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Role of efflux pumps and metabolising enzymes in drug delivery

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The impact of efflux pumps and metabolic enzymes on the therapeutic activity of various drugs has been well established. The presence of efflux pumps on various tissues and tumours has been shown to regulate the intracellular concentration needed to achieve therapeutic activity. The notable members of efflux proteins include P-glycoprotein, multi-drug resistance protein and breast cancer resistance protein. These efflux pumps play a pivotal role not only in extruding xenobiotics but also in maintaining the body's homeostasis by their ubiquitous presence and ability to coordinate among themselves. In this review, the role of efflux pumps in drug delivery and the importance of their tissue distribution is discussed in detail. To improve pharmacokinetic parameters of substrates, various strategies that modulate the activity of efflux proteins are also described. Drug metabolising enzymes mainly include the cytochrome P450 family of enzymes. Extensive drug metabolism due to the this family of enzymes is the leading cause of therapeutic inactivity. Therefore, the role of metabolising enzymes in drug delivery and disposition is extensively discussed in this review. The synergistic relationship between metabolising enzymes and efflux proteins is also described in detail. In summary, this review emphasises the urgent need to make changes in drug discovery and drug delivery as efflux pumps and metabolising enzymes play an important role in drug delivery and disposition.

Keywords: ATP-binding cassette, BCRP, C_{max}, cytochrome P450, MRPs, multi-drug resistance, P-glycoprotein, protease inhibitors, SSRIs

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1. Introduction

In the last two decades, remarkable progress has been made in the understanding of the biochemical barriers in the field of drug delivery. The most important among them are efflux pumps and metabolising enzymes. Clinical efficacy of many therapeutic agents depends largely on their ability to cross physiological barriers containing efflux pumps and metabolising enzymes to reach their target.

For example, until recently, poor oral bioavailability was generally considered to be due to either physicochemical problems (i.e., poor solubility in the gastrointestinal (GI) fluids or inability to diffuse through the intestinal membrane) or due to significant first-pass hepatic metabolism. Later it was hypothesised that poor oral bioavailability could be due to the activity of biochemical barriers, in addition to physicochemical problems. Based on a series of cellular, animal and human studies, it has been concluded that intestinal metabolic enzymes and efflux transporters may be responsible for the poor oral bioavailability of a number of drugs [1]. Multi-drug resistance (MDR) in tumour cells is a significant obstacle to the success of chemotherapy in many cancer patients. Over expression of

Table 1. Examples showing overlapping substrate/ inhibitor/inducer specificity of CYP3A4 and P-glycoprotein.

Substrates	Cortisol, cyclosporine, dilitiazem, etoposide, nicardipine, colchicine, paclitaxel, hydrocortisone,dexamethasone, ritonavir saquinavir, lopinavir, indinavir, amprenavir, erythromycin, tacrolimus, terfenadine, vinca alkaloids, lovastatin, docetaxel
Inhibitors	Ketoconazole, diltiazem, erythromycin, verapamil, grapefruit juice, lopinavir, itraconazole, ritonavir, statins, PSC-833, St John's Wort
Inducers	Dexamethasone, phenobarbital, rifampin, phenytoin, clotrimazole, reserpine, isosafrol

CYP: Cytochrome P450.

P-glycoprotein (P-gp), an *MDR1* gene product, has been linked to resistance development against anticancer drugs such as vincristine, etoposide and dactionomycin in many cancers. The expression of a wide variety of efflux proteins such as multi-drug resistance proteins (MRPs) and breast cancer resistance protein (BCRP) also accentuates the problem of drug resistance and thereby compromises drug delivery to tumourous tissues. Although these proteins tend to be overexpressed in tumours, their expression is widespread among many normal tissues, perhaps most notably in excretory sites such as the liver, kidney and intestine, where they constitute a formidable barrier against drug penetration, while providing a mechanism for drug elimination. Drug delivery to the brain and other important organs has also been shown to be hampered to a great extent by the pronounced phenotype of efflux proteins.

Excellent detoxification mechanisms exist in the form of metabolising enzymes to reduce the potential damage from xenobiotics. The biological half-life of a drug is generally determined by the extent of metabolic degradation and excretion. Although, eventual elimination of the parent drug and its metabolites from the body is desired, the metabolic processing in the early stages after drug administration is strictly unwanted. Despite the fact that liver is the primary organ of metabolism for orally administered drugs, there is now also a vast amount of evidence that metabolism in the gut wall may contribute substantially to this metabolic break down. It has subsequently been proposed that efflux proteins, specifically P-gp and metabolising enzymes, particularly cytochrome P450 (CYP) 3A4, act synergistically to reduce the xenobiotic entry (**Table 1**). Under this model, metabolites yielded by the action of metabolising enzymes may themselves become substrates for efflux pumps.

This review focuses on the ATP-binding cassette (ABC) family members that mediate drug transport, as these proteins can have a major impact on drug disposition and resistance to chemotherapy, as well as physiological homeostasis. These drug efflux proteins principally comprise the MDR and MRP-type transporters. In the metabolic enzymes section, the emphasis is on CYP3A4 and its role in drug delivery, as it accounts for 50 – 70% of drug metabolism. This review also provides an insight into the mechanisms that mediate drug efflux and metabolism relevant to drug delivery and disposition. Developing strategies to overcome these barriers in order to enhance bioavailability are also discussed in detail.

2. Efflux proteins

Efflux pumps are transport proteins mainly involved in the extrusion of toxic substances (including antibiotics and anticancer drugs) from within the cells into the external environment. These proteins were found in both prokaryotic as well as in eukaryotic organisms and are responsible for clinically significant resistance to chemotherapeutic agents in cancer cells. They also play a very crucial role in drug absorption, distribution and elimination processes.

International Union of Biochemistry and Molecular Biology currently recognises five large ubiquitous super families comprises cellular multi-drug efflux pumps. One of them is the ABC superfamily, members of which use ATP hydrolysis to drive efflux. It is no exaggeration to say that the members of the ABC transport system have been the topic of intense scientific investigation, not only due to their role in the efflux of diverse groups of drugs, but also because of their role in maintaining the body's homeostasis [2].

2.1 ATP-binding cassette transporters

The ABC superfamily is one of the 16 subfamilies that are driven by ATP energy. There are seven subfamilies classified as ABC transporters (ABCA – ABCG). Although the ABC superfamily contains both uptake and efflux transport systems; it is the efflux transport systems that are mainly discussed in this review. This family includes clinically significant MDR pumps, P-gp and MRP, all of which confer resistance to anticancer drugs. Remaining transporters are mainly found in a number of pathogenic fungi and parasitic protozoa, where they confer resistance to antimicrobial drugs [3]. Although, most ABC proteins were discovered as drug transporters, they frequently transport a wide range of substrates, including dyes, ionophoric peptides, lipids and steroids. Due to their role in limiting drug availability to the target organs, this review focuses on the role of efflux proteins, especially P-gp and MRPs, in drug delivery.

2.1.1 General structure

Members of the ABC family share extensive homology and domain organisation. The general structure of ABC transporters is composed of two homologous halves, each containing six putative transmembrane domains (TMDs) and an ATP-binding domain located towards the cytoplasm (**Figure 1A**). There are a number of exceptions to this structural arrangement. For example, MRP1 – 3 and MRP6 – 7 have a third membrane

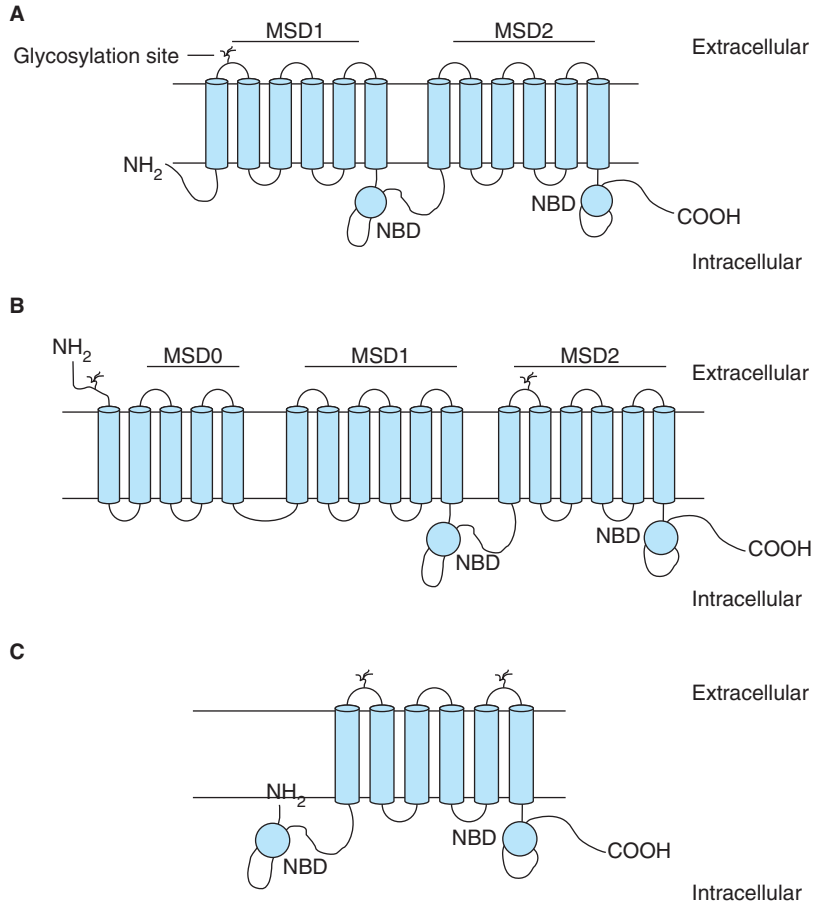

Figure 1. General structure of ABC transporters. Two dimensional structures of **A.** P-glycoprotein, **B.** MRPs and **C.** BCRP. The only distinguishing structural feature among MRPs is that four of them (MRP4, MRP5, MRP8 and MRP9) are devoid of the MSD0 domain. Functional activity of BCRP requires the dimerisation of two half transporters (BCRP).

ABC: ATP binding cassette; BCRP: Breast cancer resistance protein; MRP: Multi-drug resistance protein; MSD: Membrane spanning domain; NBD: Nucleotide binding domain.

spanning domain (MSD0) in which five transmembrane helices reside at the extracellular N terminus (**Figure 1B**) [4-6]. BCRP, in comparision, is a half transporter [7] and is believed to dimerise in order for it to participate in transport activity that results in different substrates corresponding to different dimerisation partners. Both nucleotide binding sites are necessary for the efflux of substrates (**Figure 1C**) [8].

2.1.2 P-glycoprotein (ABCB1)

P-gp is the most widely studied MDR-ABC transporter or human MDR1/ABCB1 [9], it was first discovered in the early 1970s [10] in MDR cells. This ABC transporter has been proposed to act as a 'hydrophobic vacuum cleaner' because of its ability to remove both lipids and drugs as they intercalate and diffuse through the cell membrane [11].

It is evident from the literature that P-gp can interact with a wide spectrum of chemical compounds. The substrates include not only anticancer drugs but also therapeutic agents such as HIV protease inhibitors, linear and cyclic peptides, steroids, detergents, antibiotics, immunosuppressive drugs, antihypertensives and cytotoxic agents (**Table 2**) [12]. P-gp is important not only in the excretion of drugs but also in their absorption. It has thus recently been linked to the incomplete or slow intestinal drug absorption of fexofenadine, digoxin and quinidine [13,14].

2.1.2.1 Localisation and physiological role

P-gp was initially discovered in cancerous tissues and was thought to be a barrier to anticancer agents only. However, its constitutive expression in a wide range of normal tissues demonstrates that it is also an important barrier to drug delivery in other tissues. It is predominantly located on apical membranes of the lower GI tract [15], brain, testis [16] and kidney (**Table 3**) [17]. The presence of P-gp at these localisations suggests that the role of P-gp is to serve as a barrier to the entry of toxic compounds into the circulation and as a process that enhances the excretion of drugs from the circulation.

2.1.2.2 Mechanism of action

Although a significant amount of research has been carried out to solve the structure of P-gp and its mechanism of action, there is no clear understanding at a molecular level of how P-gp effluxes a wide spectrum of compounds. Some

Table 2. Examples of P-glycoprotein substrates.

Steroid compounds	Aldosterone
	Progesterone
	Hydrocortisone
	Cortisol
	Corticosterone
	Dexamethasone
Anticancer agents	Doxorubicin
	Daunorubicin
	Vinblastine
	Vincristine
	Actinomycin D
	Epotoside
	Cisplatin
Immunosuppressive agents	Cyclosporin
	Tacrolimus (FK-506)
	Methotrexate
Protease inhibitors	Indinavir
	Nelfinavir
	Ritonavir
Antibiotics	Grepafloxacin
	Erythromycin
	Rifampicin
Cardiac drugs	Digoxin
	Quinidine
	Lovastatin
Antihistamines	Terfenadine
	Domperidone
Taxanes	Paclitaxel
	Docetaxel

researchers believe that P-gp has a common drug-binding site and the binding of unrelated substrates can be explained by a 'substrate-induced fit' by utilising residues from TMDs 4 – 6 and 9 – 12 [18,19]. It has also been postulated that P-gp possibly acts as a flippase [20], carrying its substrate from the inner leaflet of the lipid bilayer to the outer leaflet, as many of its substrates are hydrophobic and readily partition into the lipid bilayer [21]. Another model suggests that P-gp has the ability to efflux drugs not only from the lipid bilayer but also from the intracellular region [22].

The interaction of the compounds with P-gp is a complex process and interpretation is further complicated by the fact that P-gp may have two or more binding sites [23,24]. Although it is disappointing that a conclusive structure–activity relationship (SAR) is not available, the future success of this work mainly depends on the development of more specific models [25].

2.1.2.3 P-glycoprotein role in drug disposition

2.1.2.3.1 P-glycoprotein in cancerous tissues

MDR in tumour cells is a significant obstacle to the success of chemotherapy in many cancer patients. Over-expression of P-gp has been linked to the development of resistance against anticancer drugs such as vincristine, etoposide and dactionomycin. Elevated P-gp expression has been reported in leukaemias, breast and ovarian cancers, gastric cancer [26] and sarcoma [27-29]. P-gp-positivity was revealed in 30 – 50% of acute myeloid leukaemia cases, and this protein was more often detected during chemotherapeutic regimen in the patients resistant to the treatment [30]. Multiple myeloma is an excellent example of a disease in which P-gp-MDR developed from the treatment regimen. Although only 6% of the diagnosed patients are found to be P-gp-positive before therapy; ≤ 85% patients treated with vincristine, doxorubicin and dexamethasone were shown to over-express P-gp [30]. Even though acquired or drug-induced over-expression of P-gp (e.g., in leukaemias, lymphomas, myeloma and breast and ovarian carcinomas) is the main source of MDR, the role of intrinsic or constitutive over-expression in various cancers (e.g., in colorectal and renal cancers) should also be appreciated when treating those cancers [31-33]. The mutant p53-dependent regulation and other pathways may have been involved in regulating this intrinsic expression [34,35]. Recently it has been proposed that P-gp plays an important role in the regeneration mechanism of cancerous tissues after extensive chemotherapy [36].

2.1.2.3.2 P-glycoprotein in oral drug absorption

Intestinal P-gp is localised extensively on the villus tip of enterocytes [37] (i.e., the main site of absorption for orally administered compounds; **Figure 2**). It is, therefore, ideally positioned to limit the absorption of compounds by pumping them back into lumen. Evidence of P-gp involvement in drug absorption was first demonstrated *in vitro* with Caco-2 cells [37,38]. Later, direct evidence of the role of P-gp in drug absorption was derived from *in vivo* studies with *mdr1a*$^{(-/-)}$ knockout mice [39]. When paclitaxel was administered orally to *mdr1a*$^{(-/-)}$ knockout mice, a sixfold increase in area under the curve (AUC) was observed [39]. After oral administration, plasma concentrations of HIV protease inhibitors (indinavir, nelfinavir and saquinavir) were elevated two- to fivefold in *mdr1a*$^{(-/-)}$ mice. Thus, the above data demonstrated that P-gp limits the oral bioavailability of these agents [40]. Intestinal absorption of both acebutolol and vinblastine increased 2.6- and 2.2-fold, respectively, when cyclosporin, a P-gp inhibitor, was administered intravenously [41]. These findings demonstrate that P-gp plays a role as an absorption barrier by transporting several drugs from intestinal cells into the lumen.

2.1.2.3.3 P-glycoprotein at blood–brain barrier

P-gp was extensively localised on the luminal side of brain capillary endothelial cells and involved in the exclusion of various drugs from the capillary endothelial cells, blocking their entry into brain. HIV protease inhibitors are the most potent therapeutic moieties developed so far for the treatment of HIV.

Table 3. Tissue distribution and cellular polarisation of efflux pumps.

	Subcellular localisation							
Tissue	**P-gp**	**MRP1**	**MRP2**	**MRP3**	**MPP4**	**MRP5**	**MRP6**	**BCRP**
Small intestine	Epithelium, apical side of lumen	Basolateral membranes of lumen	Epithelium, apical side of lumen	Basolateral membranes of lumen	Present on both sides of the lumen	Basolateral membranes of lumen	Basolateral membranes of lumen	Epithelium, apical side of lumen
Colon	Epithelium, apical side of lumen	Not present	Epithelium, apical side of lumen	Basolateral membranes of lumen	Basolateral membranes of lumen	Not present	Basolateral membranes of lumen	Epithelium, apical side
Liver	The bile canalicular face of hepatocytes	Basolateral membrane of hepatocytes	Bile canalicular membrane (apical)	Basolateral membrane of hepatocytes	Not clear	Basolateral membrane of hepatocytes	Present both at lateral and canalicular surfaces of hepatocytes	Bile canalicular membrane
Kidney	Brush border surface of proximal tubules (apical)	Basolateral membrane of epithelial tubule cells	The apical membrane of proximal tubule cells	Basolateral membrane of epithelial tubule cells	Apical membranes of kidney tubule cells	Apical membranes of kidney tubule cells	Unknown	Not present
Placenta	Trophoblast	Trophoblast (fetal capillary)	Trophoblast (membrane facing maternal blood)	Trophoblast (membrane facing maternal blood)	Unknown	Unknown	Unknown	Trophoblast (membrane facing maternal blood)
BBB	Luminal side of brain and testis capillary endothelium	Luminal side of brain capillary endothelial cells	Luminal side of brain capillary endothelial cells	Not present	Present on both sides of BBB	Luminal side of brain capillary endothelial cells	Unknown	Endothelial cells
Other major organs	Abundant on adrenal cortex	Basolateral membrane of sertoli cells			Prostatic glandular cells			Breast lobules, apical

BBB: Blood–brain barrier; BCRP: Breast cancer resistance protein; MRP: Multi-drug resistance protein; P-gp: P-glycoprotein.

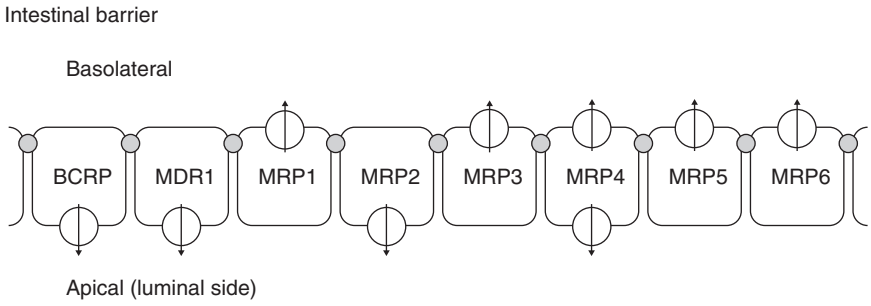

Figure 2. Cellular localisation of efflux transporters on intestinal epithelium. Some of these transporters (BCRP and P-gp) were present intracellularly depending on the tissues. This figure depicts the subcellular localisation of important efflux proteins.
BCRP: Breast cancer resistance protein; MDR: Multi-drug resistance; P-gp: P-glycoprotein.

However, being the substrates of P-gp, they are unable to cross the blood–brain barrier (BBB). As a result, the treatment of brain disorders such as HIV-related dementia is at stake [42]. Treatment failure in many CNS diseases, including Alzheimer's disease, multiple sclerosis and Parkinson's disease is primarily due to poor brain uptake of therapeutic agents [43]. P-gp involvement in the outward transport of antiepileptic drugs (phenytoin, carbamazepine, lamotrigine, gabapentin and topiramate) leading to the inadequate accumulation of drugs in the brain, can also limit the treatment of pharmacoresistant epilepsy [44]. Thus, P-gp is a functional barrier and an integral part of the collective phenomenon called the BBB by restricting access to various pharmacological agents.

2.1.2.3.4 P-glycoprotein in drug metabolism

In humans, CYP3A4 is the principal enzyme involved in the hepatic and intestinal drug metabolism, and both CYP3A4 and P-gp have broad substrate specificity (**Table 1**). There is a striking overlap of substrates between CYP3A4 and P-gp, moreover both proteins are coexpressed in the intestine, kidney and liver. Coadministration of cyclosporin with rifampin, an inducer of both CYP3A and P-gp, reduces the oral bioavailability of cyclosporin and its maximum concentration (C_{max}) [45]. Conversely, ketoconazole, a CYP3A and P-gp inhibitor, increases cyclosporin bioavailability and C_{max}. Plasma concentrations of saquinavir are decreased 80% by the CYP3A/P-gp inducer rifampin. Concomitant administration of ritonavir with saquinavir caused a five- to sixfold increase in saquinavir C_{max} indicating a significant first-pass effect in the intestine [45]. When paclitaxel was given orally together with cyclosporin, or its analogue SDZ-PSC-833, a > 10-fold increase in paclitaxel AUC was observed [46]. Thus, it can be concluded that the coordinated function of both CYP3A and P-gp can dramatically decrease oral bioavailability.

However, the magnitude of the effect of P-gp on the coordination of drug metabolism seems to be dependent on the spatial relationship between P-gp and CYP3A enzymes. In the liver and kidneys, drug molecules interact with P-gp only after their cellular uptake, intracellular distribution and metabolism because P-gp is localised at the canalicular surface of hepatocytes and at the basolateral surface of renal epithelial cells, respectively. In contrast, P-gp is localised at the apical membrane of intestinal epithelial cells. A fraction of absorbed molecules is extruded by intestinal P-gp from inside the epithelial cells into the intestinal lumen. However, a portion of the extruded drugs can then be reabsorbed into the epithelial cells. Through the repetitive processes of extrusion and reabsorption, P-gp prolongs the intracellular residence time of drug molecules and increases the exposure to drug-metabolising enzymes. Consequently, P-gp has been shown to enhance the intestinal metabolism of drugs, although such a process does not take place in the liver and kidneys. This theory has been proven by cyclosporin (a P-gp substrate) and midazolam (not a P-gp substrate) where cyclosporin undergoes extensive metabolism whereas midazolam does not [47,48].

2.1.2.3.5 P-glycoprotein in drug excretion

In addition to absorption, P-gp also plays a very important role in drug clearance [49]. Drugs are generally eliminated from the body by metabolism and/or excretion. Both the liver and kidney play an important role in the excretion of unchanged drugs and their metabolites.

During biliary excretion, the expression of P-gp on the bile canalicular membrane, suggests that P-gp may be involved in the excretion of xenobiotics from the body. This was confirmed by a 10% increase in biliary excretion of digoxin in *mdr1a*$^{(-/-)}$ mice compared with wild type [50]. The liver-to-plasma concentration ratio of antitumour drugs (vinblastin) was increased by MDR modulators [51] (e.g., PSC-833), which can be accounted for by the inhibition of excretion by P-gp [52]. Often biotransformation occurs when the drug molecules are passing through the hepatocytes, suggesting a cooperative role of P-gp and CYP3A4 in the elimination of xenobiotics. It is confirmed by the *in vivo* experiments in which rifampicin (a CYP3A4 inducer) induced the hepatic expression of CYP3A in *mdr1a* knockout mice but not in control mice [53].

During renal excretion, the luminal brush-border membrane of the kidney responsible for the last step of excretion into the urine, contains numerous active transporters, including P-gp. It was also observed that P-gp is involved in the excretion of digoxin, quinolones (levofloxacin, cinoxacin, norfloxacin and ciprofloxacin) and vincristine [54,55]. Agents that modulate the activity of P-gp, such as vinblastine, daunorubicin, reserpine and PSC-833 markedly inhibited the secretion

of these substrates. Biotransformation of the drugs may also occur during intracellular diffusion, again implicating the importance of coordination between CYP3A4 and P-gp in the excretion of xenobiotics.

2.1.3 Multi-drug resistance protein family

For > 10 years, P-gp was widely believed to be the only protein capable of conferring MDR in mammalian cells. However, it has been shown that at least one other transport protein, a 190-kDa glycoprotein named MRP (MRP1) also conferred MDR in mammalian cells [56,57]. The MRP family is comprised of nine related ABC transporters that are able to transport structurally diverse lipophilic anions and function as drug efflux pumps. Increased expression of MRPs not only cause alterations in the subcellular drug distribution [58], but also confer resistance to anticancer drugs (irinotecan) and their active metabolites (SN-38), which is associated with reduced drug accumulation [59].

2.1.3.1 Multi-drug resistance protein 1 (ABCC1)

The founding member of this family, MRP1, functions as an ATP-dependent cellular efflux pump for a variety of cytotoxic drugs. Although it is similar to P-gp in this regard, the substrate selectivity of the two pumps is quite distinct. MRP1 is able to confer resistance to anthracyclines, vinca alkaloids, camptothecins and methotrexate, but not to taxanes, which are an important component of the P-gp profile [56,59]. MRP1 transports various conjugated metabolites, including glutathione conjugates such as leukotriene C4 ($CysLT_1$) and 2,4-dinitro-phenyl-*S*-glutathione (DNP-SG), bilirubin glucuronides, oestradiol-17-β-glucuronide and dianionic bile salts, indicating a role for MRP1 in detoxification of endogenous metabolites [60-62].

2.1.3.1.1 Localisation and physiological role

MRP1 is localised on the basolateral membranes of intestine (**Figure 2**), brain, liver and kidney (**Table 3**) [56,63,64]. Importantly, the ability of MRP1 in transporting glutathione and glucuronide conjugates indicates that it is also a component of the phase III xenobiotic detoxification (see Section 4.4) [65]. Hence, this pump may play a vital role in protecting the cells from carcinogens. A protective role of MRP1 can be summarised from observations in which both free and glutathione-conjugated forms of the potent carcinogen aflatoxin B1 are transported by MRP1 [66].

2.1.3.1.2 Role of multi-drug resistance protein 1 in cancerous tumours

Numerous reports document the expression of MRP1 in cancers that are treated with anthracyclines (leukaemia and breast), camptothecins and etoposide (colorectal and germ cell) [67-69]. It is reasonable to infer that MRP1 contributes to the inherent sensitivity of cancers in which it is expressed.

2.1.3.1.3 Role of multi-drug resistance protein 1 in drug absorption and distribution

In contrast to P-gp, MRP1 is a basolateral transporter whose activity results in the movement of compounds away from luminal surfaces into the tissues underneath the basement membrane (**Figure 2**) [63]. In a study carried out by Pei *et al.* MRP1 showed an adaptive response to maintain cellular detoxification [70]. Thus, the presence of MRP1 at the basolateral membrane is an important factor in contributing to the detoxification process and may, therefore, influence overall drug disposition.

2.1.3.2 Multi-drug resistance protein 2 (ABCC2)

The substrate selectivity of MRP2 is similar to that of MRP1 with respect to glutathione and glucuronate conjugates, but recent reports indicate that the transport characteristics of the two pumps differ in detail [71,72]. MRP2 is a lower affinity transporter for conjugates, and it is subject to positive allosteric regulation by bile acids and certain other amphipathic anions [73].

The drug-resistance profile of MRP2 is similar to that of MRP1 with respect to anthracyclines, vinca alkaloids and camptothecins [71,72,74]. Another difference between MRP1 and MRP2 is that the latter is able to confer resistance to cisplatin [75]. Other drugs transported by MRP2 include HIV protease inhibitors [76], nucleoside phosphonates [77], p-aminohippuric acid [61,78], fluoroquinolone antibiotics [79] and dietary flavonoids quercetin 4-β-glucoside.

2.1.3.2.1 Localisation and physiological role

The functions of MRP2 in the body are distinct from those of MRP1 as a result of differences in expression pattern and subcellular polarity. In contrast to MRP1, MRP2 assumes apical localisation in polarised cells (**Figure 2**), and is mainly expressed in liver, kidney, gut and placenta (**Table 3**) [80-82]. Therefore, it is functionally similar to P-gp as a barrier in the gut and placenta. Previously, MRP2 was referred to as the canalicular multispecific organic anion transporter as it has the ability to extrude a range of lipophilic anions into the bile. MRP2 also plays a role in the hepatobiliary excretion of numerous pharmaceuticals [83].

2.1.3.2.2 Role of multi-drug resistance protein 2 in cancerous tumours

Although the significance of MRP2 as an *in vivo* resistance factor remains to be determined, expression has been reported in human cancers such as colorectal (camptothecins), breast and leukaemia (anthracyclines), and ovarian (cisplatin) [67,68,84-86].

2.1.3.3 Multi-drug resistance protein 3 (ABCC3)

MRP3 is the closest homologue of MRP1 and mediates the transport of organic anions toward the basolateral side of polarised membranes (**Figure 2**). MRP3 is usually expressed at low levels at the basolateral surfaces of bile duct cells, hepatocytes and enterocytes. In addition to the gut and liver, MRP3 is expressed in the pancreas, kidney, adrenal gland and gall bladder (**Table 3**) [87,88]. MRP3 transports a wide range of bile salts (taurocholate, glycocholate) and glucuronide conjugates (oestradiol-17β-glucuronide) but not glutathione conjugates. MRP3 also transports a few of the anticancer agents such as etoposide, teniposide, vincristine and methotrexate, but not doxorubicin, daunorubicin, paclitaxel, actinomycin D, mitoxnantrone, estramustin or cisplatin [89].

Table 4. Some examples of P-glycoprotein pharmacological modulators.

Calcium channel blockers	Verapamil Nifedipine Nicardapine Niguldipine Bepridil Prenyl amine Diltiazem
Steroidal agents	Progesterone Tamoxifen Toremifene Dexamethasone
Antibiotics	Cefaperazone Ceftriaxone Erythromycin Tetracycline
Alkaloids	Vindoline
Immunosuppressive drugs	Cyclosporin A 11-Methyl-leucine cyclosporine SDZ-PSC-833 SDZ-280-446 Rapamycin
Calmodulin antagonists	Trifluoperazine Prochlorperazine Fluphenazine
Miscellaneous compounds	Quinidine Rhodamine 123 Chloroquine Terfenadine Reserpine Amitryptyline Phenytoin

2.1.3.4 *Multi-drug resistance protein 4, 5 and 8 (ABCC4, ABCC5 and ABCC8)*

The absence of a third (N terminal) MSD domain (**Figure 2**) suggested that MRP4 and MRP5 may have distinct properties [87], but they seem to efflux anionic fluorochromes and have substrates similar to MRP1 [90-92]. However, in contrast to the larger members of the MRP family, MRP4, MRP5 and MRP8 can readily transport cyclic nucleotides, and, therefore, purportedly play a role in cellular signalling pathways [60,78,92]. Both MRP4 and MRP5 have been localised basolaterally in the prostate, intestine, brain and kidney [90,93]. The tissue distribution of MRP8 has yet to be described, but like MRP4 and MRP5, it can be detected in many tissues [94,95] (**Table 3**).

2.1.3.5 *Multi-drug resistance protein 6 (ABCC6)*

MRP6 is abundant in the liver and kidneys and localised mainly on basolateral surfaces of hepatocytes and proximal tubules with no expression in any other tissues (**Table 3**). MRP6 can mediate the transport of several natural cytotoxic agents such as etoposide, doxorubicin and cisplatin, but not vinblastine, vincristine and paclitaxel. MRP6 also transports $CysLT_1$ and DNP-SG but not oestradiol-17β-glucuronide [87]. Recent results show that MRP6 does not play an active role in drug resistance and that its overexpression in these cell lines is due to coamplification with the MRP1 gene, which is located immediately next to the MDR6 gene [96].

2.1.3.6 *Multi-drug resistance protein 7 and 9 (ABCC7 and ABCC12)*

Of the two recently described MRP family members, functional studies have only been reported for MRP7. *In vitro* experiments showed that MRP7 transports E217bG and to a lesser extent, $CysLT_1$ but not other MRP substrates such as cyclic nucleotides, methotrexate or bile acids. MRP7 expression was low in most tissues [97]. Functional studies on MRP9 have yet to be reported, but its structural resemblance to MRP4, MRP5 and MRP8 (**Figure 2**) raises the possibility that it may share some of the properties of the latter.

There is increasing evidence to suggest that the simultaneous activity of both the P-gp and MRPs, rather than the separate expression of either one, is the decisive factor in the resistance of tumour cells to anticancer agents [98].

2.1.4 *Breast cancer resistance protein (ABCG2)*

ABCG2 (placenta-specific ABC transporter/mitoxantrone receptor/BCRP) is a recently recognised ABC half transporter that forms a homodimer in the plasma membrane and actively extrudes a wide variety of chemically unrelated compounds from the cells. Consistent with P-gp and the MRPs, BCRP also confers resistance to a variety of drugs. Lesser, but still significant, resistance is observed with anthracyclines, daunorubicins, doxorubicins, camptothecins and their derivatives, mainly topotecan and SN-38. Lysotracker is the only known substrate that is specific for BCRP. The common substrates of all the three efflux proteins (i.e., MDR, MRP and BCRP) are daunorubicin, doxorubicin and epirubicin [99]. The substrate specificity of BCRP overlaps with MDR1 and MRP proteins, indicating these three proteins form a special network in chemo-defense mechanisms. The inhibitor, GF-120918, is perceived as a multiplex inhibitor because it is found to be highly effective at reversing both P-gp- and BCRP-mediated MDR [100].

2.1.4.1 *Localisation and physiological role*

BCRP is localised in the placenta, bile canaliculi, colon, liver, small bowel and brain. It is predominantly localised on the plasma membrane of these cell types [101,102].

2.1.4.2 *Role of breast cancer resistance protein in cancerous tumours*

BCRP reactivity was detected in colon cancers, lung cancers, endometrial cancers and oesophageal cancers [103]. BCRP has been shown to induce resistance to anticancer drugs such as topotecan, but recent work suggests that it also imparts resistance to HIV-1 nucleoside reverse transcriptase inhibitors (zidovudine). These results, if confirmed, have important implications as to the role of BCRP in clinical oncology and virology.

2.1.4.3 Role of breast cancer resistance protein in oral drug bioavailability

BCRP is highly expressed at the apical membrane of the small intestine and colon (**Figure 2**) [104]. The apical localisation of BCRP in the gut suggests a role of BCRP in reducing the uptake of orally administered BCRP substrates. When coadministered with BCRP inhibitor GF-120918, oral bioavailability of BCRP substrates is increased whereas hepatobiliary excretion is diminished. These results suggest a role of BCRP in limiting drug absorption both in intestine and liver [105].

2.2 Strategies to modify the activity or expression of efflux proteins

It can be concluded from the previous sections that the efflux proteins play a very important role in modifying drug pharmacokinetic and pharmacodynamic properties. In addition to P-gp, one or more of the other efflux proteins contribute to an MDR phenotype in tumour cell lines. Therefore, it is very important to develop effective strategies to circumvent these efflux proteins, especially P-gp.

2.2.1 Inhibitors strategy

2.2.1.1 Chemosensitisers

The developments of clinically useful inhibitors that decrease the effectiveness of efflux pumps represent a significant advance in our ability to provide successful and complete treatment. The ability of certain compounds to modulate the activity of P-gp is a well studied field and has been reviewed extensively elsewhere [106-109]. These modulators can be divided into several classes based on their structural or functional features (**Table 4**) [106-109]:

- calcium channel blockers
- calmodulin antagonists
- flavonid or steroidal compounds
- immunosuppressive drugs
- indole alkaloids
- cyclic peptides
- quinolines
- surfactants

Verapamil is one of the most effective first-generation modulators [108]. Unfortunately, the doses of verapamil that provide effective P-gp modulation in humans are sufficiently high to cause life-threatening toxicities [108]. Structural analogues of verapamil, including emopamil and gallopamil and the nonimmunosuppressant analogue of cyclosporin A, PSC-833 were developed as second-generation modulators [110-113]. These compounds appear to alter the pharmacokinetic properties of coadministered anticancer drugs, such as paclitaxel, thus producing ataxia [108,114]. The changes in pharmacokinetic properties of coadministered drugs were due to nonspecific interactions of these modulators with CYP enzymes and other resistance proteins such as MRPs and BCRP. Often as a result of these changes, there is a need to decrease the dose of the anticancer drug, which may adversely affect the outcome of therapy [115].

Clearly, modulators that are non-toxic and do not alter the pharmacokinetics of the coadministered drugs and that can be targeted specifically towards P-gp are needed. These third-generation modulators (tariquidar, zosuquidar, laniquidar, ONT-093) have effective MDR reversal concentrations that are low (i.e., 20 – 100 nM), and these modulators are highly specific towards P-gp, which is confirmed by minimum alterations in the pharmacokinetic properties of coadministered drugs. However, these compounds are yet to be rigorously tested clinically. Other chemicals from sources as diverse as flavonoids [116], green tea extracts, to commercially available chemical libraries are under screening [81,117].

However, the use of these modulators is not completely devoid of problems. The first and foremost concern is the inherent pharmacological action of these modulators (**Table 2**) as the therapeutic concentration of the modulator may be high enough to cause toxicity. Optimising drug dosage regimen due to improvement in pharmacokinetics and distribution of many drugs is another concern. Drug–drug interactions of these combinations can become complicated as P-gp modulators also modulate other transporters (MRP2, BCRP, organic anion transporting polypeptide) and further influence absorption distribution metabolism of P-gp substrates [118]. The strong clinical evidence that the drug–drug interactions can be mediated by the modulation of efflux proteins comes from the fact that the oral exposure of drugs such as fexofenadine, talinolol, quinidine, paclitaxel and digoxin has been significantly increased by the presence of inhibitors such as ketoconazole, verapamil, PSC-833 and erythromycin. These interactions can be attributed largely to the inhibition of P-gp-mediated transport. In some cases, these interactions can be fatal (e.g., digoxin–quinidine interaction) or result in unexpected side effectes (e.g., loperamide–quinidine). Therefore, utilisation of chemosensitisers or modulators should be employed under careful supervision as the protective function of P-gp can also be modulated due to inhibitor strategy. Thus far, coadministration of P-gp modulators and various drugs has had a limited clinical success (**Figure 3**) [119].

2.2.1.2 Polymers

Polymeric excipients were used to overcome P-gp in the gut with a view to improving oral delivery of anticancer agents. One novel approach involves the application of polymers to overcome MDR. In contrast to free drug, chronic exposure to polymer drug conjugates did not induce MDR in cancer cells (**Figure 3**) [120,121]. Another report by Kabanov *et al.* suggested that pluronic block copolymers sensitised MDR cells, resulting in an increase in the activity of cytotoxic drugs by two to three orders of magnitude [122]. This property was attributed to the inhibition of drug efflux proteins, abolition of drug sequestration and lowering of the glutathione/glutathione-*S*-transferase detoxification process. Furthermore, recent studies demonstrated that pluronic block copolymers induce a dramatic

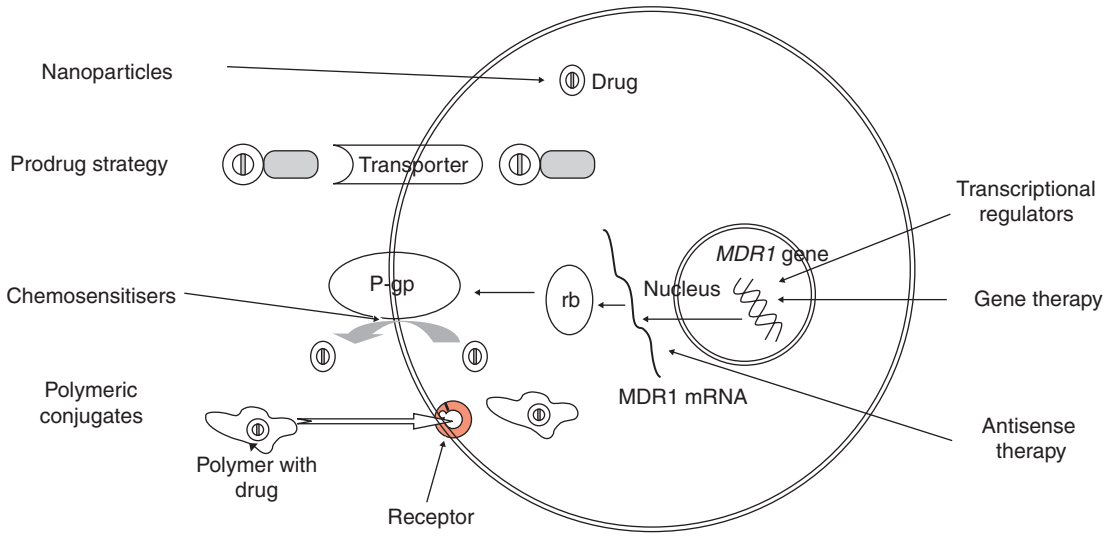

Figure 3. Strategies to A. Modulate or circumvent the activity of P-gp: i) Prodrugs: these are substrates to influx transporters but not to P-gp; ii) Nanoparticles: these are able to circumvent P-gp by encapsulating the substrates; iii) Polymers: this strategy works either by inhibiting P-gp or by carrying substrates through membrane endocytosis; iv) Chemosensitisers. **B. Modulate or regulate the expression of P-gp:** i) Antisense nucleotides: these bind to mRNA and inhibit protein expression; ii) Transcriptional regulators: these bind to promoter region of a gene and inhibit the gene expression; iii) Gene therapy: this allows delivery of cDNAs encoding the P-gp into target cells to protect them against the toxic effects of chemotherapy.
MDR: Multi-drug resistance; P-gp: P-glycoprotein; rb: Ribosome.

reduction in ATP levels selectively in MDR cells, whereas non-MDR cells are not responsive [123]. Therefore, the recognition of energy-depleting effects of pluronic block copolymers, in addition to their very high sensitisation effects and their ability to inhibit multiple mechanisms of drug resistance in MDR cells, is of considerable clinical significance.

2.2.1.3 *Monoclonal antibodies*

Monoclonal antibodies recognising P-gp have been explored as potential inhibitors of P-gp in order to directly target P-gp and to avoid the clinical side effects associated with pharmacological chemosensitisers. The studies using MRK-16, one of the P-gp monoclonal antibodies, suggested that their use, together with MDR-related cytotoxic drugs with or without chemosensitisers, may be a potential therapeutic anti-MDR therapy [124,125].

2.2.2 *Prodrugs*

Prodrugs are pharmacologically inactive compounds that result from transient chemical modifications of biologically active species. The strategy is to modify drugs so that they are no longer recognised by the efflux proteins. Furthermore, recent studies have shown that the modification of a drug to make it a substrate for the influx transporters can have a potential utility. In that direction, peptide transporters are the promising targets as they have a broad substrate specificity and rapid turnover rate. Transporter and receptor-targeted prodrug design has tremendous potential (**Figure 3**). It is very important to know the structural characteristics during the prodrug design; otherwise this can produce prodrugs, which have more affinity towards efflux proteins. For example, Borchardt *et al.* showed that both P-gp and MRP2 are responsible for the restricted permeation of the cyclic prodrug of the opioid peptide H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE) in Caco-2 cells [126].

Combination of the above two approaches (using monoclonal antibodies and prodrugs) can be highly effective wherein the substrate is chemically modified by attaching it to a monoclonal antibody so that it is no longer recognised as a substrate for the efflux transporter. This novel prodrug (antibody-substrate) can be used further to specifically target a particular organ or tissue. This strategy not only circumvents efflux proteins but can also reduce the toxic affects of the P-gp substrates [127].

2.2.3 *Nanoparticle technology*

This strategy involves the design of nanoparticles conjugated with specific ligands targeted to specific receptors. Conjugated nanoparticles carry the drugs through the receptor-mediated transcytosis. Nanoparticle technology could be used to avoid P-gp. Nanoparticle-bound loperamide and doxorubicin are able to translocate to the brain by avoiding P-gp (**Figure 3**) [128].

2.2.4 *Transcriptional regulators*

Transcriptional regulators often modulate the expression of efflux systems by monitoring the intracellular levels of the transported substrate by direct substrate binding [129,130]. In relation to efflux transporters, there is a far greater understanding of the structural interactions between regulators and their bound substrates [131-134]. It is possible to envisage a

situation in which MDR is modulated indirectly by agents that inhibit efflux pump expression by an interaction with a specific regulator. Indeed, such an approach, using K2-5F, a designed transcriptional repressor, on the expression and function of the *MDR1* gene in highly drug-resistant NCI/ADR-RES carcinoma cells was investigated and decreased P-gp levels were found (**Figure 3**) [135]. Although the utility of transcriptional regulators can cause changes in mRNA levels of non-targeted genes, designing specific transcriptional repressors can eliminate such problems.

2.2.5 *Gene therapy*

Gene therapy allows delivery of cDNAs encoding the multi-drug transporter into target cells to protect them against the toxic effects of chemotherapy. Such a strategy could be useful during chemotherapy of cancer, by protection of bone marrow and other drug-sensitive tissues. The feasibility of this approach has already been demonstrated in transgenic mice expressing the human P-gp gene [136] and in gene transfer experiments of human P-gp into mouse bone marrow (**Figure 3**) [137-139]. Vectors for delivery of P-gp have been developed and clinical trials to test this hypothesis are also underway.

3. Metabolising enzymes

Excellent detoxification mechanisms exist in the form of metabolising enzymes to reduce the potential damage from xenobiotics. Although eventual elimination of the parent drug and its metabolites from the body is desired, the metabolic processing in the early stages after drug administration is strictly unwanted. Despite the fact that the liver is the primary organ of metabolism for orally administered drugs, there is now a vast amount of evidence indicating that metabolism in the gut wall may contribute substantially to this metabolic break down [140-145]. A brief discussion of the metabolic enzyme systems, their tissue distribution and a detailed discussion of their role in drug delivery and strategies to circumvent these enzymes is provided in the following sections. As the topic of drug metabolising enzymes is so vast and far reaching, a detailed discussion of all the important interactions mediated by all the metabolising enzymes is beyond the scope of this review. Therefore, the emphasis is placed on CYP3A4 and its role in drug delivery as it accounts for 50 – 70% of drug metabolism.

3.1 The phase I system

Drug metabolism takes place in two phases. The phase I system of enzymes metabolise the xenobiotics into reactive species, which become substrates for phase II enzymes, and are further converted to soluble non-toxic metabolite conjugates. Most pharmaceuticals are metabolised through phase I biotransformation [146-149]. The phase I detoxification system, composed mainly of the CYP supergene family of enzymes, is generally the first enzymatic defense against exogenous compounds. The CYP comprises a superfamily of mixed function oxidases responsible for the oxidation of numerous endobiotics and thousands of xenobiotics [150-152]. The CYP family of enzymes is the most prominent system in both the detoxification and bioactivation of xenobiotics [153-158].

3.1.1 *Cytochrome P450*

Based on similarities of amino acid sequences, the CYP enzymes have now been classified as family, subfamily and isoform [159-161]. The major CYP isozymes involved in the metabolism of drugs or exogenous toxins are the CYP3A4, CYP1A1, CYP1A2, CYP2D6 and the CYP2C enzymes [162]. The CYP enzymes involved in drug metabolism are found, not only in the liver, but also in the kidneys, lungs, brain, small intestine, skin and placenta [163-166].

Enzymes of the CYP450 system are responsible for the oxidative metabolism of a large and varied number of compounds including the antiretroviral agents (protease inhibitors [PIs] and non-nucleoside reverse transcriptase inhibitors [NNRTIs]), many new generation serotonin-specific re-uptake inhibitors (SSRIs), psychotropic agents and endogenous substances such as steroids and prostaglandins, environmental toxins and dietary components. The primary role of these enzymes in drug metabolism is to render drugs more water soluble and less fat soluble, so that biliary excretion can proceed. As a result, the actions of these enzymes can affect the amount of active drug in the body at any given time. Such changes can be positive, enhancing efficacy, or negative, enhancing toxicity and adverse, depending on how a drug interacts with these enzymes. Drugs interact with metabolising enzymes either as substrates or inhibitors and change the levels of metabolising enzymes by transcriptional induction. The complexity of drug–drug interaction reaches a new level when a drug exhibits all three of the manifestations (i.e., substrate, inhibitor and inducer). A substrate in one situation can be an inhibitor in another when it is given in combination with a compound of lower binding affinity. On the genetic level, compounds can also be inducers and modulate the levels of metabolising enzymes. Almost all of the substrates and inhibitors can be inducers following chronic exposure (**Table 5**).

3.1.1.1 *Cytochrome P450 3A*

Of the CYP family, the CYP3A group represents the most abundant phase I drug metabolising enzymes and accounts for ~ 30% of hepatic CYP and > 70% of intestinal CYP activity. Moreover, CYP3A is estimated to metabolise between 50 and 70% of currently administered drugs [167]. A significant amount of CYP3A is expressed in the enterocytes capable of modifying xenobiotics during their transit across intestinal epithelium [168]. The major congener of the CYP3A family is CYP3A4, the most abundant form [169]. This CYP3A4 enzyme is present in the liver and enterocytes lining the small intestinal lumen [170,171]. According to recent studies, CYP3A5 is also polymorphically expressed in the small intestine and

Table 5. Examples of substrates, inhibitors and inducers of cytochrome P450 family enzymes .

P450	Substrates	Inhibitors	Inducers
CYP3A4,5 (~ 30% of liver CYP, 70% of small intestinal CYP)	Cyclosporin, nifedipine testosterone, terfenadine astemizole, azelastine midazolam, alprazolam triazolam, cyclosporin A tacrolimus, haloperidol Ca^{2+} channel blockers, diltiazem, verapamil, felodopine, cisapride, pimozide, alfentanil, sufentanil, fentanyl, erythromycin, TCA, dextromethorphan, codeine, granisetron, lignocaine, ropivacaine, hydrocortisone, dexamethasone, theophylline, ethinyl oestradiol, testosterone, tirilazad, carbamazepine, glyburide, ketoconazole, lovastatin, HIV protease inhibitors, taxol, lansoprazole	Troleandomycin, ketoconazole, gestodene, ritonavir, nelfinavir, amprenavir, indinavir, propoxyphene, saquinavir, ketoconazole, itraconazole, erythromycin, grapefruit juice, nefazodone, fluvoxamine, fluoxetine, diltiazem, verapamil, clarithromycin, omeprazole	Carbamazepine rifampin, phenobarbital, phenytoin, efavirenz, nevirapine prednisone, rifapentine, troglitazone
CYP2C8 (found in kidney, adrenal, brain, uterus, breast, ovary and duodenum)	R-mephenytoin, tolbutamide, *S*-warfarin, TCA, diazepam, verapamil	Cimetidine	Rifampicin, phenobarbitone
CYP1A2 (~ 13% of liver CYP)	Phenacitin, caffeine, aflatoxin B1, TCA, erythromycin, haloperidol, theophylline, paracetamol, ropivacaine, propranolol, naproxen, tacrine, verapamil	Ellipticine, furafylline, α-naphthoflavone, ciprofloxacin, grepafloxacin, fluvoxamine, fluoxetine, nefazodone, enoxacin	Cigarette smoke, ritonavir, omeprazole, charcoal-smoked foods, cruciferous vegetables
CYP2E1 (~ 7% of liver CYP)	Ethanol, carbon tetrachloride, dimethyinitrosamine	Diethyldithiocarbamate, diallyl sulfide, cimetidine, isoniazid, watercress	Ethanol, ritonavir, isoniazid
CYP2A6	Coumarin, dimethyinitrosmaine	Methoxalen	
CYP2D6 (~ 2% of liver CYP. Mainly in the liver, with little intestinal activity)	Debrisoquine, sparteine, bufurol, dextromethorphan, β-blockers, haloperidol, chlorpromazine, thioridazine, dexfenfluramine,flecainide, propafenone, mexiletine, procainamide, fentanyl, pethidine meperidine, SSRIs (fluoxetine), TCAs, trazadone, zuclopenthixol, *S*-mianserin, tolterodine; azelastine ,	Quinidine, ajmalicine, yohimbine ritonavir, sertraline, fluoxetine, paroxetine, quinidine, thioridazine, cimetidine, amiodarone, diphenhydramine, haloperidol, ticlopidine	
CYP2B6	Cyclophosphamide artemisinin, *S*-mephobarbital, *S*-ifosfamide	Sulphaphenazole	Phenobarbital, cyclophosphamide
CYP2C9	*S*-warfarin, phenytoin, diclofenac and other NSAIDS, tolbutamide, fluoxetine, torsemide, verapamil, dextromethorphan	Fluconazole, ketoconazole, sulphonamides (sulphaphenazole, sulphinpyrazone), amiodarone, ritonavir, metronidazole, clopidrogel, fluvastatin, fluvoxamine, fluoxetine, miconazole, metronidazole, trimethoprim	Carbamazepine ethanol, phenytoin, rifabutin, ritonavir, rifampin

CYP: Cytochrome P450; NSAID: Non-steroidal anti-inflammatory drug; SSRI: Serotonin-specific re-uptake inhibitor; TCA: Tricyclic antidepressant.

Table 5. Examples of substrates, inhibitors and inducers of cytochrome P450 family enzymes (continued).

P450	Substrates	Inhibitors	Inducers
CYP2C19 (found in duodenum and in few other extrahepatic tissues; lower hepatic expression than 2C9)	(*S*)-mephenytoin, phenytoin, diazepam, TCA, (clomipramine, imipramine), dextromethorphan, propranolol, omeprazole, progesterone, sertraline, aminopyrine	Ticlopidine, fluvoxamine, fluoxetine	Rifabutin, rifampin

CYP: Cytochrome P450; NSAID: Non-steroidal anti-inflammatory drug; SSRI: Serotonin-specific re-uptake inhibitor; TCA: Tricyclic antidepressant.

contributes significantly to drug metabolism in certain human subjects [172]. Although hepatic metabolism contributes largely to systemic drug elimination, the combination of hepatic and intestinal drug metabolism appears to have significant influence on presystemic or, first-pass drug loss.

3.1.1.2 Role of cytochrome P450 3A4 in drug delivery

CYP3A4 is the most clinically significant member of CYP3A subclass of cytochrome P450 enzymes. Its role has been reported in many clinically significant drug–drug interactions. The following examples are given to highlight its importance in drug delivery. The number of interactions cited in this article are minimal and selected to demonstrate its significance in drug metabolism. Most of the interactions were reported when two or more of its substrates were given together.

Excessive drops in blood pressure have been reported following the introduction of ritonavir into the regimen of a hypertensive subject stabilised on a calcium channel blocker such as verapamil. This could be due to the fact that ritonavir is one of the potent inhibitors of CYP3A4 [173]. Indinavir and nelfinavir exhibit the same level of inhibition, whereas saquinavir and amprenavir appear to be poor inhibitors of CYP3A4 [174-177]. Among the NNRTIs, delavirdine is a potent irreversible inhibitor of this enzyme and is presently the only drug to elevate ritonavir plasma levels, increasing its AUC by 60% in patients maintained on a regimen of ritonavir 600 mg b.i.d. [177].

Several of the medications used to treat mood and anxiety disorders are also substrates of the CYP3A4. For patients receiving these medications concomitantly, the need for close monitoring of drug–drug interactions is evident [178]. SSRIs and nefazodone are inhibitors of many CYP450 isoenzymes. When such agents are administered concomitantly with other drugs such as the PIs, the NNRTIs, or other non-antiretroviral agents, which may also be substrates, inducers or inhibitors of these enzymes, drug accumulation can occur, leading to unpredictable toxicities [179]. Inhibitors of the CYP enzymes such as the azole antifungals (i.e., ketoconazole, itraconazole and fluconazole) will cause a decrease in the clearance of drugs such as citalopram, terfenadine, midazolam and triazolam leading to cardiac arrhythmias [180-183].

Saquinavir undergoes extensive first-pass metabolism by the major metabolising isozyme CYP3A4. Ketoconazole (CYP3A4 inhibitor) inhibited the formation of all saquinavir metabolites. In addition, saquinavir inhibited the metabolism of terfenadine and the formation of the 6-β-hydroxylation products of testosterone, indicating its specificity towards CYP3A4 [184]. Metabolism of ritonavir on the other hand is caused by CYP3A4 and CYP2D6. Moreover, this drug significantly inhibits the metabolism of CYP3A4 substrates such as nifedipine and CYP2D6 substrates such as dextromethorphan, when administered in combination [185]. The major isozyme responsible for indinavir metabolism is CYP3A4, whereas the metabolism of nelfinavir is caused by several isozymes including CYP3A4 followed by CYP2C19, CYP2D6 and possibly CYP2C9 and CYP2E1 [177,186,187].

The anticoagulant effects of warfarin, as measured by the increase in prothrombin time, have been reported to be increased twofold by the presence of fluconazole and threefold by ketoconazole [188,189]. The clearance of both isomers of warfarin were reduced with a fluconazole dose of as low as 100 mg/day for 7 days. Omeprazole, another drug commonly used by patients for palliative care, has been shown to inhibit the metabolism of warfarin, an interaction that is most likely mediated by CYP3A4 and other CYP enzymes [190].

As a general rule, patients with clotting disorders, those awaiting surgical procedures and those on anticoagulant therapy should be cautioned against the use of herbs such as garlic and St John's Wort. As most of these herbs are known to interact with CYP3A4 and to a certain extent with other CYP class of enzymes, coadministration of warfarin or any other CYP3A4 substrate with these herbs is unwarranted [191].

Oestrogens and corticosteroids are substrates to the CYP enzyme system. Protease inhibitors such as nelfinavir or ritonavir can act both as inducers and inhibitors of the CYP enzyme system. These compounds have thus been shown to increase the degradation of ethinyl oestradiol, a major component of oral contraceptive pills, by their induction effect [192].

3.2 The phase II system

Phase II conjugation reactions generally follow phase I activation, resulting in a xenobiotic that has been transformed into a water-soluble compound and can be excreted through urine or bile. Several types of conjugation reactions are possible, including glucuronidation, sulfation, and glutathione and amino acid conjugation. These reactions require cofactors, which must be replenished through dietary sources. Much is known about the role of phase I enzyme systems in the metabolism of pharmaceuticals, as well as their activation by environmental

toxins and specific food components. However, the role of phase II detoxification in clinical practice has received less consideration. Furthermore, little is currently known about the role of the detoxification systems in the metabolism of endogenous compounds. But the recent knowledge that the sulfate and glutathione conjugates formed by the action of the phase II enzymes become substrates to MRPs and are removed from the cell highlights their importance in protecting the cell from foreign cells [65,66].

3.3 Strategies to bypass metabolic inactivation of drugs

This section discusses the approaches available for CYP inhibition, with particular emphasis on the potential use of antisense phosphorodiamidate morpholino oligonucleotide strategies to inhibit human CYP3A4.

3.3.1 Ritonavir boosting

Ritonavir and lopinavir were combined together into a powerful boosted PI combination that takes advantage of ritonavir's inhibition of the CYP3A enzyme system to elevate plasma levels of lopinavir ≤ 10-fold its normal AUC. This combination can cause a 10-fold increase in potency, for the most part overcoming PI resistance. Results from the few studies completed so far indicate that the profiles of drug–drug interactions are mostly similar to the combination of lopinavir and ritonavir [193-198].

3.3.2 Antisense approach

A recent study describes an antisense approach for the inhibition of CYP3A4 in two distinct model systems: primary cultures of human hepatocytes and the human colon carcinoma cell line Caco-2 stably transfected with CYP3A4 cDNA. Antisense phosphorodiamidate morpholino oligomers (PMOs) were shown to inhibit target CYP3A4 gene expression by preventing ribosomal assembly, thus preventing translation [199,200]. The same approach was applied to deactivate CYP3A2 wherein antisense PMOs were applied topically to adult male rats. This study demonstrated that the topical application of antisense PMOs in rats is a feasible delivery strategy for gene targets in liver and underlying skin [201,202]. The application of antisense technology for the inhibition of specific CYP enzymes can provide significant therapeutic benefits, including:

- a reduction of first-pass drug metabolism
- lowering in drug dosage
- selective reduction of toxic metabolites
- increased oral/topical drug bioavailability

The use of antisense morpholino oligonucleotide strategies to target CYP enzymes may result in safer and more effective therapy [203,204].

3.4 Maybe a phase III?

Recently, the efflux activity of P-gp or MRP has been defined as the phase III detoxification system. The phase II process generates conjugated metabolites to facilitate their removal out of the cells. Some of those conjugates (glutathione and glucoronide) have been shown to be effluxed by MRPs. This facilitatory function of MRPs in drug metabolism was presented under the section of MRPs. Both MRP1 and MRP2 are involved in the transport of glutathione-*S*-conjugates, which is essential for the elimination of such conjugates from the cell. Synergism between CYP enzymes and P-gp may occur in the intestine when metabolites produced by CYP enzymes, such as CYP3A, are better substrates for transport proteins [205], such as P-gp, than the parent drug, or when P-gp prolongs the duration of absorption by necessitating the repeated entry of the drug into the enterocytes. This process would increase exposure to CYP enzymes and could also prevent kinetic saturation of these proteins [203,204]. Close chromosomal location of *P-gp* and *CYP3A4* genes and the observation that these two proteins share similarity among several substrates and inhibitors (**Table 1**), can explain the composite nature of pharmacokinetic drug–drug interactions resulting from the interplay of both systems [1,206]. These coordinated functions of metabolism and active efflux in the small intestine can result in poor oral absorption [1,207-209]. Simultaneous inhibition of P-gp and CYP3A has been postulated to be responsible for the effects of ketoconazole on the bioavailability of digoxin. The appropriate addition of blocking agents, which are substrates for either CYP3A4 or P-gp or both, can result in higher bioavailability. Low-dose ritonavir, when combined with lopinavir, inhibits lopinavir's efflux and metabolism and produces synergistic antiviral activity.

4. Pharmacogenomics of efflux pumps and metabolising enzymes

Due to the vastness of this topic, the following paragraphs briefly highlight the importance of the topic and references for excellent reviews in this field are provided [210-212].

4.1 Pharmacogenomics of P-glycoprotein

Interindividual variability in the expression of P-gp can be one of the major contributing factors for the variation in drug absorption. *MDR1* gene is highly polymorphic with > 50 single nucleotide polymorphisms and insertion/deletion polymorphisms have been reported [213]. Clearly, genetic polymorphism is a major source of the interindividual variability in intestinal P-gp expression. Genetic polymorphisms of drug metabolising enzymes may be responsible for interdividual differences in pharmacokinetics and, thereby, can alter clinical efficacy [213-218].

4.2 Pharmacogenomics of metabolising enzymes

The pharmacokinetics of many drugs often vary considerably among individuals, largely because of variations in the expression of different CYP enzymes in the liver and other tissues [219]. Extensive population differences in the frequencies of various *CYP3A4* alleles were noted [220]. Inter-individual variations of 10- to 50-fold have been reported in the activity of

the CYP3A4 enzyme, which contributes to the metabolism of > 50% of all clinically relevant drugs [221].

5. Induction of metabolic enzymes and efflux pumps

Recent studies demonstrated that a nuclear receptor, pregnane X (PXR, also called steroid and xenobiotic receptor), plays a central role in the induction of P-gp activity [222,223] and also regulates CYP3A4 transcription [224]. Interestingly, the nuclear receptor PXR and P-gp are co-expressed in a number of tissues (i.e., liver, intestine, kidney and placenta) [225,226]. The question as to whether the tissue-dependent differences in the P-gp induction are attributed to the tissue differences in the expression level of PXR, or to different mechanisms in different tissues, remains to be explored. Although it is evident that the nuclear receptor PXR plays a central role in P-gp induction, the pattern of induction is not clear. In addition, other nuclear receptors such as the constitutive androstane receptor and the aryl hydrocarbon receptor have been shown to be involved in the induction. Therefore, a detailed knowledge of the inductive processes for each inducer is required in order to understand its implications and consequences. The topic of drug induction is so vast that it is beyond the scope of this review [149,227-239].

6. Expert opinion and conclusion

Efflux pumps and metabolising enzymes have been receiving great attention as the most important cellular barriers to effective drug delivery. This review thus outlines their physiological role and their influence on the final output of drug delivery irrespective of the route of drug administration. Based on a series of *in vitro*, *in vivo* and clinical studies, intestinal metabolic enzymes and efflux transporters have been shown to be responsible for the poor bioavailability of a number of drugs. CYP3A, the major phase I drug metabolising enzyme family in humans, and multi-drug efflux pump, P-gp, are present at high levels in the villus tip of enterocytes in the GI tract, which can severely limit drug absorption. Even though, these two have occupied the centre stage as the most important biochemical barriers, a lot of effort has been put into investigating the other critical systems and pathways involved in precluding the efficiency of drug delivery and disposition. MRPs, BCRP and other major metabolising enzymes have consequently been found as the new cellular barriers.

As knowledge about the role played by efflux pumps and metabolising enzymes begins to emerge, many models of how these barriers can be circumvented or nullified were proposed. Inhibitors (chemosensitisers, polymers and monoclonal antibodies), nanoparticles, transcriptional regulators and gene therapy have been proposed as the viable strategies to overcome this challenge. However, both the advantages and disadvantages of such strategies should be considered before implementing them in clinical practice.

As the notion and theme of drug delivery is changing, the unavoidable truth of the importance of efflux pumps and metabolising enzymes should be kept in mind during drug design and optimisation. As the structural requirements decide the fate of a drug (i.e., if it can be a substrate to efflux pumps and metabolising enzymes), the design of new drugs or the modification of the existing drugs should be carried out such that the modified molecules will not be recognised by efflux proteins and metabolising enzymes. Structural alterations that reduce the affinity of a drug towards the efflux pump without compromising its therapeutic activity may lead to the development of more potent compounds and studies have already been carried out in this area with promising results [240-242]. Such drug discovery efforts may lead to agents with improved overall efficacy and less likelihood of developing resistance. Understanding the fundamental mechanisms underlying drug secretion and metabolism will allow the development of optimal dosing protocols, including the use of agents to specifically enhance absorption, by reducing the activity of efflux systems.

However, the strategy of structure-based inhibitors has met with failure because of the scarce amount of structural data pertaining to efflux transporters and metabolising enzymes. Despite progress being made, relevant structural data on efflux pumps such as P-gp is so far unavailable. The currently available structural information has failed to delineate the dynamic conformational changes involved in substrate translocation. As a result, the precise nature of the substrate interaction is not yet known. Undoubtedly, a key breakthrough will be needed, particularly from structural determination studies of MDR transporters and metabolising enzymes. In the near future, more information will be available on the structural dynamics of efflux proteins and metabolising enzymes, which will lead to a better understanding of the disposition of drugs and pharmacokinetic interactions.

Individualised pharmacotherapy (i.e., so-called tailor-made or order-made pharmacotherapy) can be provided to the patients by investigating the genetic polymorphisms of drug metabolising enzymes and efflux proteins. All of this can be achieved using the huge amount of knowledge from the human genome project. One of the practical approaches to accomplish this is the application of antisense technology for the inhibition of specific drug metabolising enzymes and efflux proteins.

Although rational drug design may lead to the development of new molecules, we must also look at new methods of empirical high-throughput screening to identify compounds that modulate the activity of multidrug pumps. Such an approach may involve the development of

drug-binding biosensors based on proteins (i.e., components of transcription factors). Such information could be utilised to quickly and economically screen chemical libraries for new drugs.

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