgo et al in 1989[13]. Starting with a stable clone of Caco-2 cells, which express three drug efflux transporters – P-gp, BCRP, and MRP2 – on their apical surface (which corresponds to the lumenal surface of an intestinal epithelial cell), one can then use RNAi to knock down the expression of an individual gene. RNA interference works by targeting a specific gene transcript for destruction, thereby preventing it from being translated into a protein. With this technique, it is possible to create a BCRP knockdown Caco-2 cell line in which BCRP expression is reduced by more than 90% compared to unmodified, or parental, Caco-2 cells, greatly reducing the presence and function of BCRP in this cell model (**Figure 4**).

These BCRP knockdown Caco-2 cells can form the basis of a bidirectional transport assay to identify compounds that interact with P-gp. Because P-gp expression is relatively unaffected, there is greater certainty that any observed efflux is a result of P-gp activity; interference by BCRP is less of a problem than with parental Caco-2 cells. Reduced efflux of a test compound in the presence of

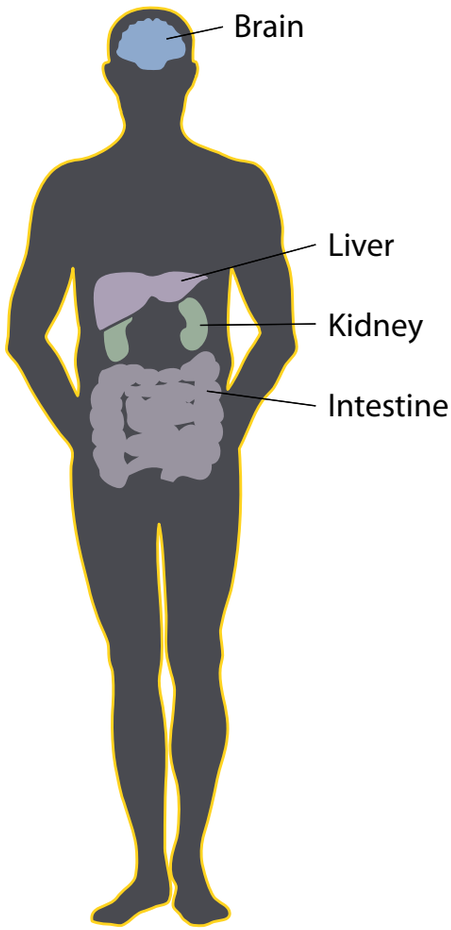

Figure 3
These are major organs in which transporters are involved in drug absorption, distribution and elimination and, therefore, are pharmacokinetically important sites of transporter-mediated drug-drug interactions (DDIs)

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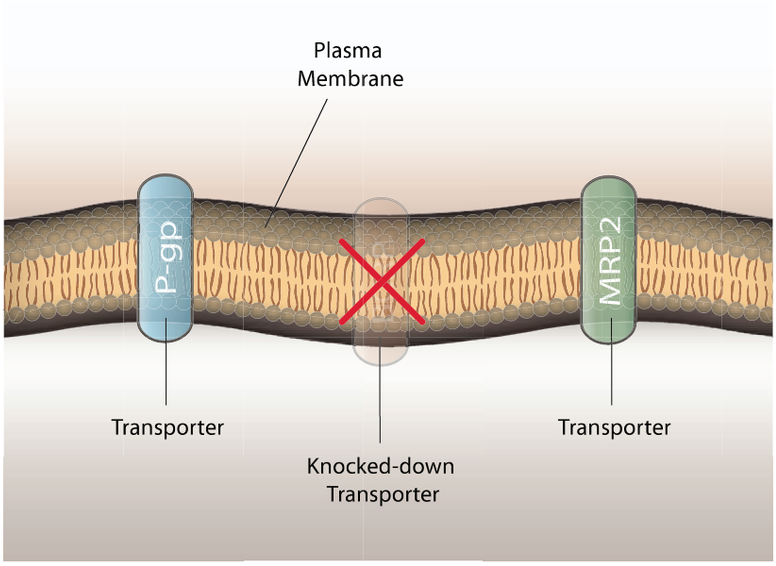

Figure 4: The 'transporter knockdown' approach to creating *in vitro* cell-based assay systems results in a versatile model that can be used in two different ways, as described in the text

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cyclosporine A (CsA), a known inhibitor of P-gp, provides further confirmation that the test compound is a P-gp substrate.

The BCRP knockdown cell line can not only be used to identify P-gp substrates without BCRP interference, it can also be used to identify compounds that are substrates of the BCRP transporter. This can be achieved by performing parallel bidirectional transport assays using the BCRP knockdown cell line in combination with parental Caco-2 cells and comparing the results. Efflux of compounds that are BCRP substrates will be higher in the parental cells than in the cells with a silenced BCRP gene.

An all-human *in vitro* cell-based assay system offers a clear advantage over other cell-based models in which human transporters are over-expressed in non-human cell lines. Interpretation of the results in non-human cell-based systems is complicated by the potential for interference from animal transporters. This is not an issue using human Caco-2 cells.

Conclusions

The ability to manipulate the expression of transporters and to identify the activity of a single transporter in a human cell line has led to the development of a series of cell-based assays for use in preclinical testing to characterise the transporter mechanisms affecting experimental drugs and to screen each drug against the major transporter proteins. This yields a profile that can be used to predict drug interactions that would otherwise not be suspected until the drug is tested in clinical trial participants or even post-marketing when it is routinely prescribed to patients.

The FDA and the pharmaceutical industry recognise the need for more predictive *in vitro* model systems that can evaluate the ADME properties of experimental drug compounds before they are tested in human subjects. *In vitro* systems that mimic human physiology can overcome some of the problems associated with studying drugs in non-human cell lines or in live animals and then using the data to predict how the drugs will be handled in humans. Assays that accurately represent the processes in the human body and can predict how drugs will interact *in vivo* offer a reliable means of determining a drug's ADME properties in the laboratory. Using this information to predict the possibility of poor drug efficacy, poor permeability, diminished bioavailability, unwanted side effects, and toxicity can prevent costly and dangerous drug failures in the later stages of clinical development.

Drug-drug interactions are an important contributor to drug toxicity and the failure of experimental compounds in development. The ability to screen for DDIs during preclinical studies in model systems that are predictive of human outcomes is an important advance toward the goal of predictive *in vitro* testing. Recognition of the role that transporter proteins play in DDI risk and the potential for multi-drug interactions involving both transporters and CYPs has led to a critical need for new *in vitro* screening systems capable of predicting DDIs involving transporters. DDW

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