Section I: The Role of Transporters in ADME

CHAPTER 1

Membrane Transporters: Fundamentals, Function and Their Role in ADME

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1.1 Introduction

In the middle of the 20th century, the absorption, distribution, metabolism and elimination (ADME) of pharmaceutical drugs was considered to be mediated primarily by simple diffusion and metabolism. The concept that membrane transporter proteins existed and could facilitate the flux of molecules across eukaryotic cell membranes was still in its infancy, but as knowledge and information expanded, it was recognised that transporter proteins could play an important role both in the movement of endogenous

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compounds within the body and in the ADME of drugs. Two superfamilies of transporters, the ATP binding cassette (ABC) and the solute carrier (SLC), comprising between them over 500 members, have now been identified in the human genome, although only a few transporters of specific interest to the pharmaceutical industry are described here.

This chapter summarises the discovery of transporters, their function in cellular processes, location and mechanism(s) of action, as well as outlining the key transporters currently considered to be clinically relevant. A description of how and why they are evaluated within drug discovery and development is included, outlining some of the key pharmacokinetic (PK) concepts useful to transporter scientists, and briefly discussing the methods and strategies used. While there are many different transporters within the body, this overview concentrates primarily on those transporters currently known to influence drug ADME and will not cover the transport of oligonucleotides or proteins. Links to current transporter databases and reviews are also included throughout the text for those wishing to pursue the area further.

1.2 The History of Transporter Science

1.2.1 The Discovery of Transport Processes

The basic functional unit of eukaryotic organisms is the cell, with each cell enclosed by a plasma membrane that forms an inherent physical barrier to the free transport of solutes. While small hydrophobic molecules are able to move freely across these phospholipid membranes by simple diffusion, the more water-soluble molecules require the presence of membrane proteins or channels embedded within the plasma membrane to gain access into and out of cells. This concept of transport *via* membrane proteins ('transporters' or 'drug transporters') and their involvement in the ADME of small drug molecules was first noted in the 1950's, although there were much earlier indications that transport processes may be present within the body.

Bile salts are highly amphipathic molecules that are synthesised and secreted by the liver and considered as model compounds for enterohepatic circulation.[1,2] At the turn of the 18th century, Mauritius Reverhorst and Alfonso Borelli reported that the amount of bile recovered in faeces was much less than the amount of bile produced by the liver, and they coined the term "*motus circularis bili*" (or enterohepatic circulation, the cycling of compounds between the intestine and the liver).[3] This insight into the selective preservation and recycling of bile can be viewed as an early but major step towards the appreciation of transport processes for solutes. Focusing on specific mechanisms, Tappeiner noted in the 19th century that bile salts are preferentially absorbed in the ileum of dogs, but not in the upper part of the small intestine, suggestive of a transporter-mediated process.[4] In 1923, Eli Kennerly Marshal demonstrated the active secretion of

phenolsulfonephthalein in the kidney, which may be considered the first demonstration of active (rather than passive) transport.[5] Prior to this, phenoltetrachlorophthalein was used as a liver function marker.[6] This work illustrated not only that both the liver and kidney are important drug clearance organs, but also that they can be selective, indicating the presence of specific, active mechanisms in these organs.[7] However, it was not until 1958 that Crane published his observations on active transport, demonstrating that 6-deoxyglucose can be transported against a concentration gradient[8] and subsequently that sugar absorption is dependent on the presence of sodium[9] and ultimately that the process is electrogenic.[10]

Applying the knowledge from these early findings, the very short half-life of penicillin in patients, concomitant with its rapid appearance in urine,[11] was believed to be caused by active tubular secretion of the drug in the kidney. It was therefore reasoned that *para*-aminohippurate, also known to be secreted by the kidney, may interfere with the secretion of penicillin. The subsequent finding that co-administration of *para*-aminohippurate with penicillin resulted in a marked retention of penicillin in the blood of dogs[12] may well be considered the first application of transporter research to ADME. These findings led to the development of probenecid, which inhibits penicillin secretion in the kidney and thereby prolongs its plasma half-life,[13,14] although the specific transporter proteins involved were not identified until many decades later. At the same time, it was discovered that probenecid enhanced renal urate elimination,[15,16] and probenecid is still used today as an uricosuric drug.[17]

In the 1960s, following the development of the first chemotherapeutic drugs for the treatment of cancers, it was noted that some tumours developed resistance against these drugs. This was followed by descriptions of cross-resistant cell lines[18] and the isolation and characterisation of several multidrug resistant cell lines, as reviewed by Gottesman and Ling.[19] For example, a Danish group reported on active drug export from Ehrlich ascites tumour cells[20] and Ling and Thompson, whilst isolating cells resistant to colchicine, observed that these cells were resistant to other chemotherapeutic drugs with different pharmacodynamic (PD) properties. The authors concluded that this cross-resistance was caused by a reduced permeability of the cell lines to drugs[21] and identified a glycoprotein of about 170 kDa that was only expressed in colchicine resistant and not in revertant colchicine sensitive cells. They named this protein P-glycoprotein (P-gp), whereby ''P'' designated permeability. Sequencing of a mouse multidrug resistance protein (MDR) sequence[22] demonstrated that P-gp is a member of the ABC superfamily of transporters, subfamily member B1 (*ABCB1*), which had previously been identified.[23] Purification and reconstitution of human multidrug resistance protein 1 (MDR1) finally demonstrated that MDR1 displayed drug-stimulated ATP hydrolysis[24] and drug transport.[25] Hence, P-gp is also known as MDR1 and ABCB1. These discoveries led to a series of programmes over many decades testing clinical *in vivo* inhibitors of P-gp to overcome multidrug resistance in chemotherapy, with limited success.[26]

1.2.2 The Development of Transporter Science in Industry

Drug transporters first came to the attention of drug discovery and development specialists in the drug formulation and drug metabolism and PK (DMPK) fields, as these are disciplines that characterise how the body handles pharmaceutical compounds. Arguably, the earliest appreciation of differential drug–tissue distributions (and later of drug transport mechanisms) in the DMPK field arose with the development of semi-quantitative radiolabelled drug–tissue distribution studies and later quantitative whole body autoradiography in rats.[27,28] Distribution studies revealed that, for some drugs, the tissue concentrations of drug related material were much greater than blood concentrations, sometimes by many orders of magnitude, whereas in others, drug related material in tissues was virtually undetectable. These observations were presumed to be due to the physicochemical properties of the molecules (lipophilicity, charge at pH 7.4, solubility, *etc.*) and/or biological phenomena such as metabolism, and plasma and tissue protein binding.[29,30] However, with the discovery of P-gp, these assumptions began to be re-evaluated. Over a period of time, a body of evidence emerged, demonstrating that many marketed drugs were in fact both *in vitro* substrates and inhibitors of P-gp. This raised concerns that P-gp could influence drug absorption, distribution and elimination (ADE), and therefore be a potential mechanism driving both systemic and target organ exposure, as well as drug–drug interactions (DDIs). Given its abundant localisation at the blood–brain barrier, P-gp was most often evaluated as a modulator of drug penetration of the central nervous system (CNS),[31–35] becoming the first drug transporter routinely investigated during drug discovery and development in the pharmaceutical industry.

As the science, understanding and discovery of drug transporters and their function continued to develop, it became clear that a number of pharmaceutical products were substrates, inhibitors and inducers of multiple drug transporters of different types. These included widely prescribed drugs such as digoxin, transported by P-gp and the organic anion transporting polypeptide member 4C1 (OATP4C1);[36–38] metformin, transported by organic cation transporter member 1 (OCT1) and multidrug and toxin extrusion proteins (MATEs);[39–43] 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA) inhibitors, transported by OATP1B1;[44] fexofenadine, transported by OATPs and P-gp;[45,46] and acyclovir, transported by organic anion transporters (OATs),[47] to name just a few. One notable example is that of rosuvastatin, a HMG-CoA reductase inhibitor (also known as a 'statin') with a high distribution into the liver that was shown to be mediated *via* transporter dependent mechanisms.[48] Subsequent work showed that rosuvastatin and other statins were substrates of some of the OATPs, members of the SLC organic anion transporter (*SLCO*) gene family.[49] This and other work led to an explosion of interest in hepatic transporter systems, as it became clear that a number of DDIs between statins and other molecules could be readily explained by considering hepatic OATPs and other drug transporters, such

as breast cancer resistance protein (BCRP; the protein product of the *ABCG2* gene), multidrug resistance associated protein 2 (MRP2; the protein product of the *ABCC2* gene), and the organic anion and cation transporters (OATs and OCTs; protein products of the *SLC22* gene family).[50–54] Even some molecules ultimately cleared by metabolism were discovered to be clinical substrates of drug transporters, which could result in the restriction or enhancement of systemic exposures and thereby influence the drugs' PK. For example, atorvastatin is a substrate of OATPs and P-gp, is primarily eliminated as metabolites, but is subject to DDIs when co-administered with transporter and/or metabolism inhibitors.[55]

Several findings regarding other transporter-mediated processes were also noted, and there are now many examples of the PK of marketed drugs being influenced by the action of drug transporters.[56] The clinical consequences of many of these interactions are still being investigated, but continue to result in changes and warnings on labels for drug use. This topic is discussed in more detail in Chapter 11. As knowledge of transporter tissue distribution and localisation grew, particularly for barrier tissues such as the gastrointestinal tract (GIT) and in the major clearance organs (kidney and liver), there was a growing appreciation that drug transporters could significantly influence drug PK.[57–60] This was particularly true for molecules with low simple diffusion (see Figure 1.1a), as this limits their transfer across cellular membranes. In these cases, drug transporters were found to be an important mechanism for oral absorption,[61,62] systemic clearance,[63] and renal and faecal excretion.[40] Furthermore, evidence that drug molecules were substrates, inhibitors or inducers of multiple transporters and drug metabolising enzymes (DMEs), challenged commonly held assumptions that associated clinical DDIs were due to a single mechanism, and led to the re-evaluation of these DDIs.[64–67] However, identifying the precise contributions of multiple mechanisms to overall clinical PK remains a remarkably challenging goal. This is due in part to limitations of the current preclinical toolkit, as well as the reliance of PK on measurement of drug concentrations in the blood compartment rather than within organs or tissues.[57,58,63,68] While subtle changes in systemic drug concentrations can be measured, they may not always reflect much larger concentration changes in tissues and organs, which may result in unpredicted toxicology and/or pharmacology.[69]

These findings raised many questions for drug discovery and development scientists, as they alerted the scientific, medical and regulatory communities that they could have relevance to drug safety, efficacy and toxicity. A good example of the depth and breadth of a typical investigation now is that of bosentan DDIs and drug induced liver injury (DILI). Bosentan is a non-peptide dual endothelin receptor antagonist that is used to treat pulmonary arterial hypertension.[70] It is metabolised in the liver to three major oxidative metabolites,[71] undergoes biliary elimination[72] and was shown to be a substrate for OATPs expressed in human hepatocytes.[73] Studies in rats indicated that cyclosporine inhibited the uptake of bosentan into hepatocytes *via* members of the rat OATPs (rOATPs),[74] and *in vitro* studies identified

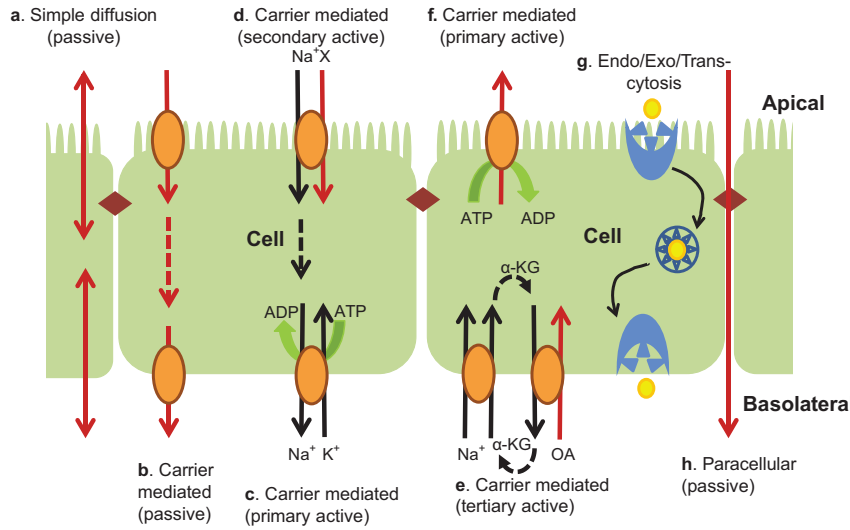

Figure 1.1 Mechanisms of transepithelial permeability. Ovals: transporter proteins; diamonds: tight junctions between cells; black arrows: direction of transport of solutes providing the driving force(s) for substrate transport; red arrows: direction of substrate (drug) transport; α-KG: alpha-ketoglutarate; OA: organic anion; X: solute/drug substrate. See text for further details.

rifampicin as an inhibitor of rat and human OATPs expressed in hepatocytes.[75,76] Several drugs were subsequently tested for PK interactions on co-administration with bosentan,[73,77] including cyclosporine[77] and rifampicin.[78] Both of these compounds increased the minimum blood concentrations of repeat-dose bosentan, suggesting inhibition of hepatic uptake and/or metabolism of bosentan by these drugs. Additionally, long term treatment of patients with rifampicin led to a reduction in serum concentrations of bosentan, which could be explained by the induction of cytochrome P450 (CYP450) 3A4 (CYP3A4),[78] which generated a metabolism-driven sink for bosentan in hepatocytes, stimulating its OATP mediated uptake.

In terms of DILI, elevated liver enzymes,[79,80] preceded by an increase in serum bile salts, were observed in patients receiving bosentan, which resolved on withdrawal of the drug. Interference with bile salt elimination, possibly through modulation of the bile salt export pump (BSEP; protein product of the *ABCB11* gene) was suspected. In addition, in a chronic heart failure trial, bosentan and glibenclamide were found to act synergistically on liver injury, elevating serum bile salt levels. This was also confirmed in rats.[80] *In vitro* investigations using isolated rat canalicular plasma membrane vesicles and vesicles from the insect cell line Sf9 over-expressing the rat bile salt export pump (rBSEP) demonstrated that bosentan and its three metabolites competitively inhibited rBSEP and human BSEP mediated

Table 1.1 Human SLC and ABC transporters currently considered of relevance to drug disposition.

Gene symbol	Protein name	Other/old protein name	Regulatory recommendations
SLCO1A2	OATP1A2	OATP-A, OATP	
SLCO1B1	OATP1B1	OATP-C, OATP2, LST-1	EMA/FDA/PMDA
SLCO1B3	OATP1B3	OATP8	EMA/FDA/PMDA
SLCO2B1	OATP2B1	OATP-B	
SLC10A1	NTCP	—	
SLC10A2	ASBT	ISBT	
SLC15A1	PEPT1[a]	Oligopeptide transporter 1	
SLC15A2	PEPT2	Oligopeptide transporter 2	
SLC22A1	OCT1	—	Consider for EMA, PMDA
SLC22A2	OCT2	—	EMA/FDA/PMDA
SLC22A4	OCNT1	ETT	
SLC22A5	OCTN2	CT1, CDSP	
SLC22A6	OAT1	PAHT, ROAT1, NKT	EMA/FDA/PMDA
SLC22A8	OAT3	—	EMA/FDA/PMDA
SLC22A12	URAT1	OAT4L, RST	
SLC29A1	ENT1[a]	—	
SLC29A2	ENT2[a]	—	
SLC47A1	MATE1[a]	—	PMDA, consider for EMA/FDA
SLC47A2	MATE2[a]	—	PMDA, consider for EMA/FDA
SLC51A	OSTα	OST alpha	
SLC51B	OSTβ	OST beta	
ABCB1	MDR1	P-gp	EMA/FDA/PMDA
ABCB4	MDR3	Phospholipid floppase	
ABCB11	BSEP[a]	SPGP	Consider for EMA, FDA, PMDA
ABCC1	MRP1	GS-X	
ABCC2	MRP2[a]	cMOAT	Consider for PMDA
ABCC3	MRP3[a]	MOAT-D	
ABCC4	MRP4[a]	MOAT-B	
ABCG2	BCRP	MXR	EMA/FDA/PMDA

[a]Emerging transporters, as referred to by the ITC.[103] Although not specifically recommended within the guidance for evaluation (apart from MATEs), many are now being 'considered'. Guidelines can be found on the following websites: EMA: www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf; FDA: www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf; PMDA: www.pmda.go.jp/english/. For a more complete list of transporters, readers are referred to the following websites: SLC transporters: http://www.genenames.org/cgi-bin/genefamilies/set/752 or www.bioparadigms.org/slc/intro.htm; ABC transporters: http://www.genenames.org/cgi-bin/genefamilies/set/417; PMDA: Japanese Pharmaceuticals and Medical Devices Agency.

transport of the bile salt taurocholate.[80,81] Glibenclamide also competitively inhibited rBSEP[82] and, as the serum markers for liver injury in patients spontaneously resolved after discontinuation of bosentan, the molecular mechanism of liver injury was linked to drug-induced cholestasis (*i.e.* the slowing or blockage of bile flow).[80,83] *In vivo* studies with bosentan in rats indicated no change in bile salt output, but there was stimulation of bile flow by bosentan mediated by rat MRP2 (rMRP2).[84] This was confirmed

in vitro using human MRP2 and BSEP expressed in insect cells, indicating that bosentan inhibited BSEP but activated MRP2 transport.[85] Such a functional MRP2–BSEP interaction has also been observed for estradiol-17β-glucuronide[82] and gives an indication of the complexity of interactions that can occur for compounds that are substrates and/or inhibitors of membrane transporters.

Recently, it has been asserted that all solute transport across cell membranes is *via* transporter proteins, and that simple diffusion does not happen.[86,87] It should however be kept in mind that cells are unable to control simple diffusion and hence are vulnerable to the entry of potentially toxic substances, unless they possess protective mechanisms. Efflux transporters may limit the entry of such toxins, and indeed MDR1 has been dubbed a ''vacuum cleaner'', because of its ability to efflux substrates from the cell membrane to the external environment, thus protecting the cell.[88,89] Such considerations may support the concept that all solutes enter (or are expelled from) cells by transporter proteins. This is disputed by other workers in the field[90,91] and remains a matter of debate.

The interplay between metabolic enzymes and transporters is also considered to be of potential importance, as discussed in *Drug Transporters: Volume 2: Recent Advances and Emerging Technologies*, Chapter 4. It has been highlighted in regulatory guidance for DDIs[92–94] and is an active area of study for both academic[95,96] and industrial scientists.[63,97,98]

A list of drug transporters of current (2016) interest to the pharmaceutical industry is given in Table 1.1. The reader is advised that this list is not comprehensive and will almost certainly be updated as further advances and discoveries are published. It is recognised that as well as their direct impact on PK, drug transporters also influence other clearance mechanisms of ADME, such as the action of DMEs. Additionally, their expression in multiple barrier and clearance organs presents a major technical and logistical challenge to the pharmaceutical industry and to clinicians.

1.3 Transporter Form and Function

1.3.1 Transporter Families and Nomenclature

Transporters are large proteins (40–200 kDa) located in the plasma membrane of cells and organelles. They normally span the membrane many times and modulate the transfer of xenobiotics (including nutrients, micronutrients and pharmaceuticals), and endogenous substances such as neurotransmitters, hormones, signalling molecules, vitamins *etc*. across cellular membranes, tissues or organ barriers. There are two transporter superfamilies, the ABC transporters and the SLCs, numbering in excess of 500 members between them. Until the late 1990s, gene and protein nomenclature of transporters was haphazard and sometimes conflicting, resulting in multiple names for the same transporter, and occasionally the same name for different transporters. The Human Genome Organisation

(HUGO) Gene Nomenclature Committee (HGNC)[99] is now responsible for approving unique gene symbols and protein names; however, older publications still carry redundant names. Symbols for human and rodent proteins are given in all capitals (*e.g.* MDR1, OATP2B1, OCT1) while their corresponding gene symbols are always in italics and in all capitals for human genes (*e.g. ABCB1*, *SLCO2B1*, *SLC22A1*) and in lower case with an initial capital for rodents (*e.g. Abcb1*, *Slco2b1*, *Slc22a1*).

The transporters given in Table 1.1 include both the transporters quoted in the current regulatory guidance[93,94,100] and those considered to be of emerging importance in the pharmaceutical industry in 2016, which may be incorporated into future regulatory guidance. Additional information may be obtained by consulting white papers and the outputs from professional collaborations such as the International Transporter Consortium (ITC), which are regularly updated.[101–104]

1.3.1.1 ABC Superfamily

In mammals, there are seven families of ABC transporter genes (*ABCA* to *ABCG*), encoding 48 individual transporters (see the HGNC website for further details).[99] They are responsible for transporting a wide range of endogenous substrates, including conjugated bile salts, steroid hormones, cholesterol and unconjugated bilirubin. For drug transport, the *ABCB* family with MDR1 (*ABCB1*; also known as P-gp or ABCB1) and BSEP (*ABCB11*), *ABCC* family with the MRPs (*ABCC1* to *ABCC6*), and *ABCG* family with BCRP (*ABCG2*) are the most clinically relevant. All mammalian ABC transporters are efflux transporters and transport is driven by ATP hydrolysis. A functional ABC transporter consists of two transmembrane domains (TMD1 and TMD2), each fused at the C-terminus to a nucleotide binding domain (NBD1 and NBD2). These two units may be fused as a single protein or combined as homo- or hetero-dimers to form functional transporters. In addition, some members of the *ABCC* family contain an extra TMD (TMD0) at the *N*-terminus, spanning the membrane five times, which is connected by the cytoplasmic loop L0 to the TMD.[105]

1.3.1.2 SLC Superfamily

SLCs are classified into 52 different families based on their amino acid identities. Full listings of the families and their known or suggested function can be found in the HGNC and Bioparadigms websites.[106,107] They have recently been summarised in a special issue of *Molecular Aspects of Medicine*.[108] The different families are responsible for the transport of a hugely diverse range of biologically important molecules, including sugars, amino acids, peptides, inorganic ions, organic anions and cations, metal ions, electrolytes and neurotransmitters, *etc*. They also transport, or are inhibited by, many drug molecules. Families that have been identified as important for drug transport include *SLCO*, *SLC22* and *SLC47*. The *SLCO* family

members, including OATP1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*), *SLC22* members OCT1 (*SLC22A1*), OCT2 (*SLC22A2*), OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*) for drug uptake, and *SLC47* members MATE1 (*SLC47A1*) and MATE2K (*SLC47A2*) for drug efflux, are the most relevant in the study of clinical transporter-mediated DDIs, as they transport a number of important therapeutics. It should also be noted that other family members not listed here may be of clinical importance for specific compounds. All solute transporters are integral membrane proteins with 7–14 predicted membrane-spanning domains. Members of the *SLC5* and *SLC7* families are predicted to have 14 such TMDs,[109,110] while members of the *SLCO* and the *SLC22* family have 12 TMDs.[111] Members of the *SLC3* family span the membrane only once but they heterodimerise with a transporter of the *SLC7* family to form a functional transporter.[110] Most of these predictions are based on hydrophobicity analyses but, *e.g.* in the case of SGLT1, the topology has been deduced from the crystal structure of the related *Vibrio parahaemolyticus* sodium/galactose symporter.[112] With additional crystal structures to be solved in the future, the topology and number of TMDs of many more transporters will be elucidated.

1.3.2 Driving Forces for ABCs and SLCs

Transport can be defined as the movement of solutes from one aqueous compartment to a neighbouring compartment that is separated by a phospholipid bilayer containing transport proteins or by tight junctions between cells. Different mechanisms of transport are illustrated in Figure 1.1. It has been known for a long time that small molecules (*e.g.* NO and O_2) can move across cell membranes without a transport protein. Whether this is true for all small and lipophilic molecules is increasingly questioned.[87] This movement is always along the concentration gradient and is called ''simple diffusion'' (Figure 1.1a). It is worth noting that the phrases ''passive permeability'' or ''passive membrane permeability'' are very often used in the literature to describe simple diffusion processes. If solutes are hydrophilic or carry a net charge, they are less able to penetrate cell membranes without interacting with a membrane transporter protein (Figure 1.1b–f). With respect to energy requirements, protein-mediated transport can occur in two ways: either along the electrochemical gradient of the solute and thus be passive (Figures 1.1b and 1.2A); or against the electrochemical gradient and therefore be active (Figures 1.1c–f and 1.2B). Members of the SLC family of transporters can belong to either of these classes of passive or active transport. In contrast, the ABC transporters are all primary active transporters because they directly hydrolyse ATP to pump their substrates out of the cell (Figures 1.1f and 1.2B). The driving forces for some transporters have been demonstrated and, although the principles of primary, secondary or tertiary transport discussed below are valid, the precise mechanisms for many transporters remain unknown, particularly for SLCs. Passive protein-mediated transport, also called facilitated diffusion, is always along an

electrochemical concentration gradient (Figures 1.1b and 1.2A), whereas active transport may work against an electrochemical gradient and can be primary (Figures 1.1c and 1.2B), secondary (Figures 1.1d and 1.2B) or even tertiary active (Figures 1.1e and 1.2B).

A primary active transporter is one that directly generates the energy required to move solutes against their electrochemical concentration gradient by hydrolysing ATP, such as ABC transporters (Figures 1.1f and 1.2B) or Na^+/K^+ ATPase (EC 3.6.3.9) (Figure 1.1c). Secondary active transporters often use the out-to-in sodium gradient, which is maintained by a primary active Na^+/K^+ ATPase, and couple sodium movement down its electrochemical gradient to provide substrate uptake against its electrochemical gradient (Figures 1.1c and 1.1d combined). Examples of such transporters include the different sodium dependent co-transporters responsible for cellular uptake of *e.g.* sugars,[109] amino acids,[113] dicarboxylates[114] or bile acids.[115] The organic anion transporters OAT1 and OAT3 are examples of tertiary active transport systems (Figure 1.1e). They mediate the uptake of organic anions against their electrochemical gradients into, for example, proximal tubule cells in exchange for α-ketoglutarate, which in turn is taken up into the cells *via* sodium dependent dicarboxylate transporter 3 (NaDC3; *SLC13A3*, a secondary active process).[116] The driving force is the sodium gradient, which is

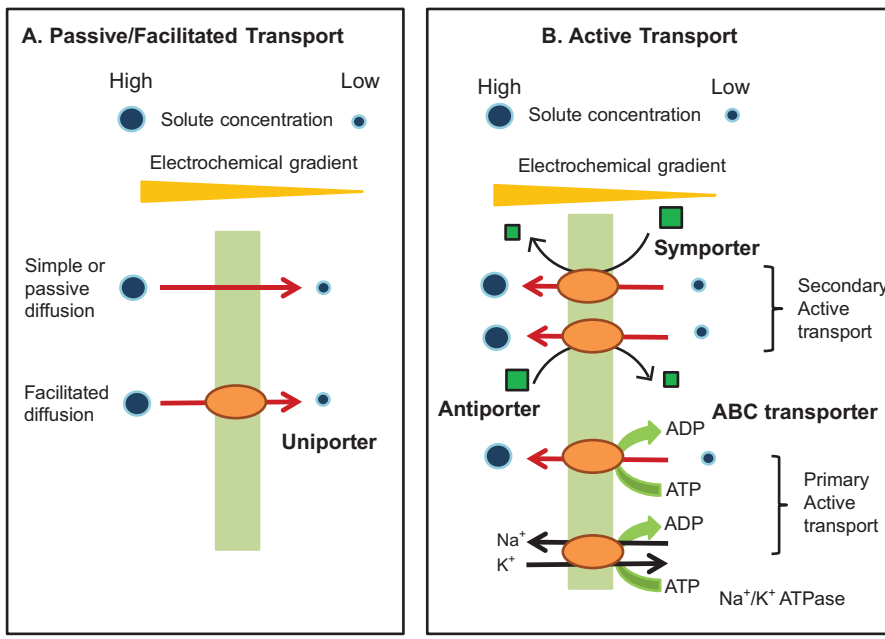

Figure 1.2 Energetics of transepithelial transport. Ovals and straight arrows: transporter proteins and the direction of solute transport; red arrows: solute/drug transport; curved arrows: co-transported solutes for symport and antiport transporters; large circles: high solute concentrations; small circles: low solute concentrations. See text for further details.

ultimately maintained by the Na^+/K^+ ATPase. All of these transporters are located in the same basolateral membrane of proximal tubule cells.[117] Although Na^+/K^+ ATPase is mentioned here, it is not in fact a member of the ABC superfamily and is not a recognised drug transporter, *per se*.

Endo- and exo-cytosis (Figure 1.1g) are primarily relevant to the transport of large biomolecules, such as polypeptides, and as such are not discussed further in this book. In addition, small ions such as Na^+ or Cl^- can move along their electrochemical gradient across tight junctions in a process called paracellular transport (Figure 1.1h). However, because this route is not an important route of drug transport under normal physiological conditions it will also not be discussed in this book.

1.3.3 Uptake, Efflux and Bi-directionality

Besides the classification based on driving forces outlined above, transporters are also classified according to their direction of transport, *i.e.* uniporters (Figure 1.2A), symporters and antiporters (Figure 1.2B), the latter two being co-transporters. Uniporters are found in most cells and mediate the uptake of sugars,[118] amino acids,[110] nucleosides[119] and other small molecules along their electrochemical concentration gradients without any coupling of this transport to that of other molecules or ions. Symporters mediate transport by coupling the movement of one molecule or ion against its concentration gradient with the simultaneous movement of another molecule or ion in the same direction down its concentration gradient. Examples of symporters are the sodium dependent glucose transporter[109] or the sodium dependent amino acid transporters.[113] Antiporters couple the transport of two different molecules or ions in opposite directions across the membrane and include for example the Na^+/Ca^{2+} exchanger in cardiac muscle cells[120] or the Na^+/H^+ exchanger involved in regulation of cytosolic pH.[121]

Under normal physiological conditions, SLC transporters mediate the uptake of solutes into cells. Exceptions to this are the MATE1 and MATE2K transporters,[122] which efflux solutes out of cells, and exchangers that can mediate the uptake of one solute coupled to the efflux of another. Examples include OAT1 mediating the exchange of *para*-aminohippurate for α-ketoglutarate,[117] or OATPs, which exchange substrates for bicarbonate.[123] In contrast, all of the mammalian ABC transporters are efflux pumps and export solutes out of the cell.

1.3.4 Substrate Specificities and Binding Sites

Membrane transporters are capable of transporting a wide range of structurally diverse substrates, often with overlapping substrate specificities, and are known as polyspecific drug transporters. Consequently, there are very few, if any, specific probes available for *in vitro* transporter studies, making

the true substrate specificity of a transporter *in vivo* difficult to delineate. However, it is possible to assess the contributions of individual transporters in the disposition of a new molecular entity (NME) using methods such as relative activity factors[124] and PK modelling.

The overlap of substrate specificities may allow transporters to compensate for the loss of another that is either absent or non-functional (*e.g.* through disease or inhibition), so-called transporter redundancy. For example, the impaired biliary elimination of bilirubin in MRP2 deficient rats can be compensated for to some extent by its removal from the liver by MRP3, although the animals remain jaundiced.[125] However, this may not always be the case; the inherited mutation in *ABCB11*, which encodes the bile salt exporter BSEP in hepatocytes leads to severe liver disease in humans in childhood. This is despite the fact that bile salts can also be exported by other hepatic transporters, such as MRP4 and the organic solute transporter (OST; *SLC51*) α/β dimer (OSTα/OSTβ), as evidenced from *in vitro* data,[126] which might be expected to compensate for the loss of BSEP.

The issue of substrate specificity is further complicated by the fact that some transporters such as the OATPs have multiple substrate binding sites.[127] For example, OATP1B1 is reported to have two binding sites for estrone-3-sulfate, but only one binding site for fluvastatin.[66] Similarly, some MRPs transport certain substrates only in the presence of glutathione,[128] hence glutathione could be considered a modulator of the substrate specificity of these transporters. In addition, the kinetics of some transporters have been shown *in vitro* to be strongly influenced by membrane composition, particularly cholesterol content, with both MRP2 and P-gp showing altered transport kinetics in different lipid environments *in vitro*. MRP2 demonstrates allosteric kinetics with a low cholesterol content and Michaelis–Menten type transport with a high cholesterol content.[129,130] The reasons why this occurs, and the precise impact *in vivo,* are unclear, but appear to be related to the interaction of substrates with the lipids in the plasma membrane. What is clear is that the kinetics of substrate–transporter interactions are likely to be more complex than for DMEs.

To date, the structure of two mammalian ABC transporters (mouse P-gp[131] and human ABCB10, a mitochondrial transporter[132]) and one mammalian SLC transporter, human glucose transporter 1 (GLUT1; protein product of the *SLC2A1* gene[133]) have been crystallised. However, of these, only mouse P-gp is important for drug transport. In addition, numerous prokaryotic ABC transporters and several prokaryotic homologues of SLC transporters[134] have been crystallised. These bacterial crystal structures have been used to predict the structures of related mammalian transporters by comparative or homology modelling (*e.g.* for OATPs,[135] OAT1[136] and OCT1[137]). It has to be emphasised, however, that such models can have major limitations when homologues with less than 30% amino acid sequence identities are used, and therefore should only be used to formulate hypotheses, *e.g.* with respect to the location of charged amino acids that can then be tested experimentally using site-directed mutagenesis and functional assays.[138]

1.3.5 Transporter Localisation and Interplay

Membrane transporters are located in every cell in the body, although not necessarily just on the plasma membranes (Figure 1.3). They may also be found intracellularly (*e.g.* on mitochondrial membranes), but from an ADME perspective it is usually the plasma membrane transporters that are the main focus of interest, as reflected in the current regulatory guidance.[93,94,100] Some transporters can be exclusively expressed in one organ (*e.g.* OATP1B1 in the liver), some ubiquitously (*e.g.* OCT3) and others can have a restricted distribution (*e.g.* MATE1 in the liver and kidney) (Figure 1.3). Thus, to fully understand their role in drug PK, the location and expression of transporters at the organ, cell and sub-cellular levels needs to be determined. This in itself is challenging, for example: transporter expression levels are low relative to other proteins (*e.g.* DMEs), making quantitation difficult; specific antibodies are not routinely available for

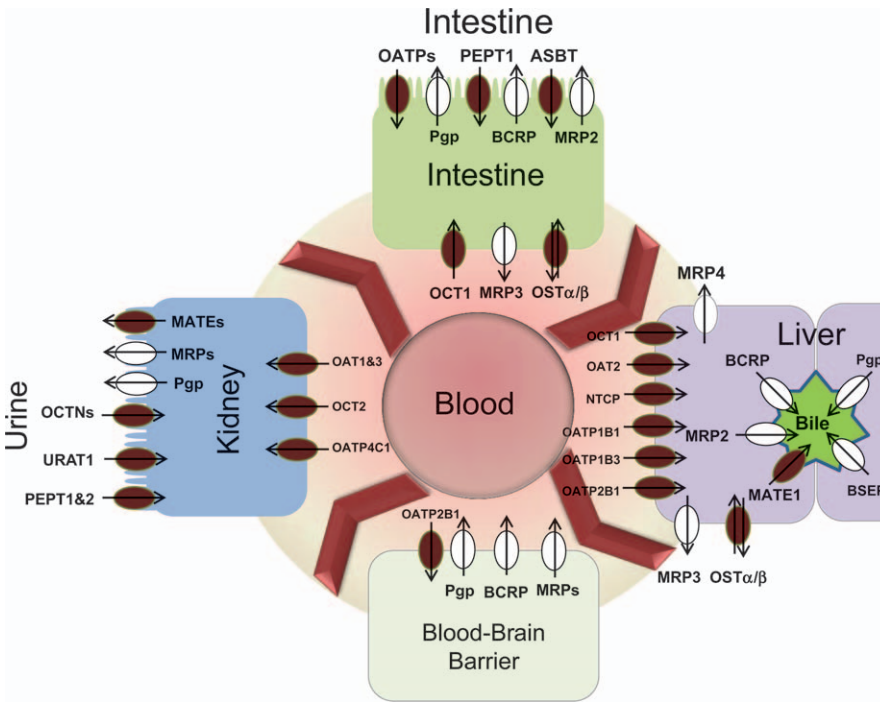

Figure 1.3 Schematic showing type, orientation, localisation and direction of transport of some major drug transporters in selected tissues. Transporters on the blood side of the cell concentrate substrates from the blood (SLC) or efflux substrates from the cell (ABC). Transporters on the non-blood side of the cell eliminate substrates from the cell (ABC and MATEs), or in the case of the GIT and kidney, facilitate uptake or re-uptake of substrates from the lumen (SLCs). Arrows: direction of transport of substrates; white ovals: ABC transporters; brown ovals: SLC transporters. See text for further details.

immunoquantification; and low yields of transporter protein in the membrane isolates used for quantitative techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS) can result in poor estimates of mass balance.[139]

The localisation of some key ADME transporters in human tissues are outlined schematically in Figure 1.3, which gives an indication of their distribution and orientation on specific membranes (apical or basolateral) of polarised cells such as hepatocytes or proximal tubule cells. This localisation of transporters is key to their biological function within those cells, tissues or organs.

For example, P-gp is expressed on the apical surface of cells and effluxes its substrates into the lumen of the organ where it is expressed. This means that in the enterocytes of the GIT, it effluxes substrates back into the GIT, thereby potentially limiting oral absorption. By contrast, in the liver, P-gp delivers substrates into the biliary tract from the hepatocytes, maintaining low intrahepatic concentrations of substrates extracted from the blood. At the blood–brain barrier, P-gp effluxes substrates into the blood, thus protecting the brain from excessive drug exposure. Thus, a substrate of P-gp may have restricted oral absorption, enhanced biliary elimination and restricted CNS distribution. It is important therefore to consider transporter tissue distribution when evaluating the significance of a transporter–drug interaction.

The spatial distribution of transporters within or along the tissue or organ (*e.g.* small intestine to colon) is also relevant to their net impact. There are numerous instances of differential tissue distribution of transporters, for example P-gp, BCRP and OATP expression along the GIT,[140–142] and OATs, OCT1 and MATEs along the kidney proximal tubule.[143] This type of distribution along the length of luminal structures facilitates the selective uptake, efflux, re-uptake and recirculation of transported substrates, such as creatinine,[143,144] bile acids and salts,[145] and uric acid.[117,146] In addition, the balance of expression of different transporters acting in the same or opposing directions (*e.g.* MRPs and OATPs in the basolateral membrane of hepatocytes) may be relevant to their overall physiological impact.[46,147–149]

In the liver and GIT, transporters such as P-gp are co-expressed with DMEs, notably CYP3A4.[96,140,150] This has relevance in drug absorption, metabolism and elimination, and the DDI potential in those organs, as there is an apparent synergistic impact of these proteins on some processes. For example, the rate of appearance of a drug substrate in the enterocyte (and therefore presentation to the metabolising enzyme) will be decreased and/or delayed due to the influence of P-gp activity on drug permeability across the luminal membrane of the GIT; thus, intracellular drug concentrations may remain at sub-saturating levels for CYP3A4. A similar process is proposed for hepatic elimination, albeit that localisation of P-gp and CYP3A4 relative to the sequence of exposure to the drug is reversed.[96,97,150] Although the precise relationship and therefore the net impact on drug ADME is a matter of some debate, it has been demonstrated in genetically modified mice that the absence of either P-gp, Cyp3a or both results in substantial changes in the PK of drugs that are substrates for both mechanisms.[95]

1.3.6 Transporter Expression in Animal Species

In preclinical ADME studies, several different animal models and/or primary or immortalised cell lines from animal tissues can be used to assess drug–transporter interactions. Rodents, particularly mice, are a frequently used model.[39,101,151,152] Other species have also been used, although not routinely (*e.g.* dog[153] and monkey[154]). A typical objective of preclinical species studies is to aid in understanding human drug–transporter interactions, which may otherwise be difficult or impossible to study clinically. It is therefore important to be aware of differences between human and animal transporter expression, tissue distribution and function.

In vitro experiments suggest that transporters from different species have broadly similar substrate activities, and possibly affinities, although it has been reported that intrinsic transporter activity may differ between species (*e.g.* MRP2[155]). Genomic and *in vivo* data demonstrate some fundamental differences in transporter expression and distribution profiles across species, resulting in profoundly different PK between species for some molecules. This can have important implications for predicting the impact of human transporters on drug PK using data from preclinical species.

MDR1A and MDR1B in rodents are represented by MDR1 (P-gp) in humans, and both transporters must be eliminated (genetically engineered out, so called ''knockout'' mice, or chemically inhibited, so-called ''chemical knockout'') to reflect the impact of P-gp on human PK in mice.[31] The dual MDR knockout mouse model (*Mdr1a*$^{-/-}$/*Mdr1b*$^{-/-}$) has been used routinely and successfully to investigate, for example, P-gp-modulated CNS penetration of drug molecules, and has been extended to include BCRP and MRP2, creating triple knockout mouse models.[156–158] However, this relatively straightforward correlation is not applicable for all transporters. For instance, OCT1 and OCT2 are predominantly expressed in human liver and kidneys, respectively, whereas murine and rat orthologues are highly expressed in both tissues, resulting in substantially different excretion profiles in rodents *versus* humans for substrates of these transporters.[39]

A more complex scenario again is OATP and Na–taurocholate co-transporting polypeptide (NTCP; the gene product of *SLC10A1*) expression in hepatocytes. These transporters are responsible for the uptake of bile acids from the blood into the hepatocyte, and therefore important components in bile homeostasis, as well as being notable drug transporters. Although NTCP is expressed in both rodent and human hepatocytes, two members of the OATP1A family (OATP1A1 and OATP1A4) and OATP1B2 are expressed in rodents. Conversely, in humans, the OATP1B family predominates (OATP1B1 and OATP1B3) and OATP1As are absent.[159] Knockout of all of the rodent/murine *Oatp* genes is required to create a model that will give some indication of the net impact of contribution, loss or inhibition of these transporters in humans. However, ablation of the genes may not result in an otherwise ''normal'' animal, as these manipulations may lead to unpredictable alterations in the expression or function of other (transporter)

proteins, as illustrated by van de Steeg *et al*.[160] and Slijepcevic *et al*.[161] Van de Steeg *et al*. showed that *Oatp1a*$^{-/-}$ and *Oatp1b*$^{-/-}$ knockout mice have elevated unconjugated bile acids compared with their wild-type littermates, indicating that NTCP is unable to compensate for the loss of the *Oatp* genes.[160] Similarly, characterisation of a NTCP knockout mouse strain showed changes in the expression and functional levels of a number of other bile acid transporters in the GIT, liver and kidney, including OATPs, impacting enterohepatic bile acid homeostasis *in vivo*.[161] This is also observed in humans, *e.g.* in a patient with non-functional NTCP, serum bile salt levels above 1 mM were found, demonstrating that OATPs cannot compensate for the loss of NTCP function despite their *in vitro* capacity to transport bile salts.[162]

Transgenic models, where the human gene has been added (''knock-in'') or has replaced the rodent version(s) (''humanised'') are increasingly available.[151] However, to demonstrate their utility in predicting human outcomes, they require careful validation. This is expanded upon in Chapter 8. Although preclinical species and genetically modified animals may have limited utility for clinical predictions or translations, and are highly dependent on the transporter(s) being tested, they are nonetheless useful tools to establish the *in vivo* impact of transporter interactions.[31,58,147]

1.3.7 Other Factors Affecting Transporter Form and Function

1.3.7.1 Regulatory Mechanisms

As for all membrane proteins, transporters are synthesised at the endoplasmic reticulum (ER), where post-translational modification begins. From the ER, transporters are shipped *via* the Golgi, where further post-translational modifications occur prior to delivery to their final destination, which is often the plasma membrane. Exit from the ER may be regulated for some transporters, hence affecting their expression levels at the plasma membrane, for example, the cystic fibrosis transmembrane conductance regulator.[163] Functional transporter activity may be conferred during trafficking to the plasma membrane, as has been demonstrated for Na^+/K^+ ATPase, which is not fully functional until it arrives at the cell surface.[164] Post-translational modifications, *e.g.* by phosphorylation or dephosphorylation of proteins, are important regulators of the activity of transporters. For example, the state of phosphorylation/dephosphorylation determines the protein levels of the bile acid transporter NTCP at the basolateral membrane of hepatocytes by cycling the transporter between an endosomal compartment and the plasma membrane.[165] The phosphorylation status of NTCP is regulated by several signalling pathways, including cyclic adenosine monophosphate, nitric oxide, Ca^{2+} and others.[165] Similarly, phosphorylation of OATPs may, depending on the transporter, lead to down-regulation of its

activity or to its internalisation from the plasma membrane.[166] However, intracellular transporters may not always be functionally inactive; *e.g.* in the vinblastine-resistant human cervical carcinoma cell line KBV1, MDR1 is functionally expressed in lysosomes.[167] In addition, ABCA3 (involved in surfactant expression) is expressed in lysosome-like structures in alveolar type II cells,[168] while in BCR/ABL-positive leukemic cells, it is expressed in the lysosomal membrane, regulating imatinib sequestration.[169]

Cells can adapt protein expression levels for DMEs and transporters at the transcriptional level in response to extrinsic factors. Typically, this is achieved by nuclear receptors sensing intracellular ligand levels. These ligands can be drugs, environmental chemicals or endogenous ligands such as bile salts. The key nuclear receptors involved in regulating drug disposition are the pregnane X receptor (PXR), the constitutive androstane receptor (CAR)[170,171] and, for sensing environmental chemicals, the aryl hydrocarbon receptor (AhR).[172] The farnesoid X receptor (FXR) constitutes the bile salt sensor.[173] In order to activate transcription, these receptors need to bind a ligand and form, in the case of FXR, CAR and PXR, a heterodimer with the retinoid X receptor (RXR), and in the case of AhR, with the aryl hydrocarbon nuclear translocator.[172] This is discussed further in *Drug Transporters: Volume 2: Recent Advances and Emerging Technologies*, Chapter 2.

1.3.7.2 Pharmacogenetics

Several genetic polymorphisms (gene sequence variations that occur within a population) of transporter genes are known, some of which may cause disease, while others may have no obvious impact but are capable of influencing drug response (by increasing or decreasing the activity of the transporter protein, or by lack of expression). Those that cause disease are relatively rare (as they decrease evolutionary fitness), but do still occur, *e.g.* the genetically inherited Dubin–Johnson syndrome, where individuals have a non-functional MRP2 transporter and subsequent impairment of biliary secretion of both endogenous substrates and drugs.[174] Other genetic diseases associated with transporters include Rotor syndrome, associated with OATP mutations,[175] and respiratory distress syndrome, associated with ABCA3 mutations.[176] It should be noted that Dubin–Johnson and Rotor syndrome are benign human syndromes, meaning that such patients usually do not present with clinically relevant symptoms.

PK can be influenced by genetic variations in transporter genes (caused by single nucleotide polymorphisms (SNPs), insertions/deletions, or a gene or sequence copy number variation), but the field has not been as extensively evaluated as that of the metabolising enzymes. Recent studies have shown that some genetic variations in drug transporters can lead to changes in systemic exposure, as well as potentially affecting local (target) concentrations, although the latter is much more complex to monitor and its impact remains uncertain. Certain transporter polymorphisms appear to have only a minimal impact on drug ADME, including those of the P-gp

transporter,[177] although some studies suggest that the SNP C3435T in the MDR1 gene may impact the expression of P-gp in some tissues and be a risk factor for certain diseases.[178,179] Other genetic variants, however, appear to have a substantial impact on drug PK, particularly those of the hepatic uptake transporter OATP1B1 (SNPs c.388 A > G and c.521 T > C)[44,180–182] and the efflux transporter BCRP (SNP c.421 C > A).[183] In some instances, this may cause an increased risk of adverse drug reactions (ADRs), as has been suggested for simvastatin induced myopathy.[184] Muscle toxicity (myopathy), and its extreme form rhabdomyolysis, can occur in association with statin therapy, especially when given at high doses and with certain other medications. The SEARCH genome-wide study identified a strong association between a common variant of OATP1B1 with reduced transport activity and the incidence of muscle myopathy, suggesting that genotyping for these variants could improve the safety of statin therapy. Several other studies have linked genetic variations in transporters to variations in drug exposure and clinical response, albeit on a much smaller scale. For example, genetic variants of the organic cation transporter OCT1 have been linked to changes in the PK and PD of metformin.[185–187] Recent work using PK/PD modelling of *in vitro* data for three genetic variants of OATP1B1 suggests a way forward for predicting the effect of polymorphisms for NMEs during pharmaceutical development.[188]

It has also been noted that the frequency of genetic variants can vary between different ethnic populations, suggesting that certain populations are more susceptible to variations in drug response for some xenobiotics. This has been particularly noted for variants of *SLCO1B1*,[189] and may be responsible at least in part for the observed differences in effect of statin drugs in different ethnic populations.[190,191]

As it has become apparent that some transporter polymorphisms can be a key determinant in inter-individual variability, the more recent pharmacogenetic guidelines from regulatory authorities specifically include references to drug transporter polymorphisms, as well as for metabolising enzymes. For example, the guideline from the European Medicines Agency (EMA) recommends that differences in exposure due to pharmacogenetic factors need to be evaluated during drug development, and provides a useful framework for when further investigations may be required or recommended for NMEs.[192] Papers have also been published on the industrial pharmaceutical perspective, with references to the transporter polymorphisms thought to be most relevant to drug development at present.[193,194]

The field of transporter pharmacogenetics is an emerging and expanding area, and there are ongoing areas of research to further the available knowledge base. One institution of note is the Pharmacogenomics of Membrane Transporters project at the University of California (USA), which has obtained DNA from many different sources for further investigation of functional variants, as well as forming a group of individuals willing to be called back for future pharmacogenetic studies (the SOPHIE cohort).[195]

1.3.7.3 Age, Gender and Disease

Knowledge of the effects of age, gender and disease on human transporter expression is limited in comparison to preclinical species. Many drug transporters have been observed to have age and/or gender specific expression in rodents, which if translatable to humans, could influence the PK and may explain age or gender related PK differences. For example, mouse OATP1A1 (*Slco1a1*) and OATP1A4 (*Slco1a4*) show both developmental and gender differences in expression in mice.[196]

Gender differences in expression have been observed for many more transporters and are summarised in a number of references.[197–199] It should, however, be kept in mind that transporter expression and function, as well as gender differences, are species dependent.[200,201] For example, liver expression of *Abcg2* messenger RNA (encoding BCRP) shows gender differences in mice but not in rats.[200] Expression of OATP1A1 in the apical membrane of the proximal tubule in the rat kidney is much higher in males than females, which may explain the much higher renal elimination of taurocholate and dibromosulfophthalein in females.[202] However, the same transporter is expressed at similar levels in rat liver, irrespective of gender. Similarly, expression of messenger RNA of *Slco1a1* and *Slco1a4* in mice shows gender differences in the liver and kidney, which is also transporter specific.[196] In humans, information on gender differences in expression of both transporters and DMEs is scarce.[203,204] Such differences in expression levels may well be the molecular basis for gender differences in PK.[205]

As DME levels change radically during growth and development from the newborn to adult, so do the expression levels of transporters during ontogenesis.[206,207] In humans, information on expression changes in drug transporters is very limited.[208,209] However, considerable knowledge has been obtained for species used in drug development, such as mice and rats. Interestingly, uptake transporters take longer to achieve adult expression levels than efflux transporters in both rats and mice.[199,210]

In disease states, transporter expression levels may be affected secondary to an underlying disease. Due to its central role in drug disposition, transporter expression in various forms of liver disease has been extensively studied in animal models.[211] In humans, various forms of liver disease lead to altered expression of drug uptake and efflux transporters.[209,212,213] Due to its devastating effect on patients with cancer, expression of MDR1 has been particularly well studied in clinical oncology.[214]

1.3.7.4 Dietary, Environmental and Lifestyle Factors

Dietary effects on drug PK are well documented in the literature. Mechanisms responsible for food–drug interactions may include physiological alterations in intestinal motility, gastric pH, gastric emptying or modulation of disposition pathways by dietary constituents. Clinical food–drug interactions have been attributed mainly to hepatic drug metabolism, with

CYP3A appearing to be particularly sensitive to dietary constituents, including grapefruit juice, garlic and St John's Wort.[215] However, the contribution of drug transporters in food–drug interactions is increasingly being recognised. For example, grapefruit juice can inhibit the activity of both P-gp[216] and some OATPs,[45] and has been reported to alter the PK of cyclosporine and fexofenadine.

Little is known about the impact of environmental and lifestyle factors on transporter form and function. A recently published article describes altered expression levels of DMEs and transporters in some alcoholics,[217] although the underlying mechanism has not been elucidated. Another recent review cites epigenetic and other factors that affect the expression and function of transporters in the placenta during gestation.[218] Finally van der Doelen *et al.* suggest that early life stress and the serotonin transporter (SERT or 5-HTT, *SLC6A4*) genotype are instrumental in mediating DNA methylation of corticotrophin releasing factor, leading to altered responses to stress in adult rats.[219] This area of research will undoubtedly develop, particularly regarding the impact of epigenetic factors on gene expression.

1.4 The Transporter Toolkit

1.4.1 *In Situ* and *In Vitro* Models: Basic Concepts, Limitations and Translation

The function of transporters can be investigated using several different experimental systems.[220] Many transporters have been sequenced,[221] a number have been identified and cloned,[134,222] and they have been characterised in different recombinant expression systems including *Xenopus laevis* oocytes, insect and mammalian cell lines, and even in humanised and knockout mice.[223] Consequently, because these systems are relatively specific for the transporter of interest, and good quality controls are available, the function of individual transporters can be carefully characterised.

Across academia and industry there is a heavy reliance on *in vitro* tools in a range of different formats to evaluate transporter interactions.[223] Immortalised human cell lines such as the colonic adenocarcinoma cell line (Caco-2),[224] and animal or insect cells stably or transiently transfected with one or more human genes [*e.g.* Madin–Darby canine kidney type II cells transfected with MDR1 (MDCKII-MDR1)] are common,[225–227] forming the workhorses for transporter investigations. They are also strongly advocated by regulatory agencies because of their relative ease of use, wide availability and, in the case of transfected cells, range of transporters expressed.[92,93] Notwithstanding the above advantages, these tools require careful and thorough characterisation and validation with suitable methodologies and controls to realise their full potential.[104,225,226,228,229] Isolated primary cells (*e.g.* hepatocytes[230] and kidney proximal tubule cells[231,232]) in a number of different formats can also yield useful and sometimes very elegant data, but are more variable, challenging and expensive than cell lines. Isolated plasma

membranes (vesicles) containing the transporter of interest are a non-cell-based variant that can also be helpful, as the medium on both sides of the membrane can be accurately defined by the researcher, and concentration gradients can be established.[233,234] Some assay formats using end-points such as inorganic phosphate from the hydrolysis of ATP (ATPase assay) and a fluorescent assay, measuring intracellular production of calcein from calcein–acetoxymethyl (AM) following inhibition of P-gp efflux (Calcein–AM assay), are also available. The results are difficult to interpret, and often conflict with other more holistic assays.[235–238]

Experiments using whole organ perfusion, where transporters are working close to their native environment, or isolated cells grown on special supports or matrices, maintain many of the complex functions and interactions between proteins, but the contribution of individual transporters can be difficult to assess due to the influence of other mechanisms active in the system.[147]

Most routine *in vitro* assays measure the inhibitory potency of the NME against a well-characterised probe substrate. This approach can be very robust, and gives a good indication as to whether the NME will alter the PK of another drug *in vivo* (*i.e.* be a perpetrator of a DDI), but provides no information as to whether the NME itself is a substrate, and therefore a potential victim of a DDI. Establishing whether the NME is a substrate of a drug transporter can be challenging for a number of reasons, not least because of experimental and post-experimental variables that cannot easily be controlled. For example, the contribution of simple diffusion (Figure 1.1a) to overall transport is compound dependent, can be substantial and is difficult to correct for during data manipulation. Similarly, non-specific binding of the test drug to apparatus, cells or proteins may be difficult to monitor, making data unreliable. Post-experiment, the sample assay requires rigorous control, because analyte concentrations in donor and receiver samples of the relevant *in vitro* system may differ by many orders of magnitude, thus requiring both high sensitivity assays and rigorous sample isolation.[69]

One major problem with *in vitro* systems, and with the results obtained and published, is that not all experiments are performed under optimal conditions that allow determination of the substrate kinetics of transport.[101] For example, kinetic determinations have to be performed under so-called zero-*trans* conditions, where the function of the transporter is not limited by the external substrate concentration.[101] Thus, in instances where insufficient substrate is present, the apparent rate of transport may decrease, but only because the substrate has been depleted. Similarly, if the disappearance of the extracellular substrate concentration is measured, it is normally limiting, and the resulting data are not obtained under initial linear rate conditions. In addition, an uptake transporter may be inhibited by the increasing intracellular concentration of its substrate created by its own activity, reducing the transport rate compared with that observed under optimised conditions.[223,239] Furthermore, transporter functional expression levels should ideally be comparable across the different expression systems;

otherwise, a substrate for a given transporter that is not very efficiently transported might be classified as a non-substrate in a system with low functional transporter expression (*e.g.* estrone-3-sulfate for OATP1B3),[240] but classified as a substrate in one with much higher expression levels.[241]

It is also important to acknowledge that although many substrates and/or inhibitors can be identified using *in vitro* assays, they do not necessarily reflect what will happen *in vivo*. The presence of many other membrane-bound and soluble proteins, the (pharmaco)dynamic environment *in vivo*, and the nature of the extra- and intra-cellular matrices may be profoundly different *e.g.* hepatocytes bathed in albumin rich, circulating whole blood *versus* an albumin free uptake buffer. Therefore, it is necessary to establish and accurately record/report the general conditions under which the *in vitro* transporter assays are performed, as well as the data generated, in order to avoid misinformation due to suboptimal experimental design and/or misleading interpretation.[223]

Regardless of the technique used to generate data, interpretation is challenging, as there are few well-validated quantitative clinical translation methods available. However, physiologically based PK (PBPK) modelling tools (*e.g.* SimCyp and Gastroplus, among others) are rapidly improving and offer a realistic way forward to permit the integration of multiple-compartmental, multi-mechanistic data to model and predict clinical outcomes.[2,57,58,97,150,242,243] Nonetheless, scaling factors for transporters remain a major hurdle, as evidenced in a recent meta-analysis of OATP protein abundance across different hepatic *in vitro* models and whole liver.[244]

1.4.2 *In Vitro* Transporter Inhibition Studies

In order to determine whether a NME is an inhibitor of a particular transporter and to estimate its inhibitory potential (*e.g.* using the concentration of inhibitor required to inhibit transport by 50% (the IC_{50}) or the dissociation constant (K_i)), carefully designed experimental conditions must be maintained. For instance, probe substrate uptake should be measured under initial linear rate conditions in the absence and presence of a potential inhibitor.[223,225,233] These aspects are covered in more detail in Chapter 7. In order to characterise inhibition kinetics for a given transporter protein, substrate and inhibitor are co-applied to the *in vitro* or *ex vivo* system. This will give the most accurate estimation of inhibition kinetic constants. However, there are circumstances, *e.g.* high throughput assays, where it is not feasible to mix the inhibitors with the substrates before addition to the test system. In these cases, any subsequent secondary screens should be performed by co-administration of both inhibitor and substrate. Furthermore, it has become clear recently that several drug transporters (*e.g.* MRPs and OATPs) exhibit substrate dependent modulation; *i.e.* certain small molecules will inhibit the transport of a given substrate but they might not affect or might even stimulate the transport of other substrates.[127,129] The recent US Food and Drug Administration (FDA) guidance recommends

testing the inhibitory potential of NMEs against at least one of several prototypical substrates, but given the practical and scientific challenges outlined above, it might be more appropriate to consider testing NMEs as inhibitors of several substrates to give the greatest chance of identifying an *in vitro* inhibitor.[245]

1.4.3 *In Vitro* Transporter Substrate Studies

Substrate studies rely on the same tools as inhibition studies, but are often more challenging to execute than inhibition studies, primarily because it is not possible to optimise experimental conditions in advance to guarantee robust experimental data for every NME. Solubility, non-specific and protein binding, cell toxicity and analytical sensitivity are all compound specific aspects and are generally unpredictable, requiring careful consideration and control in the chosen assay format. Substrates are identified and characterised by comparing drug transport in the presence and absence of a chemical inhibitor or against a non-expressing control, or both.[101,223]

Substrate assessment studies fall into two groups: the binary (positive or negative interaction) assay approach, where the molecule is defined as a substrate or a non-substrate (usually in one to three concentrations);[246,247] and the kinetic approach, where a kinetic constant such as the Michaelis constant (K_m) is determined using a wider range of drug concentrations.[223,248,249] Binary assays are traditionally used for P-gp and sometimes for other ABC transporters such as BCRP, where the transporter is expressed in a cell line. They are usually reasonably robust and sufficiently high throughput to permit a basic ranking of molecules. These assays are also often used to determine simple diffusion of molecules across the plasma membrane. Binary assays are also often used to make a preliminary assessment of substrate activity prior to embarking on a more comprehensive kinetic assessment. In practice, kinetic parameters for transporters are not often generated, as the experiments are time and resource consuming. As for inhibition experiments, substrate experiments require careful design to ensure that measurements are made during the linear phase of uptake and under optimal conditions.[223]

Alternatives to traditional Michaelis–Menten approaches for characterising transporter–substrate interactions have been published. These are generally more data-rich methods, and may provide more robust and translatable data.[226,228,250,251]

1.4.4 *In Vitro* Transporter Induction Studies

The mechanisms of induction of transporter expression appear to be closely linked with those of DME induction, as many of the same receptors (CAR, PXR, RXR, *etc*.) that are implicated in DME induction also induce transporter proteins.[127,252,253] Methodologies to investigate transporter induction therefore tend to be very similar to those employed for DMEs, *i.e.* polymerase

chain reaction assays of liver samples, functional and expression assays in cultured hepatocytes, *etc*. Induction in humans has been observed clinically for some transporters.[98,254–257]

The transcriptional activation of an enzyme or transporter of interest can be studied by using the promoter of the gene of interest fused to a readout system such as luciferase in a transactivation reporter assay. Ideally, findings are confirmed using *in vivo* models, whereby the upregulation of the protein of interest is demonstrated. Transcriptional regulation can also occur indirectly by modulating the half-life of a messenger RNA.[258] Understanding the role of microRNAs in the regulation of key mechanisms of drug disposition is a very active and rapidly evolving research field.[259]

1.4.5 *In Vivo* Studies in Preclinical Species and Humans

The utility and limitations of preclinical *in vivo* models has been discussed previously (Section 1.3.6). When clinical investigations of transporter interactions are considered, they usually focus on DDI liability. However, other clinical PK data may yield additional information that can be used to drive and refine a transporter investigation strategy (*e.g.* non-linear PK with an increasing dose or on repeat dosing is indicative of saturation of a mechanism, which could be drug transporter related).

Selecting a clinical probe for transporter investigations is challenging, as there are currently few known probes (either as substrates or inhibitors) that are specific to a given transporter.[101,104,260] Most available clinical probes interact with multiple transporters, or transporters and DMEs. It may also be necessary to investigate the same transporter in different organs. Hence, multiple clinical investigations, and PBPK modelling, may be required in order to reach a point of clarity on the major DDI liability.[256,260] These investigations are necessary to support the safe clinical use of the NME and for inclusion in drug labels.

There is also an increasing trend to use PD or biomarkers such as creatinine clearance (OCTs and MATEs),[144] or levels of unconjugated bilirubin (OATPs)[261] or bile acids (BSEP)[262] as end-points in clinical investigations, acknowledging the fact that transporter interactions influence PD and toxicology as well as PK.[263–266]

1.4.6 Metabolite–Transporter Interactions

Most drug metabolites are more hydrophilic than the parent molecule and because of this are more likely to have a reduced ability to cross plasma membranes solely by simple diffusion. Therefore, drug transporters may be important in the cellular clearance of metabolites, for example, BSEP is inhibited by bosentan and its metabolites[80] or by troglitazone and its sulfated metabolite.[267] This is an area that has received relatively little investigation by academic or industrial transporter scientists to date. However, recent revisions of the regulatory DDI guidance have focused attention on the

contribution of metabolites to drug safety and efficacy, obliging sponsors of new drug applications (NDAs) to include an evaluation of these in their submission data.[93,94,100]

1.5 Drug Transporters and PK

In order to understand the role of transporters in drug ADME, a basic understanding of the more common PK parameters can be useful to help determine the relevance, timing and suitability of transporter-related studies. For example, optimising drug clearance remains one of the most common and challenging DMPK activities in the discovery phase before selection of candidates for further investigation (often called candidate selection), as it needs to be low enough to enable an appropriate half-life and bioavailability of the drug candidate, yet not so low as to prolong exposure unnecessarily. Drug clearance is also an important consideration in the later stages of drug development, in order to understand how transporter interactions may affect overall drug disposition in health and disease. The following section discusses various aspects of drug permeability, absorption and clearance, and includes some brief descriptions of the PK parameters that are often considered when deciding upon an overall transporter strategy plan for NMEs.

1.5.1 Permeability

The plasma membrane is composed of a phospholipid bilayer containing a hydrophobic interior, which acts as a barrier to the free transport of solutes into and out of cells. Simple diffusion of drug molecules through the membrane is a key determinant of not only how they will enter the bloodstream, but also their susceptibility to clinically relevant transporter DDIs, and can be described by applying Fick's first law. This states that simple diffusion of a solute is the product of the concentration gradient of the solute across the cell membrane and the diffusivity of the uncharged form of the solute. The flux of a solute across a membrane can be expressed as:

$$J = D_{\mathrm{m}} \frac{dC_{\mathrm{m}}}{dx} \tag{1.1}$$

where J is the flux of the solute in mol cm^{-2} s^{-1}, D_{m} is the diffusivity of the solute within the membrane in cm^2 s^{-1} and the concentration gradient, dC_m/dx, is the difference in solute concentration inside and outside of the cell across a cell membrane of width dx.[268]

Simple diffusion is often measured early in the drug discovery process. The parallel artificial membrane permeability assay (also known as PAMPA) is suitable for primary screening in high throughput mode and measures the rate of simple diffusion (passive transmembrane) only, but it is possible to add a calculated passive paracellular component by using a biophysical approach[269] (see Figure 1.1). Primary screening may then be followed by

lower throughput cell based models such as Caco-2 cells (a human colonic cell line possessing many intestinal transporters), wild-type MDCK cells or MDCKII-MDR1 cells. These cell line models afford an *in vitro* assessment of drug permeability and/or drug efflux potential[270–272] and can be considered to more closely reflect the *in vivo* situation. In order to categorise experimental outputs from these models, it is necessary to calibrate each system using molecules with known permeability characteristics, as the outputs can be variable from laboratory to laboratory and system to system.[271,272] Validation of the permeability method used is also important, in order to establish the rank order relationship between *in vitro* permeability and the human intestinal absorption values for a range of low- to well-absorbed compounds.[270–272]

Permeability is also a key factor that influences routes of drug absorption and elimination, and is used in the Biopharmaceutics Drug Disposition Classification System (BDDCS; covered in Sections 1.5.2 and 1.5.3).

1.5.2 Oral Absorption and Bioavailability

Absorption of a drug into the body will depend on its physicochemical properties, formulation and route of administration. Oral dosing in tablet or capsule form is the preferred route of dose administration unless another route is more appropriate (*e.g.* nasal/inhaled sprays for lung disorders, or creams or patches for dermal application). It requires an in-depth understanding of the NME (for example its solubility and dissolution profile) and the oral absorption process in order to achieve adequate levels of the drug in the bloodstream. As drugs need to be in solution to be absorbed, tablets or capsules must disintegrate and dissolve in the GIT in a reasonable timeframe and in a challenging environment.

Bioavailability describes the systemic availability of a drug. It is defined as the fraction of unchanged drug that reaches the systemic circulation following an extravascular (*e.g.* oral) dose. Bioavailability is the product of drug solubility, permeability, and GIT and hepatic transport/metabolism processes, and can be considered a key determinant of a successful oral drug product. Identifying the biological factors influencing bioavailability, essentially those associated with absorption and metabolism, is therefore important.[273] For example, efflux transporters present at the intestinal membrane may limit the passage of substrate NMEs at sub-saturating concentrations across the GIT, even if they have otherwise high simple diffusion. Conversely, soluble substrate NMEs at high concentrations in the GIT may saturate efflux transporters, thus minimising the impact of transporter interactions on bioavailability. Alternatively, absorptive transporters may enable the absorption of poorly permeable NMEs that are substrates. Compounds with low aqueous solubility, poor simple diffusion and/or high first pass hepatic extraction are usually associated with poor oral bioavailability.

Predicting oral bioavailability is challenging, however a number of different *in vitro*, *in vivo* and *in silico* approaches and systems have been reported over many years.[274–280] One system that has gained considerable acceptance in industry was developed by Amidon and co-workers in 1995.[281] They recognised that the fundamental parameters controlling the rate and extent of oral absorption of drugs were solubility and permeability, and proposed a Biopharmaceutics Classification System (BCS) that categorised drugs accordingly into four classes that could be used to predict the extent of oral drug absorption (Figure 1.4). In this system, class 1 molecules are considered most desirable, as they exhibit the highest levels of permeability and solubility, and are therefore likely to have good oral bioavailability. A modified version of this system, the BDDCS, was proposed some 10 years later to serve as a basis for predicting the importance of drug transporters and DMEs in determining disposition[282] (Figure 1.4). The premise of this system is that highly permeable, lipophilic drugs that are capable of crossing biological membranes are usually good substrates for CYP450 enzymes, and metabolism therefore is the major route of drug elimination. However, for compounds with low permeability that are less lipophilic, metabolism only plays a minor role in drug elimination, and these compounds are primarily eliminated unchanged in the bile and urine. Thus, class 1 compounds are predicted to be well absorbed at the GIT with minimal transporter effects, whereas for class 2 compounds, the GIT efflux transporter effects may predominate, affecting their rate of absorption and extent of oral bioavailability due to their lower solubility. For class 3 compounds, efflux transporter effects at the GIT will be minimal due to their good solubility, but absorptive transporter effects will be prominent due to their low permeability. Class 4 compounds are predicted to be substrates for both absorptive and efflux

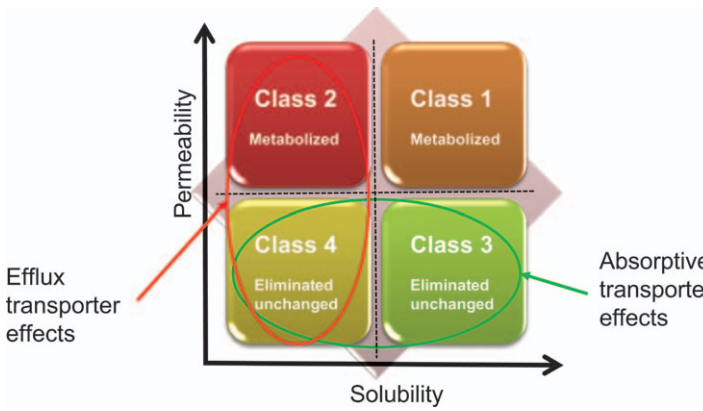

Figure 1.4 The combined BCS and BDDCS. The BCS categorises drugs into four classes according to their solubility and permeability to predict the extent of oral drug absorption as developability criteria. The BDDCS also considers routes of drug elimination and transporter effects on drug ADME. See text for further details.

transporters at the GIT due to their solubility and permeability deficiencies. It is necessary to validate the *in vitro* systems used to test these models with well-characterised molecules in order to establish optimal ranges for each class, otherwise classification of NMEs will be flawed.

In addition to the *in vitro* approaches described above, several experimental and computational models to simulate oral absorption have been developed, including commercial offerings from TNO Pharma[283] (the *in vitro* gastrointestinal model; TIM) and PBPK modelling tools from Simulations Plus Inc.[284] (GastroPlus) and Certara[285] (SimCyp). These models combine drug physicochemical properties (*e.g.* aqueous solubility/dissolution, permeability, *etc*.) with GIT physiology (*e.g.* blood flow, GIT transit time, pH, *etc*.) and biological mechanisms (*e.g.* drug uptake and efflux transporters and DMEs), and can be useful tools in understanding absorption mechanisms.

1.5.3 Drug Clearance

In addition to oral dosing, drugs can be delivered by other routes such as intramuscular, intravenous, subcutaneous, intranasal, inhaled and dermal. Whatever delivery route is used, once the drug has reached the bloodstream and/or tissues, it will ultimately be removed (*i.e.* eliminated or cleared from the body) unless bound covalently to tissues. The description and calculation of clearance is covered extensively in the literature,[286–288] but in its simplest form it can be considered as the rate at which a drug is removed from the blood (or plasma) primarily (but not exclusively) *via* the liver or kidneys. The mechanism by which clearance is achieved in these organs can be *via* metabolic transformation to more hydrophilic metabolites, transporter-mediated uptake and/or efflux, or by passage of the unchanged drug into bile or urine (or a combination of these processes).[289] The total clearance (CL) is the sum of all these clearance routes:

$$\text{Total CL} = \text{hepatic CL} + \text{renal CL} + \text{other CL} \tag{1.2}$$

Drug clearance can be defined as the volume of blood that would contain the amount of drug eliminated in a given time interval (usually per minute). For first-order kinetics, the rate of elimination of a drug is proportional to its blood concentration, and clearance is described by:

$$\text{CL} = \text{elimination rate constant} \times \text{V}_D \tag{1.3}$$

Where volume of distribution, V_D, is a proportionality constant between the total amount of drug in the body and the concentration of the drug in the blood.[290] Each clearance route is described by an equation specific to that organ and is influenced by the extraction ratio of the drug (*i.e.* the relative efficiency of elimination of the drug after a single pass through the organ). The rate of clearance will be influenced not only by the physicochemical properties of the drug (lipophilicity, protein binding ability, ionisation, *etc*.) but also by intrinsic factors (*e.g.* blood flow through the organ) and thus variations can occur if organs are impaired (*e.g.* by disease, age or

environmental factors) or if specific genetic polymorphisms of enzymes and/or transporters are present.

In the kidney, renal clearance reflects the elimination of the drug through a combination of glomerular filtration, active tubular secretion and tubular reabsorption (passive or active).[291] For ionisable drugs, urine pH may also be a factor. Transporters are present on both the uptake (basolateral) and efflux (apical) membranes of the renal tubular cell (Figure 1.3) and, hence, for drugs that undergo active tubular secretion and/or reabsorption, drug clearance can be affected by inhibition or induction of these transporters.[288,292] For some drugs, this transport is the rate determining step in overall clearance.

For the liver, the main factors that determine hepatic clearance are hepatic blood flow (delivery of the drug to the liver, which in turn may be affected by the portal concentration of the drug), uptake of the unbound drug into hepatocytes, metabolic transformation by microsomal or other enzyme systems, and the rate of biliary secretion.[293,294] Thus, if a drug is a substrate of uptake and/or efflux transporters, inhibition or induction of these transporters may influence the overall clearance of the drug. For some drugs, transport either into or out of the hepatocyte is the rate determining step in the overall process and any change will have a direct impact on drug clearance, with potentially significant clinical effects. The statin drug class is the best documented in this respect, although there are several other examples in the literature.

Given the importance of clearance in drug disposition, attempts to predict human clearance from preclinical findings using *in vitro* techniques were developed more than 20 years ago following seminal publications on the well-stirred liver model.[295,296] This model describes the inter-relationship between hepatic clearance, intrinsic clearance (CL_{int}), blood flow and the unbound fraction of drug in the blood, with the term ''intrinsic clearance'' referring to the innate ability of an organ to excrete a drug when no limitations or barriers (blood flow restrictions, protein binding, *etc.*) exist. The measurement of CL_{int} in drug discovery and development has proved pivotal in the extrapolation of *in vitro* DMPK data to the *in vivo* situation. Prior to extrapolating metabolic hepatic clearance *in vivo*, the enzymatic CL_{int} from microsomes and hepatocytes is first ''normalised'' with scaling factors to account for the microsomal protein per gram of liver and/or hepatocellularity per gram of liver, as well as liver weight.[297] The relationship between the predicted (extrapolated) clearance *in vitro* and observed clearance *in vivo* is then determined using a mathematical model, referred to in the literature as either *in vitro–in vivo* clearance correlation (IVIVC) or *in vitro–in vivo* clearance extrapolation (IVIVE). Under-prediction of clearance has frequently been associated with the well-stirred model, and more recent extrapolation approaches have been developed to remove this systematic bias.[298–300] The widening gap between predicted and actual drug clearance in recent years has also been attributed to the changing molecular chemistry of pharmaceutical drugs, leading to a greater proportion of drugs with

reduced permeability that are substrates of drug transporters.[301] As discussed in Section 1.5.2, the BDDCS system offers a framework to predict elimination routes, with more lipophilic class 1 and 2 compounds cleared by extensive metabolism and less permeable class 3 and 4 compounds primarily eliminated unchanged in bile and urine (Figure 1.4). The less permeable compound classes may therefore be good substrates for absorptive membrane transporters at major organs of elimination, raising free intracellular drug concentrations and facilitating the elimination processes (*e.g.* DMEs or efflux transporters at the hepatocyte sinusoidal or bile canalicular membranes, or renal tubules), resulting in the extensive elimination of unchanged drug in bile and/or urine.

Improvement in clearance predictions for substrates of hepatic transport requires accurate measurement of the intrinsic uptake and/or efflux processes in the hepatocyte using detailed kinetic study designs. Kinetic data may be incorporated into more complex equations to extrapolate and predict clearance, accounting for all five of the hepatobiliary pathways: simple diffusion, transporter-mediated uptake, sinusoidal efflux, metabolism and biliary efflux.[302–304] These can be combined with comprehensive mechanistic compartmental or PBPK models to predict *in vivo* clearance.[242,243,250] In most published cases, successful predictions have been obtained. These methods require detailed, time- and resource-intensive investigations of the mechanisms involved and are therefore impractical during drug discovery where multiple candidates may need to be assessed in a truncated time period. They are more readily applied during the later stages of drug development, where an in-depth understanding of the mechanisms of ADME is required, and a greater body of information is available. However, if there is reason to believe that hepatic uptake and/or efflux transporters may be the rate determining step in hepatic clearance for a class of NMEs, transporter assays with adequate throughput may sometimes be incorporated routinely into early drug discovery programmes,[104] so-called 'frontloading'.

1.6 Evaluating and Interpreting Drug Transporter Interactions in Drug Discovery and Development

Given the complexity of transporter expression, function, location and diversity in the body, it has often proved difficult to establish the precise impact of transporters on the ADE of drugs. This is further complicated by the potential changes in ADE due to interactions of transported drugs with DMEs, additional synergistic or opposing transport processes, or interaction with co-administered or endogenous molecules. Nonetheless, substantial progress has been made in recent years in understanding how transporters can affect the safety and efficacy of drugs and their importance in the clinical situation.[102,305–307] One consequence of this has been the routine incorporation of transporter assays into drug discovery and development strategies.[100,102,308] While such strategies vary between pharmaceutical

companies, the overall objectives of the drug discovery and drug development roadmaps are generally the same (Figure 1.5). The initial aim of such strategies, once potential targets for drug action have been identified, is to test a library of compounds against the proposed targets using high throughput combinatorial chemistry approaches to find suitable ''hits'' (the ''hit-to-lead'' phase). This approach can test 10^3–10^6 different molecules, of which the ''leads'' (hits with undesirable characteristics filtered out) are then entered into the lead optimisation and identification (LOID) phase to further refine the process and rank their suitability for further development. By the end of this stage, which may test a few hundred molecules, a shortlist of NMEs is put forward for candidate selection, an important phase of the process where key decisions are made regarding which compounds will progress further. Only a few compounds from each drug class are chosen, with several tests being used to determine basic toxicokinetic (TK) and DMPK properties. Generally, only one NME is nominated for preclinical development, with others being kept as back-up compounds in case the lead compound fails to meet safety and efficacy criteria during the preclinical work. The procedures and methods used in preclinical development become more time consuming, concentrating mainly on *in vitro* methods and animal *in vivo* work, to try to predict ADME and any potential toxicity in humans. If suitable candidates are identified, they transition to the clinical phase and testing on humans can begin—a costly process taking many years as NMEs progress from first time in human (FTIH) in Phase 1 with healthy volunteers, through to Phase 2 and 3 clinical trials to assess their safety and efficacy in human patients. Proof of concept (PoC) studies in Phase 2 are a key phase of the process, to demonstrate clinical efficacy within a small group of patients and eliminate potential failures from the drug pipeline. The few compounds that do reach registration and launch continue to be monitored for unexpected side effects and DDIs; post-marketing (Phase 4) studies can be of benefit in obtaining further information on the risks/benefits of the drug and how to optimise its use.

Selection of drug candidates is a balance between adequate target potency/effect and optimised DMPK properties, to ensure the eventual elimination (clearance) from the body, with minimal DDI potential or ADRs. In this context, transporters are often considered in the later stages of drug discovery (late LOID and candidate selection), after other more critical considerations have been met, *e.g.* optimal molecular chemistry for target effect, minimal toxicity, *etc*. Investigations then generally take a stepwise approach, often with higher throughput assays in the earlier LOID discovery phase followed by more in-depth investigations as molecules progress towards clinical development. The methods used are outlined in more detail in Chapter 7.

1.6.1 Drug Discovery Approaches

The routine incorporation of permeability, oral absorption and bioavailability assessments of NMEs in the early phases of drug discovery have

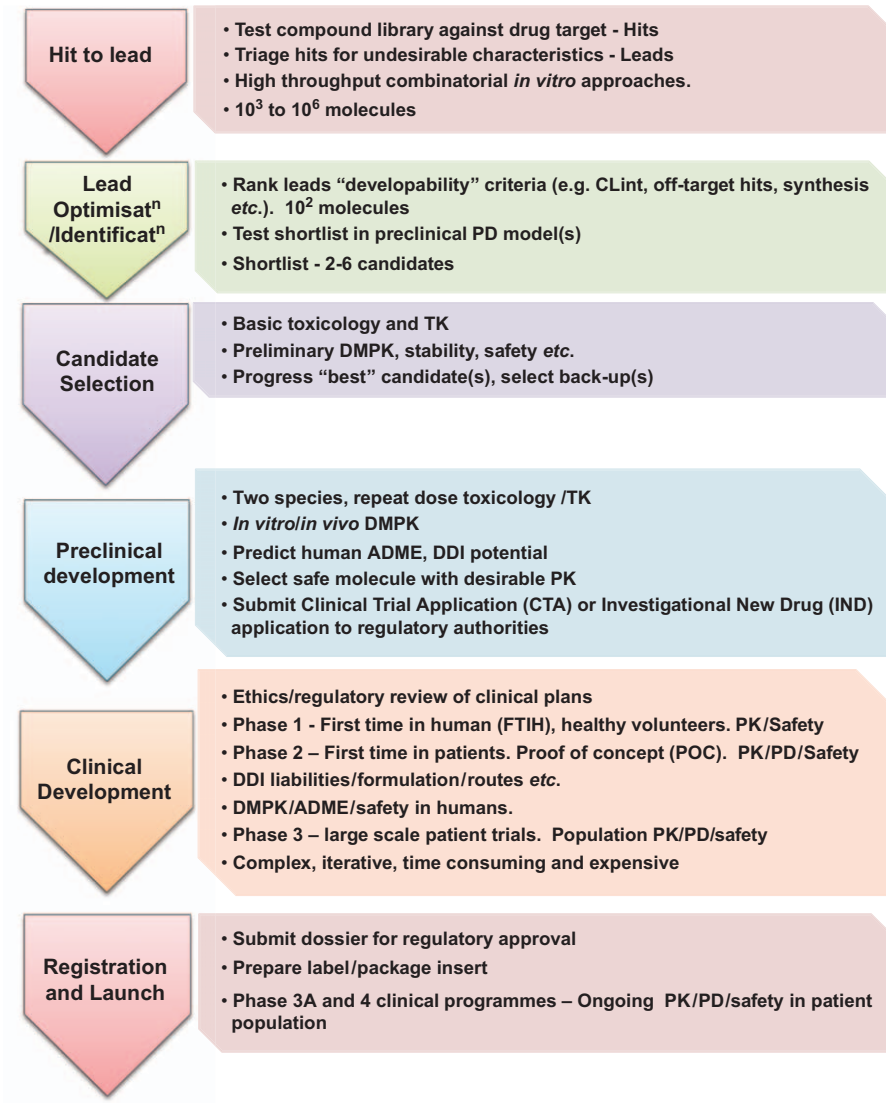

Figure 1.5 Overview of the drug discovery and development process.

already been mentioned in Section 1.5 and they are an important part of drug screening cascades. Thus, the solubility associated factors known to limit the oral absorption of drugs, which include high lipophilicity, high crystal lattice energy and low ionisation potential, can be optimised for some drug classes in early discovery to improve absorption. Optimisation of these factors can be productive, since simple diffusion is considered to be the most common mechanism by which compounds pass across the intestinal membrane, although this is disputed, as discussed elsewhere.[86,87]

Investigation of clearance is also a routine part of drug discovery assessments and is aimed at eliminating high clearance compounds. Historically, approaches focused primarily on the impact of hepatic first-pass CYP450 metabolism. This remains a central tenet of drug discovery and development as CYP450 metabolism is the most likely mechanism of drug clearance for many clinical drugs and NMEs.[309] However, screening out high CYP450 clearance molecules increasingly resulted in NMEs occupying a chemical space divergent from the historical common drug space. For example, on average, drugs approved after 2002 are typically larger but not necessarily more lipophilic than older drugs, and contain a higher number of hydrogen bond donors and acceptors.[310] This shift in the chemical space has led to increasing numbers of NMEs with low rates of simple diffusion and therefore greater reliance on transporter mechanisms and/or non-CYP450 metabolic pathways for their disposition.[294,305,311] Because of this, and in parallel with the advances in transporter science and its integration into PK, transporter clearance mechanisms now receive greater attention in industrial drug discovery and development approaches, although it is challenging to incorporate them into the high throughput drug screening cascades in the LOID phase preferred by many pharmaceutical companies. Although metabolic clearance can be efficiently screened using standard metabolism protocols with microsomes or hepatocytes,[312] giving a good indication of the major DMEs involved, these methods do not specifically include an evaluation of drug transporter clearance mechanisms. Consequently, *in vitro* methods have been adapted and developed in the last decade to explore and evaluate drug transport in primary hepatocytes.[66,250,313] Primary hepatocytes have the major advantage of intact structural integrity and are widely used to assess whether transporter-mediated drug uptake into the hepatocyte could be the rate limiting step in hepatic elimination.[294] Substrates of hepatic uptake transporters with low rates of simple diffusion will have elevated intracellular drug concentrations relative to the blood concentration, which may modulate cellular clearance by increasing drug exposure to DMEs and canalicular transporters.[304,314] However, it is known that, following isolation of primary hepatocytes, efflux transporters on the bile canaliculi lose their functionality, although this can be re-established to some extent if the cells are cultured in a collagen sandwich configuration or another three-dimensional cell culture system.[220,315–322] The sandwich-cultured hepatocyte model is useful for studying both uptake and biliary efflux transport of substrates *in vitro*.[323] Human hepatocytes in particular are subject to donor-to-donor and batch-to-batch variability. They are also more costly to obtain than cell lines, and this influences their routine use in industry.

Other clearance routes, *e.g.* renal elimination, usually receive little attention at these early stages of drug discovery unless there are known issues with a specific class of compounds. In these instances, specific screens or *in silico* approaches may be used to evaluate potential transporter issues during LOID (*e.g.* OCTs in the kidney for cisplatin, or OATs for non-steroidal anti-inflammatory drugs).

The investigation of specific transporter DDIs is often limited to projects where there is a historical precedent of transporter-mediated DDIs, which may be actively managed, or screened out. However, considering that certain widely prescribed drug classes are notable clinical substrates of drug transporters (*e.g.* statins and OATP1B1 and OATP1B3, digoxin and P-gp), there is an increasing trend towards early evaluation and screening out of these liabilities, and many pharmaceutical companies now include routine screening for P-gp and OATP1B1 inhibition in LOID.

1.6.2 Drug Development Approaches

Investigation of NMEs during development focuses mainly on understanding the properties and interactions of individual molecules, with a view to ensuring that drug candidates are both safe and efficacious in clinical use (Figure 1.5). There is a greater emphasis on evaluating the impact of transporter DDIs on drug PK, especially when there are known liabilities associated with a specific class of compounds, ensuring that NDAs are compliant with the current regulatory guidance.

1.6.2.1 Regulatory Landscape

Membrane transporter interactions were first included in regulatory guidance in 2006, as the importance of P-gp in clinically relevant DDIs became apparent.[324] As knowledge expanded, there was a growing awareness that the PK of a number of widely prescribed drugs (*e.g.* statins, metformin, digoxin and methotrexate) could be influenced by a range of drug transporters. In order to identify these transporters, and propose recommendations to guide preclinical and clinical studies, a consortium of industrial, regulatory and academic scientists with expertise in drug metabolism, transport and PK, named the ITC was formed. Its recommendations were subsequently incorporated into new regulatory guidance, which provides transporter investigation proposals and decision trees based on current knowledge.[93,94,100] Transporter-mediated DDIs now receive substantial attention in the drug development process[103,104] and regulatory guidance is often used to justify experimental strategies. The guidance relies heavily on a decision tree approach to develop an investigational strategy, making recommendations on appropriate selection and use of *in vitro* tools, and of clinical strategies. Although useful, it should be noted that decision trees can encourage a simplistic approach, which may not always be appropriate. In particular, the guidance recommends the use of cut-off values based upon a ratio of *in vitro* inhibitory potential (using IC_{50} or K_i values) *versus* circulating drug concentrations, assuming that *in vitro* inhibition values are wholly predictive of *in vivo* inhibition. In practice, this may not always be the case; as discussed in Section 1.4, *in vitro* components, study design and substrate/inhibitor selection can markedly influence inhibitory constant estimates. Similarly, the decision trees do not take into account

specific drug classes; previous work may have indicated their susceptibility to interactions with additional transporters, some of which may not be included in the current guidance. However, the regulatory guidance acknowledges that transporter science is developing rapidly, and recommends that any experimental strategies should be devised with reference to the latest developments in the field.

1.6.2.2 Investigating DDI Liabilities and ADRs

Whereas transporter DDI investigations in early drug discovery focus primarily on screening out or actively managing potential liabilities, drug development approaches focus more on describing and understanding their impact on the safety and efficacy of the NME under investigation. A good drug product will have an acceptable safety, efficacy and toxicity profile, and ideally will have no ADRs, especially if the target population is one in which patients take several different medications (polypharmacy). Consequently, any transporter interaction will have the greatest relevance when it limits the clinical use of a NME because of a DDI with a co-medicated compound or endogenous compound, or if its transport through a membrane transporter leads to a response that is unpredictable or toxic. If oral bioavailability is altered, systemic exposure may be erratic, enhanced or reduced (depending on the nature and site of the interaction), which may increase the risk of unexpected DDIs and limit polypharmacy. Many drug transporters are inducible, or may have genetic polymorphisms that alter their function, which adds further complexity in predicting and understanding the impact of transporters on PK and patient safety.[43,127,253,325,326] Although in theory transporter-related ADRs could lead to the discontinuation of development of a NME, generally this only occurs as part of a wider consideration of its product profile, which can include its therapeutic target, pharmacological potency, patient population, expected co-medications and commercial considerations (Figure 1.5).

Given the complexity of possible interactions, the general approach for a NME entering the development phase is to create a transporter strategy tailored to that specific NME. While some studies are routinely executed in order to comply with the regulatory guidance, it is also necessary to take into account the physicochemical properties of the molecule, the existing preclinical data, the potential co-medications of the target population(s) and any data from previous candidates of the same compound class that exhibited specific transporter interactions. This can be done in several ways and some of the possible points that may be raised include, for example:

- What is the permeability or BDDCS class[282,327,328] of the NME? If poorly permeable, or categorised as a BDDCS class 3 or 4 molecule, the NME is more likely to interact with transporters, which will influence its ADME (see Figure 1.4).
- Do other physicochemical properties of the NME indicate the possibility of transporter involvement? For example, anion or cation

transporters may be implicated if the NME carries a net charge at physiological pH.

- Are there indications of non-linear or erratic PK that cannot be fully explained by other factors such as poor solubility/dissolution, poor simple diffusion/permeability or DME interactions, *etc*.?
- Is the NME systemically cleared predominantly by metabolism or eliminated unchanged? If unchanged, transporters may be an important clearance mechanism, particularly if non-linear PK is observed. Drug metabolites may also be substrates or inhibitors of transporters and may need to be considered if they have high circulating or tissue concentrations.
- What is the major route of clearance of the NME—is it eliminated hepatically or renally? For example, an anionic drug may interact with OATs in the kidney (*e.g.* non-steroidal anti-inflammatory drugs) or OATPs in the liver (*e.g.* rosuvastatin).

In addition to reviewing the properties of the NME, it may also be appropriate to consider other drug development aspects, for example:

- What are the common co-medications in the target population and are any transporters implicated in their PK or DDI profiles? What is the therapeutic dose level or concentration of the NME? The higher the dose level and/or systemic concentrations, the greater the possibility that the NME will cause a DDI.
- What is the therapeutic window of the NME (and any co-medications, if relevant)? If the margin between the effective drug concentration and the toxic concentration is small, then ADRs may occur more readily in the event of a DDI.
- What are the demographics of the target patient population (*e.g.* the young or elderly, ethnically biased)? Older patients often take many medications, leading to a greater risk of DDI liabilities; some drug transporter polymorphisms are more prevalent in certain ethnic populations, which can restrict the use of specific drugs in these populations.

Once all of the data have been reviewed, the individual plans for the NME are prepared and will include specific, in-depth assays of any transporters that may give rise to an ADR (as a substrate and/or inhibitor). Regulatory guidance is also consulted to ensure that the investigations will be compliant with current thinking, especially if the preclinical results indicate that clinical interaction studies are warranted. Many pharmaceutical companies now work with the regulatory authorities on these issues well before product launch. In practice, there are few if any hard and fast rules governing investigation of transporters in drug development. Discussions and plans should consider current transporter knowledge, including a risk–benefit analysis. Further discussion around this topic can be found in Chapter 10.

1.6.2.3 "Liability" Transporters

The list of drug transporters associated with DDI or ADRs (so-called liability transporters) continues to expand. Currently, regulatory authorities recommend that a minimum of seven transporters be considered as drug interaction targets (Table 1.1). These are P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3 and OCT2, with some regulatory bodies suggesting the inclusion of OCT1, BSEP, and possibly MATE1 and MATE2K (as detailed in Chapter 11). The guidance clearly advises the sponsors of NDAs that the list is not comprehensive, rather that investigations should be driven by the science and knowledge of the NME. Future published guidelines are likely to include other transporters such as MATE1, MATE2K and MRP2 in their recommended lists.

Liability transporters are assigned that status because there is a considerable body of evidence, usually a combination of *in vitro* mechanistic studies and clinical investigations, to implicate a given transporter in an ADR or DDI. For some transporters, such as P-gp and OATPs, the body of evidence overwhelmingly supports the designation. For others, particularly those more recently investigated, the body of evidence may be less conclusive. The common consideration for all liability transporters comes down to patient safety, which must take precedence for the pharmaceutical industry, for clinicians and for those responsible for approving medicines. However, it remains difficult to undertake definitive clinical investigations prior to the launch of the product due to the current lack of specific transporter probes for use in clinical trials.

1.7 Toxicity and Transporters

Toxicity due to transporter interactions is of concern and investigations are often a result of accumulated data/observations from drug discovery and development, and from drugs in clinical use. If toxicity is observed routinely for a given drug series, tests may be incorporated into the discovery programme to reduce or screen out the liability. For example, repeat dosing studies are routinely conducted in preclinical species to investigate PD, efficacy and tolerability in LOID or during candidate selection. These studies often include basic clinical chemistry assessments to evaluate and monitor potential organ toxicity and may give an indication of potential transporter mediated interactions. For example, bilirubin, a by-product of heme catabolism from red blood cells that is mainly eliminated in the liver, is a readily monitored circulating biomarker that can indicate hepatotoxicity. It enters hepatocytes *via* OATP1B1 and OATP1B3, and is conjugated by uridine 5′-diphospho-glucuronosyltransferases before being effluxed into bile *via* MRP2. Changes in plasma bilirubin (conjugated and unconjugated) levels may occur because of adaptive non-toxic changes or alternatively severe toxicity. Hyperbilirubinaemia induced by OATP and/or MRP2 is considered benign, so long as no other clinical chemistry findings or evidence of more severe

hepatotoxicity are observed. However, these observations become critical for potential ''Hy's cases'' (hepatocellular DILI with jaundice), which would result in withdrawal of the drug from clinical use.

Once hyperbilirubinaemia has been observed *in vivo*, studies are performed during drug discovery to identify the mechanism, by investigating inhibition of hepatic transporters and/or enzymes, to gain a better understanding of the risk. Putting *in vitro* inhibition potency (IC_{50} or K_i) into context with *in vivo* exposure, using parameters such as the maximum observed drug concentration in blood (C_{max}) or the inhibitor concentration (I), may be useful to predict and understand the risk. The 2012 FDA guidance[92] is a useful reference to classify the magnitude of potential DDIs using this approach. If transporters are implicated, drug discovery activities will focus on reducing the inhibition liability in the chemical series through structure–activity relationship cycles.

BSEP, which constitutes the rate-limiting step in the biliary clearance of bile salts, is also considered to be of potential concern in terms of toxicity because its inhibition has been proposed to play a role in DILI.[329,330] However, DILI as a consequence of BSEP mechanisms may be complex and involve both direct and indirect inhibition of BSEP.[83] In recent years, *in vitro* BSEP inhibition assays[331] have been introduced into drug discovery screening strategies. However, *in vitro* BSEP inhibition data alone can be misleading, as they appear a poor predictor of DILI severity. Rather, it is suggested that consideration of the dosing regimen, route of administration and efficacious exposure (*e.g.* unbound plasma C_{max}) in the context of BSEP inhibition improves the assessment of DILI risk in humans.[332] The obvious challenges in making adequate risk assessments in discovery settings are related to the lack of knowledge on human efficacious exposure. There is also a need for assay panels that are physiologically relevant and can translate *in vitro* BSEP inhibition to meaningful predictions of the effects *in vivo* with respect to changes in bile acid profiles (*e.g.* in plasma, bile and urine) and increased plasma bile acids.[333]

1.8 Conclusions and Future Directions

Drug transporter science has progressed rapidly in the last few decades as the importance of transporters in human health and disease, as well as in pharmaceutical efficacy and safety, has become apparent. Given the extensive range, diversity of action and widespread tissue expression of transporters, it is likely to be some time before both the ABC and SLC superfamilies are fully characterised and their functions understood. Transporter interactions with both drugs and endogenous compounds are now widely acknowledged within the clinical and pharmaceutical sciences, and substantial efforts are being made to understand their impact on safe drug use. It is known that transporters can modulate the PK and PD of many drugs, and can influence, in negative and positive ways, all aspects of ADME, including DDIs and some toxicities. Hence, they are of specific interest to

drug discovery and development scientists and clinicians. More recent work has shown that transporters are also subject to clinically relevant functional polymorphisms and mutations, an area that will require further study to fully understand its potential impact on drug use in populations with a higher incidence of specific polymorphisms.

As the action of transporters can influence other biological (*e.g.* metabolism and secretory) and physicochemical (*e.g.* simple diffusion) mechanisms, their impact ideally should be assessed by considering them as part of a more holistic system, *e.g.* in a mechanistic PBPK model, or in a clinical setting. This requires good preclinical experimental methods, which are continually improving. However, *in vitro* techniques generally require transporters to be expressed in a plasma membrane, in a specific orientation and in closed systems such as cells or vesicles, and their investigation and extrapolation to the human situation remains challenging. Similarly, *in vivo* studies in animals can be problematic due to species differences, although work is ongoing to try to overcome this by using transgenic and humanised animal models. Despite the use of all of these experimental approaches, many aspects of transporter biology, form, function, regulation and interplay are not fully elucidated or understood. Methods of transporter investigation and parameters derived from them have yet to be standardised and, until this happens, conflicting information will continue to be presented in the literature, as well as impacting on the predictive power of IVIVE tools. *In vivo* measurement of intracellular concentrations of drugs in tissues also requires improvements to allow a more realistic prediction of the impact of drug transporters in the clinic. As the science progresses and techniques evolve, demonstrations of ever more complex interactions such as the transporter–metabolism–transporter interplay can be expected, as well as integration of transporters into biochemical and metabolic schemes, acknowledging their pivotal roles in drug and solute disposition within the body.

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