



## P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement

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### Abstract

Drug efflux pumps like P-glycoprotein (P-gp) and multidrug resistance (MDR) proteins were recognized to possess functional role in determining the pharmacokinetics of drugs administered by *peroral* as well as parenteral route. Advancements in molecular biology, to some extent, had revealed the structure, localization and functional role of P-glycoprotein and its mechanism of drug efflux. Broad substrate recognition by this protein and clinical implications of its inhibition has revolutionized cancer chemotherapy leading to design and development of novel P-glycoprotein inhibitors. In the recent times, the application of these inhibitors in improving *peroral* drug delivery has gained special interest. Inhibition of P-glycoprotein improves intestinal absorption and tissue distribution while reducing the substrate metabolism and its elimination. Eventually, various screening methodologies have been developed for determining the activity of P-glycoprotein, kinetics of drug transport and identification of substrates and inhibitors. In the present review, techniques used for screening P-glycoprotein inhibitors and the scope of these inhibitors in optimizing *peroral* drug absorption and pharmacokinetics are discussed along with a brief introduction to P-glycoprotein, its physiological function and active role in extrusion of drugs.

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**Keywords:** P-glycoprotein; Inhibitors; Intestinal permeability; *Peroral* drug delivery; Pharmacokinetic optimization; Screening

### 1. Introduction

The goal of a delivery system is to achieve and sustain therapeutic blood levels of drug, except for those targeted to specific sites. Various routes of administration are exploited for efficient delivery of drugs, in which *peroral* and parenteral routes are predominant. Unlike parenteral administration, *peroral* delivery poses many hurdles starting from drug dissolution in gastrointestinal fluid to first pass metabolism due to various physicochemical and biopharmaceutical problems. It was recently identified that drug efflux pumps like P-glycoprotein (P-gp) are playing major role in

altering the pharmacokinetics of various drugs and particularly associated with poor bioavailability in co-ordination with gut wall metabolism. Though much work has been pursued in the process of establishing the role of P-gp in multidrug resistance (MDR) in cancer cells, only recently it is gaining importance in absorption enhancement due to its selective distribution at the site of drug absorption. Thus, a deep insight and thorough understanding of P-gp, its physiological and biochemical role in effluxing drugs is worthwhile, in order to have an opportunity to improve the bioavailability of drugs restricted by P-gp. Role of other transporters such as multidrug resistance protein (MRP) 1–6 and BCRP at different biological barriers was also proved significant in determining pharmacokinetics of various clinically important drugs (refer [1] for further reading). This article touches upon the features and efflux function of P-gp and discusses in detail the role of P-gp inhibitors in improving the intestinal absorption and various screening techniques used in exploring P-gp modulation as a delivery tool in optimising pharmacokinetics.

**Abbreviations:** P-gp, P-glycoprotein; MDR, multidrug resistance; GIT, gastrointestinal tract; ATP, adenosine triphosphate; ADP, adenosine diphosphate; SAR, structure–activity relationship; CYP, cytochrome P 450; PET, positron emission tomography

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## 2. P-glycoprotein

### 2.1. Structure, distribution and function

P-gp is a type of ATPase, and an energy-dependent transmembrane drug efflux pump which belongs to members of ABC transporters [2]. P-gp, a 1280 amino acid long (molecular weight ~170 kDa) glycoprotein, is expressed as a single chain containing two homologous portions of equal length, each containing six transmembrane domains and two ATP binding regions separated by a flexible linker polypeptide region between the Walker A and B motifs [3,4].

Immunohistochemical analysis using monoclonal antibody provided evidence for localization of P-gp in a wide range of tissues, particularly in columnar epithelial cells of lower gastrointestinal tract (GIT), capillary endothelial cells of brain and testis, canalicular surface of hepatocytes and apical surface of proximal tubule in kidney [5]. Due to selective distribution at the port of drug entry and exit, P-gp has been speculated to play a major physiological role in absorption, distribution and excretion of xenobiotics. Overall P-gp function as a biochemical barrier for entry of xenobiotics and as a vacuum cleaner to expel them from the brain, liver, etc., and ultimately from systemic circulation.

### 2.2. Mechanism of drug efflux

Various models were proposed to explain the mechanism of xenobiotic extrusion by P-gp, however, the exact site of substrate interaction with the protein is not well resolved. The three prevalent models, pore model, flippase model and hydrophobic vacuum cleaner (HVC) model, explains the ef-

flux mechanism to certain extent (see Fig. 1). Among these HVC model has gained wide acceptance in which P-gp recognizes substrates embedded in the inner leaflet of plasma membrane and transported through a protein channel [6]. Recently, Rosenberg et al. [7] reported that three-dimensional conformation of P-gp changes upon binding of nucleotide to the intracellular nucleotide-binding domain. In the absence of nucleotide, the two transmembrane domains form a single barrel with a central pore that is open to the extracellular surface and spans much of the membrane depth, while upon binding nucleotides, the transmembrane domains reorganize into three compact domains that open the central pore along its length in a manner that could allow access of hydrophobic drugs directly from the lipid bilayer to the central pore of the transporter.

ATP binding and hydrolysis was found to be essential for functioning of P-gp, where one molecule of drug is effluxed at the expense of two molecules of ATP [8]. Sauna et al. [9] elucidated the catalytic cycle of P-gp, which expands the opportunity for the development of P-gp inhibitors, comprises of two cycles where drug and nucleotide binding sites coordinately function to efflux out the substrates by an ATP driven energy-dependent process. The drug and ATP initially binds to the protein at their own binding sites, where nucleotide hydrolyses to ADP yields energy for the extrusion of drug. The release of ADP from nucleotide binding site ends the first catalytic cycle followed by a conformational change that reduces affinity for both substrate and nucleotide. Further, the second catalytic cycle starts by hydrolysis of another molecule of ATP and released energy is utilized to reorient the protein to its native conformation. Subsequent release of ADP completes another catalytic cycle, bringing P-gp molecule back to the original state, where

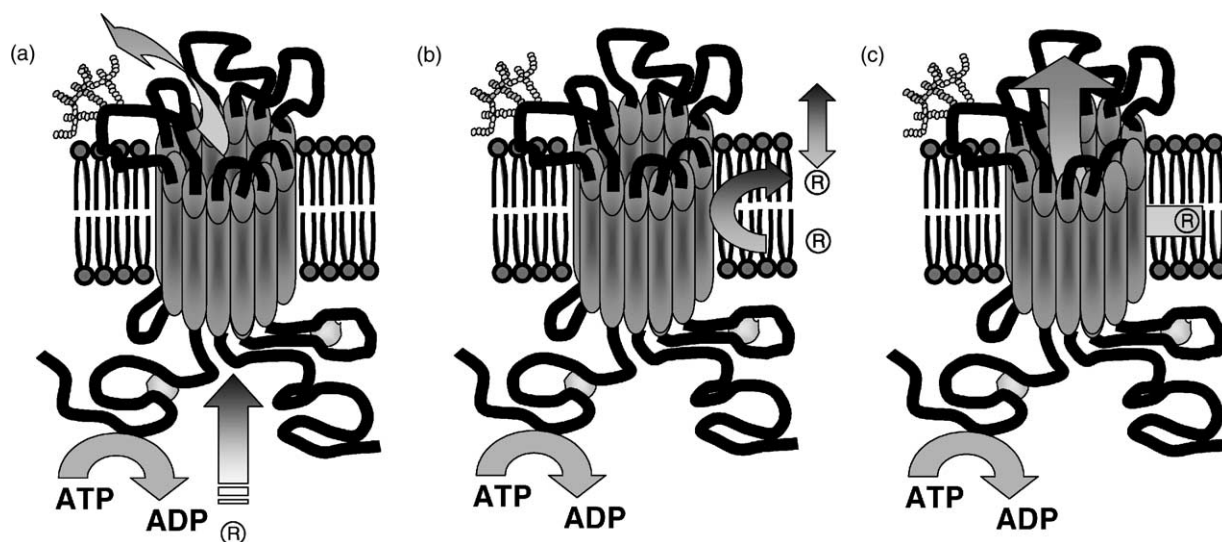


Fig. 1. Models proposed to explain the mechanism of drug efflux by P-gp. (a) Pore model, (b) flippase model and (c) hydrophobic vacuum cleaner model. In pore model, drugs associate with P-gp in the cytosolic compartment and are transported out of the cell through a protein channel. In flippase model, drugs embed in the inner leaflet of the plasma membrane, bind to P-gp within the plane of membrane and are translocated to the outer leaflet of the bilayer from which they passively diffuse into extracellular fluid. The hydrophobic vacuum cleaner model combines the features of 'pore' and 'flippase' models.

it again binds to both substrate and nucleotide to initiate the next cycle.

### 3. P-glycoprotein inhibitors: an overview

Screening studies for P-gp–drug interactions identified a number of clinically important drugs as P-gp substrates, which are as diverse as anthracyclines (doxorubicin, daunorubicin), alkaloids (reserpine, vincristine, vinblastine), specific peptides (valinomycin, cyclosporine), steroid hormones (aldosterone, hydrocortisone) and local anaesthetics (dibucaine). Even dye molecules (Rhodamine 123) and pharmaceutical excipients exhibited P-gp substrate activity (Table 1). Few of them were identified to inhibit P-gp, setting off an opportunity in MDR reversal. Improved clinical efficacy of various drugs observed by P-gp inhibition, especially drug subjected to MDR, lead to the design and development of modulators, which specifically block P-gp efflux and having improved toxicity profiles. P-gp inhibitors are gaining recognition to improve bioavailability by inhibiting P-gp in intestine, brain, liver and kidneys (Fig. 2), which has been hypothesised and emphasized by many researchers in recent years [10].

Based on the specificity and affinity, P-gp inhibitors are classified to three generations. First-generation inhibitors are pharmacological actives, which are in clinical use for other indications but have been shown to inhibit P-gp. These include calcium channel blockers such as verapamil; immunosuppressants like cyclosporin A; anti-hypertensives, reserpine, quinidine and yohimbine; and antiestrogens like tamoxifen and toremifene. The usage of these compounds is limited by their toxicity due to the high serum concentrations achieved with the dose that is required to inhibit P-gp. A

great deal of research by industrialist and academicians in the direction of improving toxicity profile resulted in second- and third-generation inhibitors that specifically modulate P-gp. Second-generation modulators are agents that lack the pharmacological activity of the first-generation compounds and usually possess a higher P-gp affinity. However, inhibition of two or more ABC transporters leads to complicated drug–drug interactions by this class of compounds, which include non-immunosuppressive analogues of cyclosporin A, PSC 833; D-isomer of verapamil, dexverapamil; and others such as biricodar (VX-710), GF120918 and MS-209. On the other hand, several other novel third-generation P-gp blockers are under development, however, primarily with the purpose to improve the treatment of multidrug resistant tumours and to inhibit P-gp with high specificity and toxicity. Modulators such as LY335979, OC144093 and XR9576 are identified to be highly potent and selective inhibitors of P-gp with a potency of about 10-fold more than the first- and second-generation inhibitors.

In general, P-gp can be inhibited (i) by blocking drug binding site either competitively, non-competitive or allosterically; (ii) by interfering ATP hydrolysis [11]; and (iii) by altering integrity of cell membrane lipids [12]. Although most of the drugs inhibit P-gp function by blocking drug binding sites, presence of multiple binding sites complicate understanding as well as hinder developing a true, conclusive SAR for substrates or inhibitors. However, the mode of handling of substrates and inhibitors are same by P-gp if the protein transport and/or inhibition are mediated only through binding sites. Then the issue to be addressed is how the substrates and inhibitors are discriminated at the molecular level. In this regard, Eytan et al. [13] proposed a plausible explanation that the modulator or inhibitor ‘flipped’ by P-gp can ‘flop’ back into the inner leaflet of the membrane, for

Table 1  
Agents that interact with P-glycoprotein<sup>a</sup>

Pharmacological category	Examples
Antiarrhythmics	Amioderone, lidocaine, quinidine
Antibiotics and antifungals	Cefoperazone, ceftriazone, erythromycin, itraconazole, ketoconazole, aureobasidin A
Antimalarials and antiparasites	Chloroquine, emetine, hydroxychloroquine, quinacrine, quinine
Calcium channel blockers	Bepidil, diltiazem, felodipine, nifedipine, nisoldipine, nitrendipine, tiapamil, verapamil
Calmodulin antagonist	Chlorpromazine, trifluoperazine
Cancer chemotherapeutics	Actinomycin D, colchicines, daunorubicin, doxorubicin, etoposide, mitomycin C, mithramycin, podophyllotoxin, puromycin, taxol, topotecan, triamterene, vinblastine, vincristine
Fluorescent dyes	BCECF-AM, Fluro-2, Fura-2, Rhodamine 123, Hoechst 33342
HIV protease inhibitors	Indinavir, nelfinavir, ritonavir, saquinavir
Hormones	Aldosterone, clomiphene, cortisol, deoxycorticosterone, dexamethosone, prednisone, progesterone analogs, tamoxifen, hydrocortisone, testosterone
Immunosuppressants	Cyclosporin A, cyclosporin H, tacrolimus, sirolimus
Indole alkaloids	Reserpine, yohimbine
Local anaesthetics	Bupivacaine
Surfactants/solvents	Cremophor-EL, triton X-100, Tween 80
Toxic peptides	<i>N</i> -Acetyl-leucyl-leucinal, gramicidine D, valinomycin
Tricyclic antidepressants	Desipramine, trazadone
Miscellaneous	Components of grape and citrus fruit juice, ethidium bromide, GF120918, ivermectin, MS-209, liposomes, LY335979, quercetin, SDZ PSC 833 (valsopodar), terfindine, tumour necrosis factor, Vitamin A

<sup>a</sup> Modified from [51].

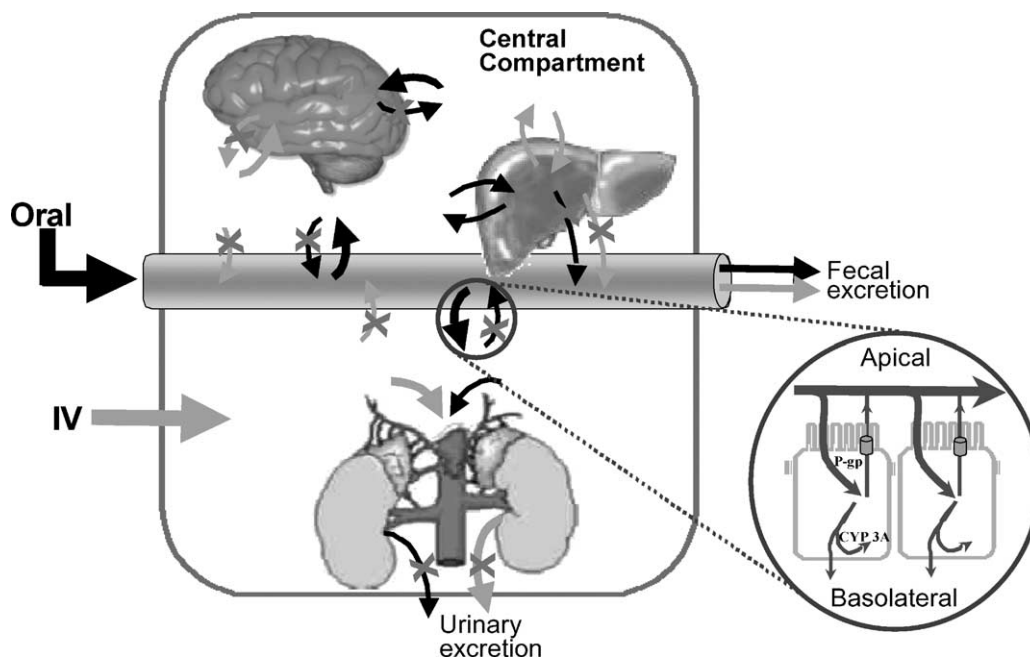


Fig. 2. Schematic representation of absorption, distribution and excretion of P-gp substrates, in the presence and absence of P-gp inhibitors. Distribution of P-gp in various organs like brain, liver, kidney and intestine affect pharmacokinetics of various drugs by (i) reducing the absorption through GIT (ii) effecting the cellular uptake into vital organs like brain, and (iii) by accelerating renal and hepatic clearance. Thus, P-gp modulators, based on their availability at these organs, inhibit the drug efflux resulting in improved bioavailability and tissue distribution. Inset shows the vectorial transport of P-gp substrates in intestinal enterocytes. Drugs which are substrates to both P-gp and cytochrome P 450 (CYP) 3A4 shows higher presystemic metabolism due to repeated access to CYP 3A4 by P-gp. Key: black arrows and gray arrows indicate drug transport after *peroral* and intravenous administration, respectively. Cross indicate P-gp inhibitors and the corresponding arrows are the pathways that may be inhibited by P-gp inhibitors, leading to enhanced drug bioavailability and drug availability at organs like brain.

further transport, which is very rapid creating a large difference between the rate of efflux of the substrate and inhibitor. Thus, the P-gp modulator is cycled repeatedly, preventing efflux of substrates, which depends on the hydrophobicity of the compound. This concept had been proved from the drug delivery point of view that absorption of high affinity drugs to the protein need not necessarily be limited by P-gp, e.g. verapamil, if it is highly permeable whereas less permeable drugs though weak substrates may undergo a substantial extrusion mediated by P-gp, e.g. tanolol [14].

Compounds inhibiting ATP hydrolysis could serve as better inhibitors, since they are unlikely to be transported by P-gp, and these kind of agents will require at low dose which is well desirable to use locally at gut lumen. Quercetin, a naturally occurring flavanoid, has been proposed to block P-gp function by an unknown mechanism but in general by interfering ATPase activity [11]. Since none of the substrates till now had been found to interact with the nucleotide binding sites to interfere the P-gp ATPase catalytic cycle, further research in exploring the detailed mechanism of inhibition of ATP hydrolysis would provide newer and better inhibitors with potent and specific activity.

Commonly used pharmaceutical surfactants are emerging as a different class of P-gp inhibitors, which act by altering integrity of membrane lipids. The change in secondary and tertiary structure is found to be the reason for loss of P-gp function due to disturbance in hydrophobic environ-

ment by surfactants. In a series of studies by Hugger and co-workers [15,16], it was observed that the change in fluidity of cell membrane facilitates influx of P-gp substrates by surfactants like polyethylene glycol, cremophor EL and Tween 80, demonstrated in Caco-2 cell line. Surfactants seem to be better choice since they were already approved for routine use in pharmaceutical formulations. However, until now it has been tested at *in vitro* level, which should be further evaluated by animal or human studies. Likewise, the clinical reality of those P-gp inhibitors, interfering ATP hydrolysis and catalytic cycle will be hampered by lack of understanding of the exact mode of inhibition which remains as a potential area for further research.

#### 4. Pharmacokinetic advantages by P-gp inhibition

Several studies have demonstrated the possible use of P-gp inhibitors that reverse the MDR phenotype-associated P-gp-mediated efflux in an attempt to improve the efficiency of chemotherapeutic agents and pharmacokinetic and pharmacodynamic profiles of a number of challenging molecules. This concept also offers new opportunities to overcome drug–drug interactions exhibited by a combination of P-gp substrates/inhibitors; and in developing clinically useful oral formulations of drugs which are, as a consequence, of poor oral absorption are administered

only by parenteral routes. Further, P-gp inhibitors may also influence absorption, distribution, metabolism and elimination of P-gp substrates in the process of improving pharmacokinetics. P-gp inhibition may reduce the clearance of intravenously administered substrates as a consequence of increased reuptake from intestinal lumen and/or a diminished intestinal secretion and increased renal reuptake.

Early studies on recemic verapamil to reverse P-gp-mediated resistance to vincristine and vinblastine [17] and its established record of safety provided the rationale for its clinical usefulness as P-gp inhibitor. In addition to this, orally administered verapamil has been shown to increase peak plasma level, prolong elimination of half-life and increase volume of distribution of doxorubicin after oral administration [18]. The total body clearance of paclitaxel and digoxin has been found to decrease substantially after co-treatment of verapamil in human subjects [19]. However, verapamil being a potent cardiovascular drug showed serious toxicities at the plasma levels needed for effective MDR1 reversal. As a result, dexverapamil emerged as a second-generation inhibitor which does not have any effect on cardiovascular system. Combination therapy of dexverapamil and paclitaxel in metastatic breast cancer patients showed increased mean peak paclitaxel concentration and delayed clearance [20].

The effects of cyclosporine A on the pharmacokinetics of etoposide have been demonstrated to be dose-dependent in a

phase I clinical trial [21], however, its use for long-term oral dosing may be hindered by the immunosuppressive effect. This led to the design of valspodar (PSC 833), an analogue of cyclosporine D with no immunosuppressive activity. In vitro studies demonstrated that PSC 833 may be as much as 20 times more potent inhibitor of P-gp as cyclosporine A [22]. In a comparative study of pharmacokinetics of paclitaxel in *mdr1a*(–/–) mice, enhanced oral absorption of paclitaxel was observed when wild-type mice were co-treated with PSC 833. Similarly, PSC 833 found to enhance oral bioavailability of anticancer drug etoposide in rats [23].

GF120918, an acridonecarboxamide derivative, was shown to be a potent blocker of P-gp in tumour cells in vitro and in vivo [24]. Further, it has been taken up to demonstrate its role in improving intestinal absorption of drugs like paclitaxel [25]. The plasma concentration of paclitaxel in wild-type mice receiving GF120918 was found to be similar to that observed in *mdr1a*-knockout mice for at least 12 h after i.v. administration suggesting that GF120918 blocks P-gp during this entire period.

Third-generation modulators, LY335979, XR9576 and OC144093 are highly potent and selective inhibitors of P-gp with less of drug interactions and toxicity. LY335979 is among the most potent modulators of P-gp. Treatment of mice, bearing P388/ADR murine leukaemia with LY335979 in combination with doxorubicin or etoposide, administered intravenously/intraperitoneally, showed a significant

Table 2

Examples for improved pharmacokinetics of P-gp substrates with coadministration of P-gp inhibitors

P-glycoprotein substrates	P-glycoprotein modulator	Experimental model	Pharmacokinetic effect
<b>First-generation inhibitors</b>			
Digoxin <sup>a</sup> [52]	Verapamil	Single-pass perfusion in rats	Increase in absorption rate
Doxorubicin [53]	Cyclosporine	Cancer patients	Inhibitor dose-dependent permeability enhancement
<b>Second-generation inhibitors</b>			
Doxorubicin [54]	PSC 833 <sup>b</sup>	Cancer patients	~50% increase in AUC
Paclitaxel [20]	R-verapamil	Cancer patients	Delayed mean paclitaxel clearance and increased peak concentration
Paclitaxel <sup>c</sup> [21]	GF120918	<i>mdr1a</i> b(–/–) knockout mice and wild-type mice	Enhanced BA
Paclitaxel <sup>d</sup> [55]	VX-710	Cancer patients	More than 50% decrease in paclitaxel clearance
Paclitaxel [56]	MS-209	Rats and mice	1.9- and 4.5-fold increase in BA in rats and mice, respectively
<b>Third-generation inhibitors</b>			
Doxorubicin [57]	LY335979	Cancer patients	~25% increase in BA at doxorubicin dose of 60 mg/m <sup>2</sup> and ~15% increase at a dose of 75 mg/m <sup>2</sup>
Paclitaxel [58]	OC144093	Cancer patients	~1.5-fold increase in AUC and ~2-fold increase in C <sub>max</sub>
Docetaxel <sup>e</sup> [59]	R101933	Cancer patients	Pharmacokinetics did not alter in the presence of inhibitor but the faecal excretion of docetaxel decreased significantly
Doxorubicin [60]	XR9576	Cancer patients	44% increase in AUC

BA, bioavailability; AUC, area under the plasma-time profile curve.

<sup>a</sup> Absorption rate of digoxin varied at different segments of GIT at different concentrations of verapamil.

<sup>b</sup> Drugs like doxorubicin are transported by P-gp and MRP2, thus non-specific inhibitors (second-generation compounds) when coadministered gives large increase in C<sub>max</sub> and AUC.

<sup>c</sup> VX-710 showed decrease in paclitaxel clearance with a maximum tolerated dose of paclitaxel (<80 mg/m<sup>2</sup>) that is roughly half of standard dose of 175 mg/m<sup>2</sup>.

<sup>d</sup> Pharmacokinetics of paclitaxel in *mdr1a*b(–/–) knockout mice was not altered by GF120918 whereas a significant increase in oral bioavailability (8.5–40.2%) was observed in wild-type mice.

<sup>e</sup> Docetaxel clearance decreased from 2.5% to less than 1%.

increase in life span without significant effect on the pharmacokinetics of these anticancer agents [26]. In contrast, LY335979 reduced paclitaxel clearance by approximately 19% when administered by infusion [27]. This may be due to its poor inhibition of four major cytochrome P450 isozymes important in metabolizing doxorubicin and etoposide. It was found that LY335979 is around 60-fold more specific to P-gp over cytochrome P450 3A4, the major metabolizing enzyme. The selectivity and potency of this modulator avoids complicated drug interactions, which make pharmacokinetic optimization difficult.

In spite of wide acceptance of MDR modulators for the cancer chemotherapy, the following issues should be addressed for a meaningful translation of MDR modulators to bioavailability enhancers. The inherent pharmacological action and higher concentration of drug required to inhibit the protein function may not be compromised for bioavailability enhancement. In addition, a local effect at GIT would be desirable rather a systemic effect and whole body burden.

Literature data (Table 2), most of the cases, demonstrated improvement in pharmacokinetics and tissue distribution for a number of drugs, when coadministered with P-gp inhibitors. Thus, it may be possible that the usual dose given will result in toxic blood levels and thus a marked reduction in dose is needed to achieve the desired blood levels, which may reduce the cost of therapy. However, complication in optimizing drug delivery demands a keen understanding of the functional and distributional features of P-gp for specificity and activity potential of P-gp modulators. Drug–drug interactions precipitating out of these combinations may be complicated as P-gp inhibitors also modulate other transporters (MRP 1–6, BCRP, LRP) and further influence distribution, metabolism and elimination of its substrates. New generation P-gp modulators are more specific to MDR1 and are found to be effective at low dose, however, demand for more and more specific P-gp modulators with low toxicity profiles, has hasten up drug discovery groups across the globe to design novel modulators. In this process, various screening methodologies evolved for effective screening of modulators with improved throughput.

## 5. Techniques for screening P-gp inhibitors

Several *in vitro*, *in situ* and *in vivo* techniques are available for screening chemical entities for their ability to inhibit P-gp and other MRPs. Each technique has their own advantages and limitations, to be used as screens in drug discovery and delivery. By drug discovery screens we mean the design of new chemical entity for inhibiting P-gp efflux whilst drug delivery screens are meant to optimize the use of inhibitors in improving the absorption or cell accumulation of challenging drugs. These techniques are also useful to test the substrate specificity to the P-gp and significance of the protein in altering pharmacokinetics, further can aid in drug candidate selection and optimization in drug discov-

ery settings. The strategic application of these approaches, based on the ultimate objective of the study, would help in high throughput and better optimization (Fig. 3). However, combinations of these assays offer the potential to address many of the bottlenecks currently encountered in screening for P-gp inhibitors and provide mechanistic information (Table 3).

### 5.1. Transport assays

Intestinal or cultured monolayers efflux assay is the most common *in vitro* technique, where the ratio of basolateral (B)–apical (A) to A–B permeability of known substrates like Rhodamine 123, digoxin and vinblastine is compared in the presence and absence of inhibitors [28]. Due to concentration-dependant inhibition of active efflux on the apical side by P-gp inhibitors, the B–A permeability decreases whilst A–B permeability increases with ratio approaching unity as the dose of inhibitor increases. The affinity of inhibitor to P-gp may be studied by calculating the active flux that can be obtained from the B–A fluxes in the absence and presence of P-gp inhibitors (Fig. 4). Inhibition potency, determined by inhibitor concentration-dependent transport assay, is usually represented as IC<sub>50</sub>, concentration that gives 50% of maximum inhibition of a known P-gp substrate. Specificity of the inhibitor to P-gp may be determined by competitive assays, which involve transport assay of a known substrate in the presence of a known inhibitors (specific to various transporters) with and without compounds under test. *Vinca* alkaloids and anthracyclines are transported by P-gp, MRP1 and MRP2 proteins, while taxanes are transported only by P-gp. MDR modulators like PSC 833, VX-710, GF120918 and MS-209 inhibit two or more ABC transporters while third-generation inhibitors like LY335979 specifically inhibit P-gp. Thus, appropriate design of competitive inhibition assays using known selected substrates and modulators will show the specificity of the inhibitors towards the efflux pump.

Expression of P-gp in the cultured monolayers (Caco-2, HCT-8, T8 and MDCK) improved upon the throughput for these screens. Caco-2 cell monolayers are widely used to demonstrate the role of P-gp inhibitors in improving intestinal absorption [29,30]. They also allow an excellent qualitative insight into kinetic and mechanistic processes involved, but careful interpretation of data is required as the level of expression varies from culture to culture, difference in passage number and does not always correlate with the expression at the site of drug absorption in GIT. Caco-2 cells take 21–28 days to reach confluency and 17–27 days for P-gp to become fully functional. Further Caco-2 cells have been found to express other multidrug resistance proteins making it difficult to elucidate inhibitor specificity towards P-gp. Madin-Darby canine kidney (MDCK) cells, which can differentiate into columnar epithelium with tight junctions in a shorter period of time (3 days), transfected with human *MDR1* gene express high level of P-gp localized on apical

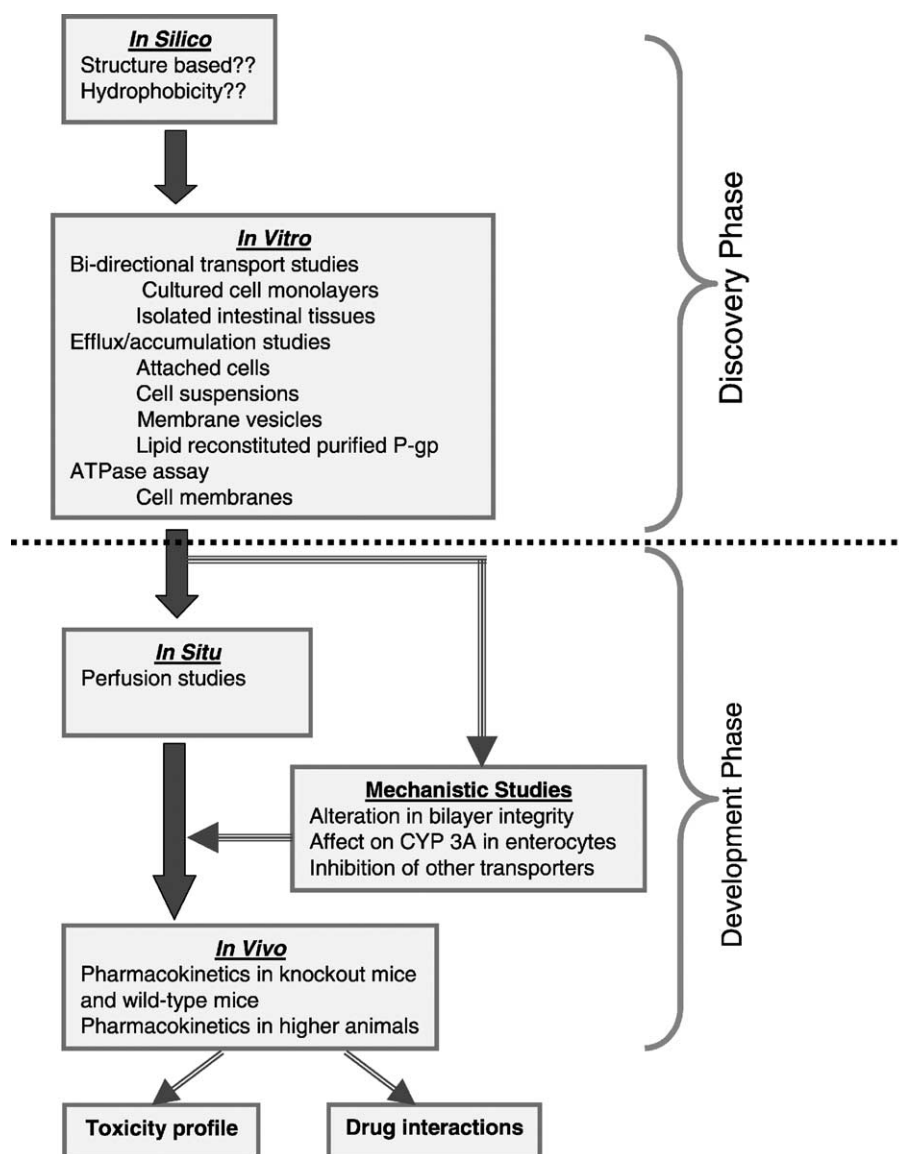


Fig. 3. Flow chart linking various models for screening of P-gp inhibitors in discovery and development phases. Models used in the early discovery phase typically allow rapid screening, whilst the highest throughput can be obtained by *in silico* methods. However, due to incomplete understanding of SAR of P-gp substrates and inhibitors, this model is of no practical utility at this stage. Transport and accumulation studies using cell cultures and isolated tissues provide good information in discovery stages; and further availability of commercial kits made these models useful for HTS. In development phase, use of *in situ* perfusion model and single dose pharmacokinetics in small animals will give more complete picture of P-gp inhibition, while accounting for intestinal metabolism and effect of P-gp inhibitor on metabolic enzymes. Mechanistic studies in the process of development provide more insights into the specificity and affinity of inhibitor towards the protein and further its effect on other transporters and metabolic enzymes.

side of polarized cell monolayers. This serves as a useful model of human P-gp in intestinal mucosa and has the potential to serve as a high throughput screening tool to detect compounds prone to P-gp-mediated intestinal secretion, for identifying P-gp inhibitors and in concluding potential P-gp related drug–drug interactions in drug discovery and development settings [31]. MDCK cells have been transfected with human *MDR1*, *MRP2* genes to elucidate the substrate specificity of these transporters [32,33]. These transfected MDCK cells expressed higher levels of P-gp or MRP2 compared to Caco-2 and MDCK wild-type cells and thus would be useful in easy identification of substrates and modulators.

The barrier function of P-gp was demonstrated using gene knockout mice, *mdr1a*( $-/-$ ), which lacks genes for P-gp expression but normal, fertile and feodal [34]. In mice, *mdr1a* and *mdr1b* genes encode two separate P-gp proteins that are analogous to the human *MDR1* gene [35]. Consequently, one or both can be knocked out to screen P-gp inhibitors and more specifically to identify the selective inhibition as these animals express other transporters and enzymes in normal levels. Use of *mdr1*-knockout mice is one of the most reliable *in vivo* approaches to investigate P-gp inhibitors where oral absorption is high for P-gp substrates. Presence of inhibitors lead to increased oral bioavailability

Table 3  
Application of screening techniques for identification of P-gp inhibitors and/or substrates

Compounds	Type of studies	Screening model	Reference
66 clinical drugs from different chemical and pharmacological classes	Transport assay	MDR1-MDCK II cell monolayers	[39]
	ATPase assay Calcein inhibition assay	Spodoptera frugiperda membrane	
Indoloquinoline derivatives	Cytotoxicity assay Accumulation assay Photo affinity labelling assay	NCI/ADR cells MCF-7 or NCI/ADR cells NCI/ADR cells	[61]
Surfactants	Transport assay	Caco-2 cell monolayers	[62]
HIV protease inhibitors	ATPase assay Photo affinity labelling assay Accumulation assay	KBV1 cells NIH3T3 cells NIH-MDR-G185 cells	[63]
Peptide chemosensitizers	ATPase assay Photo affinity labelling assay Accumulation assay	Spodoptera frugiperda membrane CH <sup>R</sup> C5 plasma membrane vesicles P-gp reconstituted proteoliposomes	[46]
Cyclopropyl dibenzosuberane modulator, LY335979	Cytotoxicity assay Accumulation assay Photo affinity labelling assay ATPase assay	MCF-7 cells and MCF-7/ADR cells 2780AD tumour cells	[26]
Cyclosporine A analogues and metabolites	Vinblastine uptake assay Photo affinity labelling assay ATPase assay	P-gp isolated from MDR Chinese hamster ovary cells reconstituted into liposomes	[64]
Isoprenoid derivatives	Cytotoxicity assay Accumulation assay Photo affinity labelling assay	KB/VJ-300 cell lines KB/VP-4 cell lines	[65]
First- and second-generation inhibitors (R- and R,S-verapamil, cyclosporine, PSC 833)	Transport assay Accumulation assay	HCT-8 cell monolayers	[66]
Fruit extracts (strawberry, orange, apricot, mint extracts)	Transport assay	Caco-2 cell monolayers	[67]
28 flavonoid derivatives of <i>N</i> -benzylpiperazine	Cytotoxicity assay Accumulation assay	K562/DOX cells	[68]

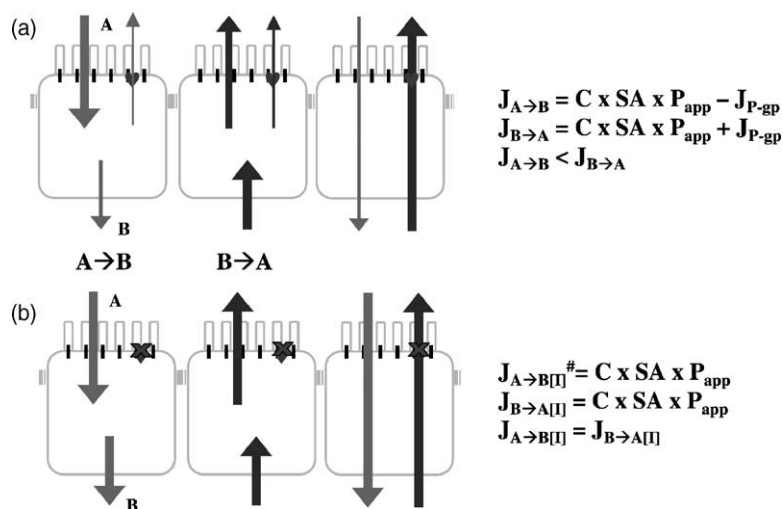


Fig. 4. Model depicting bi-directional transport (absorptive,  $J_{A \rightarrow B}$  and secretory,  $J_{B \rightarrow A}$ ) of P-gp substrates in the (a) absence and (b) presence of P-gp inhibitor. Absorptive flux ( $J_{A \rightarrow B}$ ) of a P-gp substrate will be less than the secretory flux ( $J_{B \rightarrow A}$ ), while complete inhibition of P-gp makes the ratio of absorptive to secretory fluxes near to unity. In the presence of inhibitors, transport flux is only due to passive diffusion, which is a product of concentration ( $C$ ) of the drug in donor compartment, surface area ( $SA$ ) and apparent passive permeability ( $P_{app}$ ).  $J_{P-gp}$  is given by  $C \times V_m / (C + K_m)$ , where  $V_m$  is maximum velocity of P-gp efflux per unit surface area and  $K_m$  is affinity constant. Key: A, apical; B, basolateral; #, flux in the presence of P-gp inhibitor; heavy arrows vs. light arrows indicate relative magnitudes of flux.

in wild-type mice without any change of pharmacokinetics in *mdr1*-knockout mice. This model provides the advantage of studying affinity of P-gp inhibitors under physiological conditions and their affinity in improving the bioavailability of certain drugs in the presence of metabolizing enzymes like CYP 3A. Being an elegant model for optimizing drug delivery using P-gp inhibitor, this technique is not suitable for high throughput screening and further includes tedious procedures and biological variability involved in animal experimentation. Use of intestinal sections of such transgenics for *in vitro* studies will be useful in early screening with efficient throughput. Isolated tissues from *mdr1a*(-/-) animals offer several advantages over existing *in vitro* systems for studying P-gp inhibition. Firstly, P-gp inhibition activity of a compound can be quantified without competitive inhibition assays and complicated procedures and conditions as involved in cultivating cell culture monolayers. Secondly, *mdr1a*(-/-) model facilitate identification of non-P-gp transporters inhibition and their relative contribution to the overall inhibitory potency of the compounds in test with a limited number of experiments. Thirdly, they allow for direct quantification of P-gp inhibition in a physiologically relevant system, which may allow for better predictions in humans.

A mutant animal model, CF-1 mice, exhibited a genetic defect, resulting in the absence of P-gp expression [36]. CF-1 mutant mice has been used to investigate the effect of P-gp on the disposition of orally administered ivermectin and cyclosporin A [37]. Similar to transgenic models these mutants are also useful as *in vitro* and *in vivo* screening tools.

## 5.2. Drug accumulation and efflux assays

Transport inhibition potency of P-gp modulators may also be studied by substrate accumulation studies, where the amount of substrate taken up by cells expressing P-gp is compared in the presence and absence of inhibitors. In case of substrates effluxed by the P-gp, a lesser amount of drug accumulates in the absence of inhibitors, while the presence of inhibitors, facilitate increased accumulation of drug within the cells, based on passive diffusion of the substrate across lipid bilayer, until equilibrium is attained (Fig. 5). On the other hand, in inside-out membrane vesicles and reconstituted proteoliposomes, inhibition of P-gp lead to decrease in substrate accumulation. Drug efflux assays involve preloading of the selected substrate in the cells, cell monolayers [14], membrane vesicles [38] or P-gp reconstituted proteoliposomes and studying the transport of the substrate to surrounding medium. Drug efflux assays using cell monolayers or isolated tissue provide a better picture of overall transport of drug due to active efflux by P-gp (Fig. 5). The amount of substrate transported to apical medium will be due to combination of passive and active P-gp transport, while substrates transported to basolateral medium is only due to passive diffusion. Such type of studies would help in modelling of drug transport and influence of inhibitors [14].

Several fluorescent compounds (rhodamine dyes, Fure-4, BCECF) and fluorogenic dyes (calcein AM) are substrates to P-gp in intact MDR cells, and can be used as markers to generate kinetic data of efflux and P-gp inhibition [39]. The marker selected, primarily must be an efficient substrate that is generally and ubiquitously affected by other P-gp substrates and/or inhibitors and must have a 'desirable' passive diffusion, to an extent, that the transport mediated by P-gp be magnified. Fluorescence assays provide with high throughput screening as they can be quantified easily using a 96-well plate and are vulnerable to flow cytometry. Calcein acetoxyethyl ester (calcein AM), a non-fluorescent compound with sufficient lipophilicity, diffuses across the cell bilayer where it converts to hydrophilic and intensely fluorescent calcein by the endogenous esterases. P-gp rapidly remove the non-fluorescent calcein AM resulting in decreased accumulation of the highly fluorescent calcein in the cytoplasm. This principle of cell accumulation of fluorescent calcein in the presence of P-gp inhibitors has been commercialized by Molecular Probes, Eugene (Vybrant™ MDR assay kit) and SOLVO Biotechnology, Hungary (MultiDrugQuant™ assay kit); and successfully used for high throughput screening. Fluorescence spectroscopy provides mechanistic information regarding reversal of P-gp efflux transport and cell accumulation [40]. Highly sensitive probes for functional assays of the MDR1-encoded P-gp help in screening for P-gp inhibitors with high throughput.

## 5.3. ATPase assay

P-gp, an ATP-dependent active transporter, utilizes two molecules of ATP to efflux a molecule of substrate [41]. Thus, monitoring substrate stimulated-ATPase activity in the presence of substrate like verapamil, progesterone and vinblastine, which interact on different P-gp sites [42] with and without test compounds allows identification of P-gp inhibitors. ATPase activity can be determined using cell membranes, cultured cells and membrane vesicles. Cell membranes of Sf9 insect cells expressed with human *MDR1* gene via a baculovirus vector is commercially available for screening P-gp substrates and/or inhibitors (Gentest, Woburn, MA) (<http://www.gentest.com>). The drug stimulated ATPase activity determined as the difference between amount of ATP consumed in absence and presence of vanadate is used as a marker for complete inhibition of P-gp. Vanadate associates with ATP resulting in trapping of the transition state complex ADP·Vi at the active site of one of the nucleotide binding domains, completely abolishing ATPase activity [43]. ATPase activity can be estimated by quantifying ATP, ADP [44] released NADP (<http://www.spibio.com>), or by quantifying liberated inorganic phosphate [45,46]. Ion-exchange HPLC is usually used for quantifying ATP and ADP, however, reversed-phase HPLC along with the ion-pairing reagents such as tetrabutylammonium hydrogen sulphate and tetrabutylammonium dihydrogen phosphate is found to be

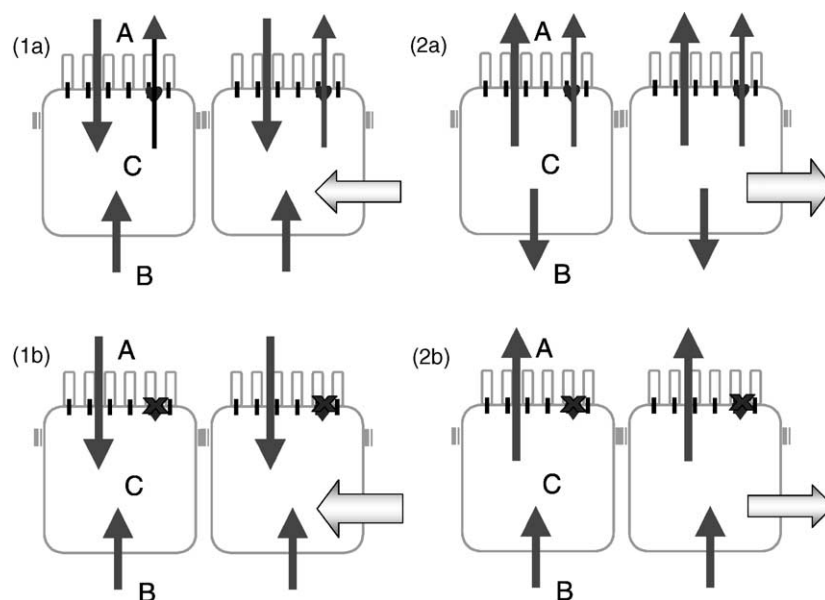


Fig. 5. Schematic of drug (1) accumulation and (2) efflux studies in cell monolayers in the (a) absence and (b) presence of P-gp inhibitor. Accumulation assays are carried out by quantifying the amount of P-gp substrates transported from extracellular medium into the cell, while in efflux studies amount of P-gp substrates transported out of preloaded cells is monitored. Key: A, apical; B, basolateral; C, cytosolic; heavy vs. light block arrows indicate relative magnitudes of the overall amount of drug transported across the cell membrane.

advantageous with better reproducibility and faster retention time [47].

Certain limitations of this screening model are as follows: (1) ATPase assay indirectly estimates efflux transport and does not provide with kinetics of drug transport and/or inhibition, (2) usually, inhibitors do not activate ATPase, however, modulators like verapamil stimulates ATPase leading to misinterpretation of results. P-gp displays constitutive ATPase activity in the apparent absence of substrate, which may be due to the transportation of endogenous chemicals and lipids. Thus, enhancement in ATPase activity is not true for all substrates as observed with cyclosporine A, daunorubicin and colchicine, which maintain the basal levels of ATPase activity [39]. In general, drugs produce a bimodal ATPase activity, with stimulation at low concentrations and inhibition at higher concentrations. These complications in ATPase activity quantification may lead to false negatives (missing of potent inhibitors) during screening.

#### 5.4. Labelling and binding assays

Inhibition of photoaffinity labelling of P-gp by  $^3\text{H}$ -azidopine or  $^{125}\text{I}$ -arylazidopropozin explore an opportunity to measure the affinity of substrates for a common binding site on P-gp [48]. However, low site occupancy and low labelling efficiency, which may change with protein conformation limits the complete reliability of this technique. In practice, photoaffinity probes incubated with P-gp enriched cell membranes in the presence of a P-gp substrate are irradiated to induce binding by UV source and the amount of radioactivity associated with P-gp is assessed by autoradiography.

Alternatively, competitive binding assays like radio-ligand binding may be used [49]. Hendrikse and Vaalburg [50] reported the use of positron emission tomography (PET) for analysing dynamics of P-gp multidrug resistance. PET, a non-invasive technology that enables visualization of cell physiology by electronic detection of positron-emitting radiopharmaceuticals quantifies the functionality of P-gp inhibitors under physiological conditions. Labelling and binding assays provide high throughput identification of substrates and inhibitors. Further, allow modelling of interaction of the drug and inhibitor with P-gp. However, the incomplete knowledge about the drug-binding site and mechanism of substrate efflux limits their use.

## 6. Conclusions

P-gp inhibitors represent an emerging class for bioavailability enhancement for a number of chemically diverse, challenging molecules. Even though a number of P-gp inhibitors are studied for MDR in cancer chemotherapy, lack of extensive studies on their role in enhancing drug absorption across various intestinal segments in human, impede their clinical utility.

The most concerning issues propelling use of P-gp modulators in bioavailability enhancement and in pharmacokinetic optimization include:

- (i) P-gp acts as barrier to intestinal absorption of a number of clinically important drugs with 'better' physicochemical properties. Further, these drugs induce P-gp

expression, which may lead to inefficient therapy on chronic usage.

- (ii) Concert role of P-gp and CYP 3A further limits bioavailability of orally administered drugs due to a great overlap between substrate specificity and intestinal distribution of both the proteins.
- (iii) Distribution of P-gp extends to the major excretory organs like kidney and liver leading to change in distribution and elimination kinetics.

Good number of P-gp modulators has been explored for bioavailability and pharmacokinetic improvement, however, modulators belonging to second and third generation are found promising because of their low toxicity profiles and high specificity towards P-gp. Few of them were proven effective in various clinical studies and are in active development phases (Table 2). But, the therapeutic benefits of these molecules are yet to be firmly established. Alternatively, use of combinations of P-gp modulators might turn out to have improved efficiency with less of toxicity. Various screening models and methodologies have evolved for identification and characterization of P-gp modulators. Strategic application of the available models provide with concluding results, however, novel models with improved throughput and confirmative results will improve the screening efficiency with less of 'false negatives' and 'false positives' during discovery settings.

Although, P-gp inhibition seems to be a complex and difficult task and much work is needed to optimise this strategy, complete understanding of P-gp efflux mechanisms, improved screening methods, and SAR studies would offer an opportunity for not only enhancing the bioavailability of life saving drugs like paclitaxel and saquinavir but also improve their pharmacokinetics, tissue distribution and cell uptake.

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