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ABC Transporters in Multidrug Resistance and Pharmacokinetics, and Strategies for Drug Development

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Abstract

Multidrug resistance (MDR) is a serious problem that hampers the success of cancer pharmacotherapy. A common mechanism is the overexpression of ATP-binding cassette (ABC) efflux transporters in cancer cells such as P-glycoprotein (P-gp/ABCBl), multidrug resistance-associated protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) that limit the exposure to anticancer drugs. One way to overcome MDR is to develop ABC efflux transporter inhibitors to sensitize cancer cells to chemotherapeutic drugs. The complete clinical trials thus far have showen that those tested chemosensitizers only add limited or no benefits to cancer patients. Some MDR modulators are merely toxic, and others induce unwanted drug-drug interactions. Actually, many ABC transporters are also expressed abundantly in the gastrointestinal tract, liver, kidney, brain and other normal tissues, and they largely determine drug absorption, distribution and excretion, and affect the overall pharmacokinetic properties of drugs in humans. In addition, ABC transporters such as P-gp, MRP1 and BCRP co-expressed in tumors show a broad and overlapped specificity for substrates and MDR modulators. Thus reliable preclinical assays and models are required for the assessment of transporter-mediated flux and potential effects on pharmacokinetics in drug development. In this review, we provide an overview of the role of ABC efflux transporters in MDR and pharmacokinetics. Preclinical assays for the assessment of drug transport and development of MDR modulators are also discussed.

Keywords

Cancer therapy; multidrug resistance; ABC transporter; Pharmacokinetics; Drug development

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CONFLICT OF INTEREST
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1. INTRODUCTION

Resistance to structurally and functionally related or unrelated medications is a major impediment to successful cancer pharmacotherapy [1–6], Intrinsic or primary multidiug resistance (MDR) occurs among a small population of tumor cells such as “cancer stem cells” or malignant tumor cells that are “inherently” resistant to anticancer drugs from the very beginning of drug treatment. In contrast, acquired or secondary MDR happens in survived tumor cells that develop drug resistance capacity during pharmacotherapy. A number of mechanisms have been identified for MDR, which could be drag-dependent, target-dependent and drag/target-independent [7]. Drag-dependent MDR is caused by the alternation of cellular drag disposition, particularly the overexpression of efflux drag transporters and drag-metabolizing enzymes in cancer cells. Target-dependent MDR is attributable to the desensitization of drug targeting, which includes mutation, translocation, deletion and amplification of the target. Drag- or target-independent MDR is due to the escape from drag targeting through genetic or epigenetic alternation of cell signaling pathways. It is noteworthy that these mechanisms are recognized for the resistance to both cytotoxic agents and targeted anticancer drags, and multiple mechanisms may be involved in drag resistant tumor cells. In addition, drug resistance is shown for other types of therapeutic agents such as antibiotics and antimalarials [8–11]. In this review, we focus on the role of ATP-binding cassette (ABC) transporters in MDR of cancer cells and the development of ABC efflux transporter inhibitors as chemosensitizing agents towards improved chemotherapy, as well as the importance of ABC transporters in pharmacokinetics and preclinical assays for the evaluation of drag transport in drug development.

2. CANCER PHARMACOTHERAPY AND MULTIDRUG RESISTANCE

Cancer disease is a leading cause of death worldwide. The number of cancer deaths is estimated to increase from 12 million new cases and 7 million deaths in 2008 to 26 million new cases and 11.4 million deaths in 2030, respectively [12]. The high incidence and mortality are likely due to the complex interactions of non-modifiable (e.g., genetic susceptibility and aging) and modifiable risk factors (e.g., tobacco, infectious agents, diet, and physical activity) [13].

2.1. Cancer Pharmacotherapy

Standard managements of cancer diseases include surgery, radiotherapy and pharmacotherapy, which may be used alone or in combination, depending upon cancer type, disease status, pathological and molecular characteristics as well as patient’s condition. Surgery represents the first line of therapy and benign tumors may be curable by surgery. Radiation therapy is commonly applied to kill or control **malignant cells** and it may be curative in some cancers localized to specific areas of human body. Pharmacotherapy is the use of a variety of anticancer drags including chemotherapeutics, hormonal and immunotherapeutic agents (Table 1) to manage cancer cell proliferation or apoptosis. Among them, the small-molecule chemotherapeutic agents exhibit a broad spectrum of cytotoxic activities and represent the most commonly used drags for cancer therapy. Based on their mechanistic actions, small-molecule anticancer drags may be divided into three

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major types, cytotoxic drags (antimetabolites, genotoxic drags and mitotic spindle inhibitors), protein kinase inhibitors and hormonal agents (Table 2) [5, 14, 15].

Antimetabolites are structurally similar to the metabolites, e.g., heterocyclic bases and nucleosides needed for the biosyntheses of DNA and RNA, so that they prevent the growth and division of tumor cells. Antimetabolite drags include folate, purine and pyrimidine antagonists (Table 2). Pyrimidine antagonists such as 5-fluorouracil, gemcitabine and arabinosylcytosine block the production of pyrimidine nucleotide or cause a premature termination when the drugs themselves are incorporated into DNA [5, 16]. This process stops DNA replication and arrests cell growth. Gemcitabine also irreversibly inhibits the enzyme **ribonucleotide reductase** (RNR) required for DNA replication and repair, and induces apoptosis [17], It is widely used in the treatment of various **carcinomas** including pancreatic, bladder, breast and non-small cell lung cancers. Likewise, purine antagonists such as 6-mercaptopurine, 6-thioguanine, fludarabine and acyclovir inhibit the syntheses of adenine and guanine necessary for DNA replication [18]. Folate antagonists include methotrexate, trimethoprim and pyrimethamine, which block the use of folic acid. In particular, these antifolates inhibit the enzyme dihydrofolate reductase (DHFR) that is required for methylation and formation of purine and pyrimidine for DNA/RNA/protein production [5, 19]. Note that methotrexate is a **competitive inhibitor** of DHFR, and overexpression of DHFR may explain the resistance to methotrexate.

Genotoxic agents bind to DNA directly or damage DNA through interfering critical enzymes in control of the changes of DNA structure [5]. Genotoxic drugs include alkylating agents (e.g., cisplatin and cyclophosphamide), intercalating agents (e.g., doxorubicin and daunorubicin) and topoisomerase inhibitors (e.g., irinotecan and topotecan) (Table 2). Alkylating agents add an alkyl group to the DNA and prevent cells from proper DNA replication and transcription. Intercalating agents bind to the DNA directly and inhibit DNA replication in cancer cells [5, 20], The inhibition of topoisomerase I or II, enzymes in control of the DNA structure, prevents the general functions such as transcription, replication and repair of DNA [21]. Topoisomerase I inhibitors include topotecan, irinotecan and rubitecan, and topoisomerase II inhibitors consist of etoposide and teniposide. Interestingly, the anthracenedione anticancer drug mitoxantrone not only acts as a topoisomerase II inhibitor but also participates in intercalation.

Mitotic spindle inhibitors are mainly natural compounds such as vinca alkaloids (e.g., vinblastine and vincristine) and taxanes (e.g., paclitaxel and docetaxel) [22] (Table 2). Mitosis is the final process in cell cycle when the chromosomes in the nucleus are separated into two identical sets for the formation of two nuclei/cells. Microtubules, assembled by tubulin components, play a central role in the separation of chromosomes to the opposite ends of a mitosing cell during the anaphase. Mitotic inhibitors block the polymerization of tubulin monomers, and thus prevent cancerous cells from division and tumorigenesis [5, 22].

Hormonal agents are a big group of anticancer drugs in treating hormone-dependent cancers (e.g., breast and prostate cancer) [15, 23–25], which interfere with the endocrine systems. Hormonal anticancer drugs include selective estrogen receptor modulators (SERMs; e.g., tamoxifen and raloxifene), aromatase CYP19A1 inhibitors (e.g., anastrozole, letrozole,

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aminoglutethimide and exemestone), androgen antagonists (e.g., flutamide, bicalutamide, cyproterone and niltamide) and gonadotropin-releasing hormone (GnRH) analogues (e.g., **leuprolide** and **goserelin**) (Table 2). Aromatase inhibitors block the action of aromatase in converting testosterone to estrogen, and they are often used for treating estrogen-sensitive breast cancers in postmenopausal women. SERMs act as antagonists of estrogen receptor in breast tissues; thus SERMs are widely used for the treatment of estrogen receptor positive breast cancers in both pre- and post-menopausal women. Androgen antagonists such as flutamide and bicalutamide inhibit the androgen receptors and suppress testosterone promoted cell proliferation. Therefore antiandrogen drugs are primarily employed to treat prostate cancers. The GnRH analogs act as pituitary GnRH antagonists and reduce the production of sex hormones (both testosterone and estrogen). GnRH analog therapy is frequently used in the treatment of prostate cancer. In addition, inhibitors of CYP17A1 (e.g., abiraterone) or 5α-reductase (dutasteride and finasteride) involved in steroid metabolism have been approved by the US Food and Drug Administration (FDA) for the treatment of prostate cancer or prostatic hyperplasia, and many others are under development.

Protein kinase inhibitors (Table 2) are a newer group of anticancer drugs that selectively block the actions of protein kinases critical for the metabolism, proliferation, migration, invasion and apoptosis of cancer cells. The approval of imatinib by FDA in 2001 as the first Bcr-Abl tyrosine-kinase inhibitor for the treatment of **chronic myelogenous leukemia** (CML) represents a successful and revolutionized "targeted" or "rational" therapy for cancer disease [26–28]. Besides Bcr-Abl, many protein kinases such the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and mitogen activated protein kinase (MAPK) involved in different or the same cell signaling pathways are commonly overexpressed or activated in cancer cells, and are proven to promote tumorigenesis. Targeting these protein kinase oncogenes with antibodies or small molecules, either by inhibiting the ATP binding sites or disrupting the kinase-substrate interactions through other means, is an effective way to manage tumor growth. Indeed, many kinase inhibitors such as nilotinib (for the treatment of CML), gefitinib (for treatment of lung and colorectal cancer), erlotinib (for the treatment of non-small cell lung cancer) and dasatinib (for the treatment of CML) (Table 2) have been developed and approved for cancer treatment in the past decade [28–30]. In addition, there are lots of other selective protein kinase inhibitors currently under clinical tests of their potential applications to cancer treatments.

2.2. Multidrug Resistance and the Common Echanisms

The ability of cancer cells that are inheritably resistant to or develop a resistance afterwards to the same drug or different drugs causes a persistent problem in cancer pharmacotherapy [31]. Since the development of the remarkable protein kinase inhibitor imatinib for the treatment of CML, MDR was once regarded as a vanishing problem specifically associated with cytotoxic drugs, and it would not be a problem for "targeted" therapeutics [32]. Soon it was found that some CML patients, especially the most advanced-phase patients, showed relapse on imatinib therapy despite many CML patients demonstrated durable responses [33–36]. So were other protein kinase inhibitors such as gefitinib and erlotinib [37–39], although some drugs (e.g., gefitinib, erlotinib and nilotinib) inhibit the function of ABC efflux transporters [40–44], Therefore, understanding how MDR occurs is critical for the

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prediction of MDR and development of possible strategies to overcome MDR towards improved pharmacotherapy.

MDR can arise as a result of the changes of cellular or non-cellular processes [45]. Non-cellular drug resistance is caused by a higher vascular permeability and the absence of functional lymphatic system that leads to a reduced nutrient, oxygen and drug access to tumor cells [46, 47]. For instance, the generation of lactic acid by hypoxic tumor cells provides acidic environments and MDRs [48], A cellular drug resistance may be classified as classical and non-classical MDR phenotypes [2]. Classical MDR, known as transport-based MDR, includes a decreased uptake of water-soluble drugs (e.g., folate inhibitors and nucleotide analogues) and increased energy-dependent efflux of hydrophobic drugs [4, 5]. Non-classical MDR is accompanied by the alteration of specific enzyme systems (e.g., glutathione *S*-transferases and/or topoisomerases) and imbalance of proteins, which control drug metabolism, apoptosis and membrane permeability [2,49–51].

Alternations of drug processing proteins and changes of drug targeted proteins or cell signaling pathways involve multiple molecular mechanisms such as gene mutation, translocation, deletion and amplification as well as epigenetic regulation [32, 52]. The mutational status of p53 was revealed to be closely related to the response of ovarian cancer to platinum-based therapy [53] and the response of ovarian cancer to platinum-based therapy [53] and the response of breast cancer to anthracycline treatment [54]. The point mutations found in the Bcr-Abl kinase domain might limit its ability to form an inactive conformation required for the inhibition by imatinib, and confer the resistance to imatinib [33, 55], Gene amplification is another common mechanism of MDR, which is exemplified by the amplification of DHFR gene in methotrexate resistance cells [56]. The aberrant epigenetic regulation such as methylation of CpG island, histone modification and noncoding microRNAs alters a large number of genes and impacts on the chemosensitivities of cancer cells [57–59].

The most commonly encountered MDRs are associated with the alternations of ABC efflux transporters that actively pump various anticancer drugs out of tumor cells [60, 61]. Overexpression of ABC drug transporters is remarkably related to gene amplification [62], transcriptional and epigenetic changes [59, 63]. In addition, the discovery of cancer stem cells in solid tumors and the finding on ABC transporter overexpression in cancer stem cells are also revolutionizing the study on drug resistance [64].

3. ABC EFFLUX TRANSPORTERS UNDERLYING MULTIDRUG RESISTANCE

The human ABC transporters consists of 48 members that are classified into seven subfamilies designated ABCA to ABCG according to the similarity of their amino acid sequences [61]. Most human ABC transporters are located on the brush border membrane of enterocytes, biliary canalicular membrane of hepatocytes, luminal membrane in the proximal tubules of kidney and the epithelium cells at blood-brain barrier. Catalyzing the ATP-dependent transport of structurally diverse compounds across cellular membranes, ABC transporters are critical elements for the cells in protection against xenobiotics [65]. Some

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ABC transporters are also responsible for the homeostasis of endogenous agents, and people carrying defected *ABC* genes may be more susceptible to specific diseases such as the Tangier's disease, Stargardt's disease and adrenoleukodystrophy [66–68].

3.1. General Properties of ABC Transporters

A typical ABC transporter is composed of two distinct domains, transmembrane domain (TMD) and nucleotide (ATP) binding domain (NBD). The hydrophilic NBD is located within cytoplasm for ATP binding and hydrolysis to harness energy for the transport of substrates across membrane [69]. The NBD is highly conservative, consisting of the Walker A (GXXGXGKS/T where X represents any amino acid) and Walker B (ΦΦΦΦD where Φ is hydrophobic) motifs that are separated by the ABC signature motif (LSGGQ). The serine residue in ABC signature sequence is critical for the interactions between Walker A and ABC signature motifs to form the so-called ATP sandwich and to warrant the consequent ATP hydrolysis. The TMD spans the membrane and forms channels. The hydrophobic TMDs are structurally diverse, which recognize and translocate a broad variety of substrates upon conformational changes. Therefore, the TMDs determine the characteristics of transported substrates. In addition, most ABC efflux transporters (e.g., P-glycoprotein or P-gp/MDRl/ABCBl) consist of two N-terminal TMDs and two C-terminal NBDs (TMD1-NBD1-TMD2-NBD2), and each TMD generally contains six transmembrane segments (α-helices). By contrast, breast cancer resistance protein (BCRP/ABCG2) is a half-transporter that only has one TMD at the C-terminal end and one NBD at the N-terminal end (NBD-TMD). Nevertheless, ABCG2 forms a homodimer through the disulfide bonds towards the extrusion of its substrates [70].

ABC efflux transporter-mediated drug translocation may be exemplified by simple kinetic mechanisms. Generally, substrate binding initiates the transport cycle and ATP binding induces NBD dimerization and configuration of the ATP sandwich. Although changes of transporter structures at different stages are not elucidated exclusively, substrates seem to be bound at the high-affinity site within the TMDs. The conformational changes by binding and hydrolysis of ATP or movement of proton via the electrochemical gradient converts the high-affinity site to low-affinity site in the membrane and the alternative side of the membrane is released [71, 72]. These conformational changes can be transmitted between domains of ABC transporters. Substrates cross the bilayer within the core of the transporter, largely shielded from the surrounding lipid phase. ABC transporters extract their substrates from the inner leaflet of the bilayer to phospholipid flippases and eventually pump them out of the cells [73], Hydrolysis of the second ATP molecule and release of Pj separate the NBDs and restore the stable conformational state for the binding of another substrate.

3.2. Multidrug Resistance ABC Efflux Transporters

The MDR phenotype is often linked to the overexpression of ABC efflux transporters such as P-gp, multidrug resistance-associated proteins (MRPs/ABCCs) and BCRP. P-gp is the first ABC efflux transporter found to be responsible for the sensitivity of cells to chemotherapeutic agents [74, 75]. The second member of ABC efflux transporter revealed to confer MDR is MRP1, which was over-expressed in cancer cells whose P-gp levels were not increased [76, 77], The third ABC efflux transporter critical for MDR is BCRP [78–80],

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which is a half-transporter exhibiting a very broad specificity for substrates like P-gp and MRP1 (Table 3). While there are many other ABC transporters important in MDR, we will limit our discussion to more extensively-studied P-gp, MRP1 and BCRP that also share the specificity for substrates.

P-gp, a 170 kDa glycoprotein, is abundantly expressed on the intestinal mucosal membrane, the luminal blood-brain barrier and the apical membranes of hepatocytes and kidney proximal tubule epithelia [81]. P-gp substrates include amphipathic compounds (unmodified drugs and drug conjugates), lipid soluble compounds (molecular weights in the range of 300 to 1000) and compounds with aromatic rings and a positive charge at physiological pH [82], P-gp transports a broad range of therapeutic drugs (Table 3) including anticancer drugs (e.g., vinca alkaloids, anthracyclines, epipodophyllotoxin and taxanes), HIV-protease inhibitors, analgesics, antihistamines, immunosuppressive agents, cardiac glycosides, calcium-channel blockers, calmodulin inhibitors, antiemetics, antihelmintics, antibiotics and steroids [83–85]. In addition, P-gp transports many endogenous compounds such as steroid hormones, lipids, peptides and small cytokines [86–88].

MRP1, a 190 kDa polypeptide, is composed of three TMDs, two NBDs and one N-terminal intracellular linker region (L0) (arranged as TMD0-L0-TMD1-NBD1-TMD2-NBD2). The characteristic TMD0 appeared to be critical for subcellular distribution of MRP1, despite it was not required for its basolateral trafficking [89]. Like P-gp, MRP I can confer the resistance to many chemotherapeutic agents such as the folate antagonist methotrexate, and MRP1 substrates comprise a variety of hydrophobic compounds, organic anion conjugates and anionic nonconjugated agents (Table 3). One difference between MRP1 and P-gp specificity for substrates is that taxanes are poor substances for MRP1 [90]. In addition, MRP1 transports many endogenous compounds including free glutathione, glutathione-conjugated leukotrienes, glucuronate and sulfate conjugates, as well as heavy metal oxyanions such as arsenite and trivalent antimonite [91, 92], which are different from those transported by P-gp.

BCRP is a 72 kDa half-ABC transporter, consisting of 655 amino acids and two TMDs. The human *BCRP/ABCG2* gene is to 532 bp) and 15 introns and spanning over 66 kb [93, 94], BCRP is mainly expressed in the gastrointestinal tract, liver, kidney, brain, endothelium, mammary tissue, testis and placenta. Overexpression of BCRP in cancer cells can confer MDR besides that BCRP affects dmg absorption, distribution and excretion [81], BCRP actively extrudes a board range of endogenous and exogenous substrates (Table 3) across biological membranes, which include sulfate conjugates, taxanes, carcinogens, glutamated folates and porphyrins [84, 85].

Clinical investigations have shown a potential relationship between these ABC efflux transporters and tumor drug responses or patient's survival, although some might not be conclusive. A meta-analysis of thirty-one clinical studies indicated that P-gp was expressed in 41% of breast tumors, and patients with P-gp-expressing tumors were three times more likely to fail to respond to chemotherapy [95], The association of P-gp expression with a poor survival rate was more striking when only considering patients whose tumor P-gp expression was measured after chemotherapy. Indeed, P-gp expression was rapidly activated

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in human tumors *in vivo* following a transient exposure to doxorubicin [96], supporting the role for P-gp induction in the acquired MDR during cancer chemotherapy.

Schaich *et al* [97] examined the utility of P-gp and MRP1 expression as prognostic biomarker in 331 adult acute myeloid leukaemia (AML) patients. While P-gp was an independent prognostic factor for the outcome of induction therapy and overall survival, MRP1 was turned out to be an independent predictor for disease-free survival. The latter is different from the observation that MRP1 was not a significant factor in MDR in 352 newly diagnosed AML patients [98]. In another study, MRP1 expression was revealed to be detectable in all of the 209 primary neuroblastoma analyzed, and high levels of MRP 1 were highly predictive of both event-free survival and overall survival of the patients [99]. In contrast, P-gp was of no prognostic significance for neuroblastoma

BCRP expression was observed in all types of tumors, and it was more frequent in endothelial cells of the adenocarcinomas of digestive tract, endometrium and lung [100]. Despite the mRNA and protein levels and function of BCRP were not correlated well, an exploratory study indicated that BCRP protein might be predictive of shorter disease-free survival for adult acute lymphoblastic leukaemia (ALL) patients [101], In another study, BCRP appeared to be a predictor of survival in patients with advanced non-small cell lung cancer (NSCLC) [102]. Further, BCRP was found to be over-expressed in 24 of a total of 73 consecutive AML patients and it was significantly co-expressed with P-gp [103]. Interestingly, P-gp expression predicts the achievement of complete remission, and BCRP-positive cases showed an increased risk of relapse and a shorter disease-free survival. The latter finding suggests that BCRP may be regarded as a prognostic factor in AML patients with normal karyotype.

4. ABC TRANSPORTERS AS TARGETS TO COMBAT MULTIDRUG RESISTANCE

One way to overcome ABC transporter-controlled MDR is to use ABC transporter inhibitors to sensitize tumor cells to chemotherapeutic agents. The rationale for combined use of ABC transporter inhibitors with anticancer drugs towards an improved drug response is clear, and large efforts have been made to develop chemosensitizers (Table 4). While the clinical trials of combined therapies showed some encouraging outcomes, no effective MDR-reversing agent has been developed and approved to date for an appreciable sensitization of malignant tumorsto chemotherapeutic drugs without toxic effects.

Combined use of the first-generation MDR inhibitors (e.g., verapamil, quinine and cyclosporine A) with anticancer drugs (e.g., mitoxantrone, daunorubicin and etoposide) produced toxic side effects and showed only limited or no benefits [4, 104, 105], Actually, the first-generation inhibitors are pharmaceutical agents themselves and they are not specifically developed for the modulation of ABC transporters. Many of the first-generation inhibitors (e.g., verapamil and cyclosporine A) are also substrates for ABC transporters (e.g., P-gp) and thus the use of high doses of chemosensitizers to inhibit the activity of ABC transporter inevitably leads to unwanted toxicities.

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The second-generation MDR inhibitors were designed to reduce possible primary toxicities. While R-verapamil and PSC-833 (Valspodar) inhibit the function of P-gp, the MDR modulators exhibit no or minimal activity in blocking calcium channel and suppressing immune system, respectively. Combined use of some MDR inhibitors (e.g., PSC-833) with anticancer drugs seemed to provide some advantage for some AML patients [106]. However, co-administration of the MDR modulators induces pharmacokinetic interactions. In particular, concurrent MDR inhibitors elevated the systemic exposure to anticancer drugs by altering the absorption, distribution, metabolism and excretion (ADME) of anticancer drugs [107] and thus led to an increased toxicity in patients [108–110]. On the other hand, VX-710 did not alter the pharmacokinetics of doxorubicin [111] and it did increase the chemosensitivity of MDR cells overexpressing ABC transporters [112], However, coadministration of VX-710 did not significantly increase the benefits of anticancer drugs, which may be due to the existence of other mechanisms of MDR besides the overexpression of ABC transporters [111, 113]

The third-generation MDR inhibitors such as laniquidar (R101933), ONT-093 (OC14–093), zosuqiodar (LY335979), elacridar (GF120918) and tariquidar (XR9576) have a high affinity to ABC transporters and a low pharmacokinetic interaction due to a limited CYP3A inhibition [114, 115]. Although many *in vitro* studies have demonstrated an enhancement of chemosensitivity by the third-generation MDR inhibitors, clinical trials revealed that the outcomes of chemotherapeutic agents were not improved by coadministration of the third-generation MDR modulators. For example, the addition of zosuqiodar did not increase the overall survival rate of older patients with newly diagnosed AML [116], and concurrent tariquidar did not appear to prolong the overall survival of patients with metastatic cancers [117], albeit a potent inhibition of P-gp did not produce any toxicity.

There are many possible reasons for the failure of clinical trials of three generations of ABC transporter inhibitors to overcome MDR, besides the inherited side effects (first-generation), unexpected drug-drug interactions (second-generation) and presence of other mechanisms of MDR in tumors. Given the fact that the tumors are highly heterogeneous, a linear distribution of drugs from blood to tumor cells might be absent. Without measuring the actual concentrations of chemosensitizers and anticancer drugs within tumor cells in a whole body system, one is merely unaware of the level of MDR modulators to which tumors are exposed, or the extent of inhibition of MDR transporters within the tumor cells. Indeed, a novel approach that employs biodegradable polymersome to carry both anticancer drug and MDR inhibitor is proven to be an effective and practical means to combat MDR [118]. In addition, the ABC transporters P-gp, MRPs and BCRP are often coexpressed in tumors and they also have an overlapped specificity for a variety of substrates (Table 3). Selective inhibition of one or two ABC efflux transporters could be compensated by the remaining transporters. Nevertheless, the notion that targeting ABC efflux transporters may overcome MDR is still strong, and new chemosensitizers as well as novel approaches such as targeted downregulation of MDR genes using small molecule dmgs or RNA interference [4, 119] are in development.

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5. ABC EFFLUX TRANSPORTERS IN PHARMACOKINETICS

ADME processes may limit or enhance the extent of dmgs to the target tumor cells. ADME is mechanistically mediated by drug-metabolizing enzymes and transporters expressed in different tissues including small intestine, liver, target site (e.g., tumor) and kidney. In particular, xenobiotic-metabolizing enzymes such as cytochrome P450 (CYP or P450) isoforms play a critical role in metabolic elimination of dmgs, and transporters such as ABC and solute carrier (SLC) transporters have high impact on dmg absorption, distribution and excretion [81, 120–122], The interplay of enzymes and transporters and the interactions between drags and enzymes/transporters ultimately determine the pharmacokinetics of dmgs and consequently, affect the pharmacodynamics (Fig. 1).

The role of P-gp in pharmacokinetics is one of the most documented. Digoxin and talinolol are two dmgs that are often used for the assessment of P-gp-mediated changes. A comprehensive analysis of 123 clinical studies [123] revealed that the degree of changes in digoxin pharmacokinetics, as manifested by AUC or C_{max} ratio, was relatively small (< 3-fold increase) when a P-gp inhibitor was co-administered. Indeed, the most strikingly change in digoxin pharmacokinetics (3.05-fold increase of AUC ratio) was caused by valspodar [107], a second-generation MDR modulator. Furthermore, induction of P-gp (and P450 enzymes) through transcriptional or other mechanisms is expected to decrease the systemic dmg exposure. The co-administration of rifampicin resulted in a 30–54% decrease in AUC of digoxin [124] and a 21–35% decrease in AUC of talinolol [125], which was associated with a 3.5- to 4.2-fold increase in intestinal P-gp protein expression. It is also noteworthy that a small change in dmg concentration or exposure may cause significant alternation of dmg response, when the dmg has a narrow therapeutic window. As an example, acute rejection in organ transplant patients was reported, due to the interaction of St. John's Wort and cyclosporin that might involve the induction of both P-gp transporter and P450 enzymes [126].

Similar to the effects of ABC transporters over-expressed in tumor cells on intracellular dmg accumulation, ABC transporters expressed on the luminal side of the endothelial cells to form blood-brain barrier may influence the blood-brain dmg distribution and consequently dmg concentration within the brain. It was shown that the AUC of ^{11}C-verapamil between human brain and blood compartment was increased around 90% after intravenous administration of P-gp inhibitor cyclosporine [127]. In contrast, the use of *Mdrla/lb* gene knockout mouse models revealed a strikingly higher extent of contribution of P-gp to blood-brain dmg distribution *in vivo* [128]. The difference between clinical and animal data may be attributed not only to the distinct physiology of animal models but also to the technique in measuring dmg concentrations. Nevertheless, it is certainly clear that ABC transporters play an important role in dmg distribution.

There are also increased clinical studies on the role of BCRP in pharmacokinetics. Co-administration of elacridar (GF120918), a potent inhibitor of BCRP and P-gp, significantly elevated the systemic exposure to orally-administered topotecan, as indicated by the increase of oral bioavailability from 40.0% to 97.1% [129]. Further investigations on BCRP pharmacogenetics also support the importance of BCRP in pharmacokinetics. For instance,

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the BCRP 421C>A polymorphism appeared to influence the pharmacokinetics of rosuvastatin (−45% lower AUC among 42ICC homozygotes) in healthy Chinese males, whereas SLCOIBI and CYP2C9 genetic polymorphism showed no impact [130]. Another study among 660 healthy Finnish volunteers showed consistent findings on the impact of 421C>A polymorphism on pharmacokinetics of BCRP substrates, atorvastatin and rosuvastatin [131]. The systemic exposure to atorvastatin was about 70% higher in subjects carrying the C.421AA allele, and the systemic exposure to rosuvastatin in subjects carrying the C.421AA allele was over 100% higher. Different pharmacokinetics may be translated into a significant change in dmg response. Indeed, the 421C>A polymorphism in ABCG2 gene was significantly associated with diarrhea in non-small-cell lung cancer patients treated with oral gefitinib [132], and irinotecan-induced severe myelosuppression [133].

6. PRECLINICAL EVALUATION OF ABC TRANSPORTER FUNCTION

A number of assays and models are available for the assessment of functions of ABC transporters, prediction of cellular and tissue drug absorption or disposition, and development of MDR modulators in the preclinical setting. Based on the model systems utilized in the assays, they are classified into *in vitro* (e.g., membrane-based ATPase activity assay and vesicular uptake assay, and cell-based monolayer drug efflux assay and uptake assay), ex vivo (e.g., everted gut sacs), *in situ* (e.g., *in situ* organ perfusion), and *in vivo* assays (e.g., genetically modified animal models) [3, 122, 134].

6.1. Membrane Vesicular Uptake Assay

This assay employs inside-out membrane vesicles to directly assess the uptake of drugs mediated by membrane transporters. The membrane vesicles may be prepared from a variety of tissues [135–138], cells [139–141] and transfected cells [142–145]. Since ATP-dependent ABC proteins do not traverse the lipid membrane, in-side-out membrane vesicles bind to ATP and pump substrates into the vesicles. Therefore, this assay directly determines transporter function and provides good estimation of kinetic parameters such as the K_m for substrates and IC_{50} or K_i for inhibitors.

Membrane vesicles require ATP-binding and -hydrolysis factors including ATP, phosphocreatine, magnesium and creatine phosphokinase to complete uptake process. It often involves the utilization of radioactive [138, 140, 141, 145] or fluorescent compounds [146, 147]. Membrane vesicular transport study is able to distinguish the inhibitors from substrates and it can be developed as high-throughput assay. It has been used to define the localization, function and regulation of ABC transporters, as well as interspecies difference [148, 149], pharmacogenetics [150, 151] and specificity of substrates or inhibitors for a given transporters [152]. One drawback of this assay is that membrane vesicle preparation is time-consuming and requires some skill. In addition, a non-specific binding or passive diffusion can lead to false negative data, highlighting the importance of critical controls for membrane vesicular uptake study.

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6.2. ATPase Activity Assay

ABC transporter-mediated substrate translocation against a concentration gradient requires the energy produced by ATP hydrolysis. Based on the transport ATPase cycle [153], the membrane ATPase activity assays have been developed to indirectly evaluate the function of ABC transporters in cellular drug disposition [143, 154–157]. Colorimetric measuring the amount of inorganic phosphate released by ATP hydrolysis, which is proportional to the rate of ATP hydrolysis, allows investigators to assess the interactions of substrates/inhibitors with ABC transporters. Similar to other AT-Pases [158–160], ABC transporters can be trapped by vanadate or beryllium fluoride. Therefore, a test in the presence and absence of vanadate or beryllium fluoride would help define the transporter selective activity.

One major drawback of ATPase activity study is that utilization of this assay is unable to distinguish between inhibitor and substrate of ABC transporter. The reason is that any compounds that can bind to transporters will stimulate the ATPase cycle. There are also some compounds that actually affect ATPase activity, whereas they do not have any direct interactions with ABC transporters; thus this indirect ATPase assay would give false positive results. Moreover, crude membrane and even purified transporter preparations are known to have a high baseline vanadate-sensitive ATPase activity. This basal ATPase activity is likely caused by endogenous lipids or other substrates, and sometimes it may not be further stimulated by substrates of ABC transporters.

6.3. Trans-cellular Drug Transport Assay

This is a direct assay of the translocation of a test compound across polarized epithelial or endothelial cell monolayer grown on permeable supports (Fig. 2) to mimic the biological barrier *in vivo*. It is regarded as a standard assay to assess drug transport and permeability. Cell monolayers are expected to form tight junctions to prevent passive paracellular transport. The test compound may be added to the apical (upper chamber) or basolateral (lower chamber), and drug concentrations in the opposite side are monitored over time, the apparent permeability coefficient (P_{app} in cm/sec) is calculated according to the equation P_{app} (cm/min) = $dQ/(dt \times A \times C_0)$, where dQ/dt is the flux rate (pmol/mon, slope of the cumulative amount (pmol) of transported drug (Q) in the receiver compartment versus time (min) curve), A (cm^2) is the surface area of the filter, and C_0 is the initial drug concentration (pmol/mL or μM) applied to the donor chamber. The efflux ratio of basal-to-apical (B-to-A) to apical-to-basal (A-to-B) is further calculated from the formula $P_{app,\ B\text{-to-}A}/P_{app}$ for various studies such as the determination of the direction of drug transport, role of specific transporter, substrate specificity and inhibition potency, the screening of transporter modulators and the prediction of drug permeability *in vivo*. For instance, a high efflux ratio (e.g., > 2) will support the potential role of an efflux transporter in transporting the test drug across cell monolayers.

The polarized, wild-type and engineered Caco-2 and MDCK cells are commonly used for cell monolayer drug efflux studies [138, 161–164], Primary cultured cells from brain endothelial cells have also been applied to monolayer efflux assay [165]. Given the complexity of intestinal drug absorption such as the interplay between metabolism and efflux, a possible involvement of carrier-mediated transport and a low or an absent

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expression of drug-metabolizing enzymes in stable cell lines, a high efflux ratio obtained from monolayer efflux study does not always mean a poor oral absorption for the test drug. Since cell culture is required, this assay is time consuming and labor intensive when compared to membrane-based ATPase activity and vesicular transport assays [134].

6.4. Cell-based Drug Uptake Assay/Intracellular Drug Accumulation

This uptake assay measures the amount of drugs accumulated within the cells following the exposure to a test compound for a certain period. Research often takes advantage of the fluorescence or radioactivity of ABC transporter substrates (or their metabolites) for the evaluation of intracellular drug accumulation by using flow cytometry or radioactivity counter. Calcein-AM is widely used in the assays for assessing the interactions of drugs and ABC transporters. Calcein-AM can easily get into the cells through passive diffusion and it is cleaved irreversibly to a hydrophilic, non-permeable and fluorescent calcein free acid by endogenous esterases. Because calcein-AM is a good substrate for ABC efflux transporters (e.g., P-gp and MRP1), intracellular accumulation of the fluorescent calcein is inversely related to ABC efflux transporter-mediated cellular drug disposition; thus calcein AM uptake assays are good for high-throughput screening of ABC efflux transporter inhibitors [157, 166], By contrast, some drugs are fluorescent compounds themselves (e.g., doxorubicin for P-gp and MRP1, and mitoxantrone for BCRP), and drug accumulation can be measured directly for assessing the function or regulation of corresponding transporters [167–169]. While this cell-based uptake assay is easy to use, studies are generally limited to the use of fluorescent or radioactive substrates, screening of inhibitors or evaluation of transporter function/regulation.

6.5. Perfusion of Isolated Tissue

The perfusion of an isolated organ such as intestine, liver and kidney has been employed for the assessment of drug uptake or disposition in a specific organ [170–173], Prediction of oral drug absorption commonly involves the use of isolated human and animal intestinal segments, which are either mounted in a diffusion apparatus (e.g., Ussing chamber) or everted to produce the everted gut sacs (Fig. 3) [174, 175]. In these studies, a test compound is administered to a donor compartment (serosal or mucosal) and drug concentrations in the receiver compartment (mucosal or serosal) are determined over time. As an example, the study with intestinal perfusion isolated from wild-type versus *Bcrpl/Abcg2* knockout mice revealed the important role for Bcrpl in the extrusion of glucuronide and sulfate conjugates formed in the enterocytes to intestinal lumen [176]. Compared to the studies with *in vitro* and whole animal models, use of excised tissues for drug transport studies, in the presence and absence of selective inhibitor, may provide a clear understanding of transporter function in the given organ. Nevertheless, a possible involvement of drug metabolism or other compounding factors can complicate the prediction of intestinal drug absorption.

6.6. *In situ* Organ Perfusion

In situ organ perfusion with live animals allows a more accurate determination of the transporter functions in intestinal absorption, biliary elimination, renal excretion and brain penetration under a physiologically relevant environment. These studies include the perfusion of intestine [177, 178], liver [179–181], kidney [173, 182] and brain [183–186].

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For example, *in situ* brain perfusion was employed to investigate the role of P-gp at blood-brain barrier in protection against xenobiotic drugs [187, 188], as well as the impact of P-gp modulators [189, 190]. This assay was also used, along with the *Mdrla* knockout mouse models, for the evaluation of P-gp-mediated drug transport across blood-brain barrier [191]. As another example, *in situ* intestinal perfusion studies in wild-type versus *Mdrla/Ib* knockout mice nicely demonstrated the impact of intestinal P-gp on the absorption of a number of P-gp substrates [178], These *in situ* organ perfusion techniques are powerful tools to define drug transport and role of ABC transporters in an intact organ system. However, *in situ* organ perfusion requires special surgical skills and sophisticated equipment. In addition, the anesthetic agents used in these studies may affect the results [192].

6.7. Genetically Modified Animal Models

ABC transporter genetically modified mouse models are unique addition to wild-type iaboratorial animals for evaluation of the role of a specific ABC transporter in pharmacokinetics in a whole body system [193–195], Mice deficient in *Mdrla* [196] or both *Mdrla* and *Mdrlb* [197] were developed, and comparative studies in wild-type versus knockout mice demonstrated the importance of P-gp in brain uptake, pharmacokinetics and toxicity of a number of P-gp substrates such as ivermectin, vinblastine, dexamethasone, digoxin, cyclosporine A, ondansetron, loperamide and rhodamine [196–199], Meanwhile, two *Mrpl* knockout mouse lines were generated through targeted disruption of the exons encoding the first NBD of mouse *Mrpl* gene [200] and part of the second putative NBD [201], Many studies were conducted with *Mrpl* knockout mouse models, demonstrating the role of MRP1 in limiting tissue distribution of MRP1 substrates [202–204], In addition, two *Bcrpl* knockout mouse models were created to investigate the *in vivo* function of BCRP/ABCG2 [205, 206]. An early study using *Bcrpl* knockout mouse models revealed a critical role for Bcrpl in protection of diet-dependent photoxocity [205]. Since then, many studies with the *Bcrpl* knockout mice have been conducted to demonstrate the effects of Bcrpl expression on absorption, distribution, and elimination of dietary carcinogens (e.g., PhIP), medications (e.g., nitrofurantoin) and other agents [207–215], These genetically modified mice are useful animal models to evaluate the importance of ABC transporters at the systemic level. Nevertheless, an altered expression of other ADME genes [196, 216] reminds that one need be cautious when translating data from transgenic mice into humans [193,217].

7. CONCLUSION

Overexpression of ABC efflux transporters such as P-gp, MRP1 and BCRP in tumor cells is recognized as an important mechanism for MDR that hampers the success of cancer pharmacotherapy, besides genetic mutations of target proteins and changes of cell signaling pathways and epigenetic regulations. Many ABC efflux transporters are also expressed ubiquitously in normal human tissues, and their interactions with drug-metabolizing enzymes and other transporters in the gut, liver and kidney may largely affect the overall pharmacokinetic properties of drugs. Meanwhile, P-gp, MRP1 and BCRP have an overlapped specificity for many anticancer drugs. These factors may complicate the development and use of ABC transporter inhibitors as MDR-reversing agents. Indeed, many

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clinically-tested chemosensitizers have been proved to be unbeneficial for cancer patients due to inherited toxicities or pharmacokinetic interactions. Nevertheless, the enthusiasm is still high for targeting ABC efflux transporters to overcome MDR, which includes the development of new chemosensitizers and novel approaches. In addition, the lessons learned from MDR modulators are helpful for the development of novel anticancer drugs that would ideally bypass transporter-mediated efflux. Therefore, understanding the impact of transporters on drug flux is critical for drug development. Presently there are a number of preclinical assays and models available for the evaluation of transporter-controlled flux, prediction of pharmacokinetic outcomes and screening of MDR modulators. Understanding the limitations as well as the benefits of individual preclinical assays and models is necessary for proper data analysis and interpretation in drug development.

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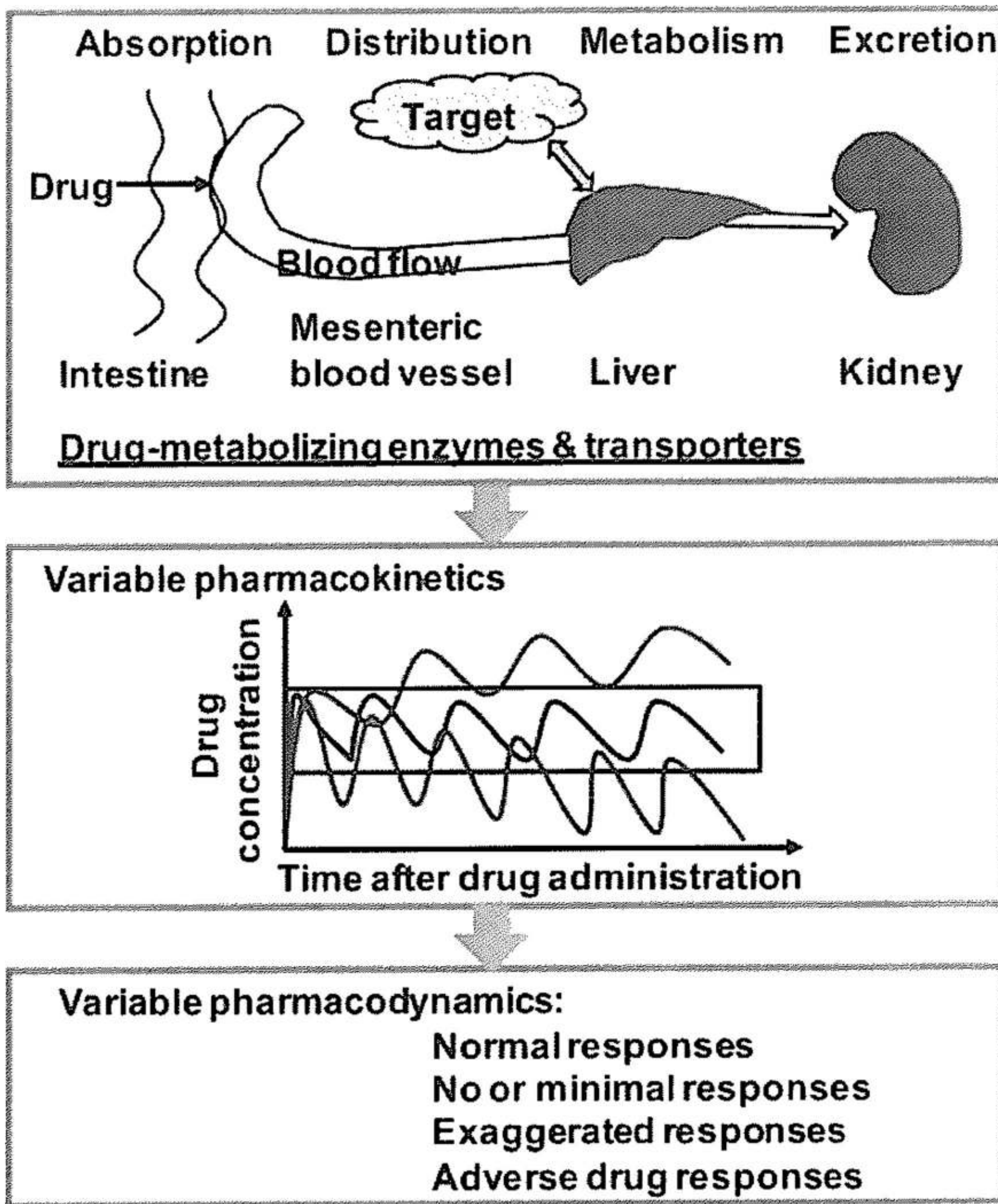

Fig. (1).

Considerable variations in pharmacokinetics and pharmacodynamics may occur when a drug is processed in different ways in humans, which are determined by the functions and extents of xenobiotic-metabolizing enzymes and transporters expressed in various tissues.

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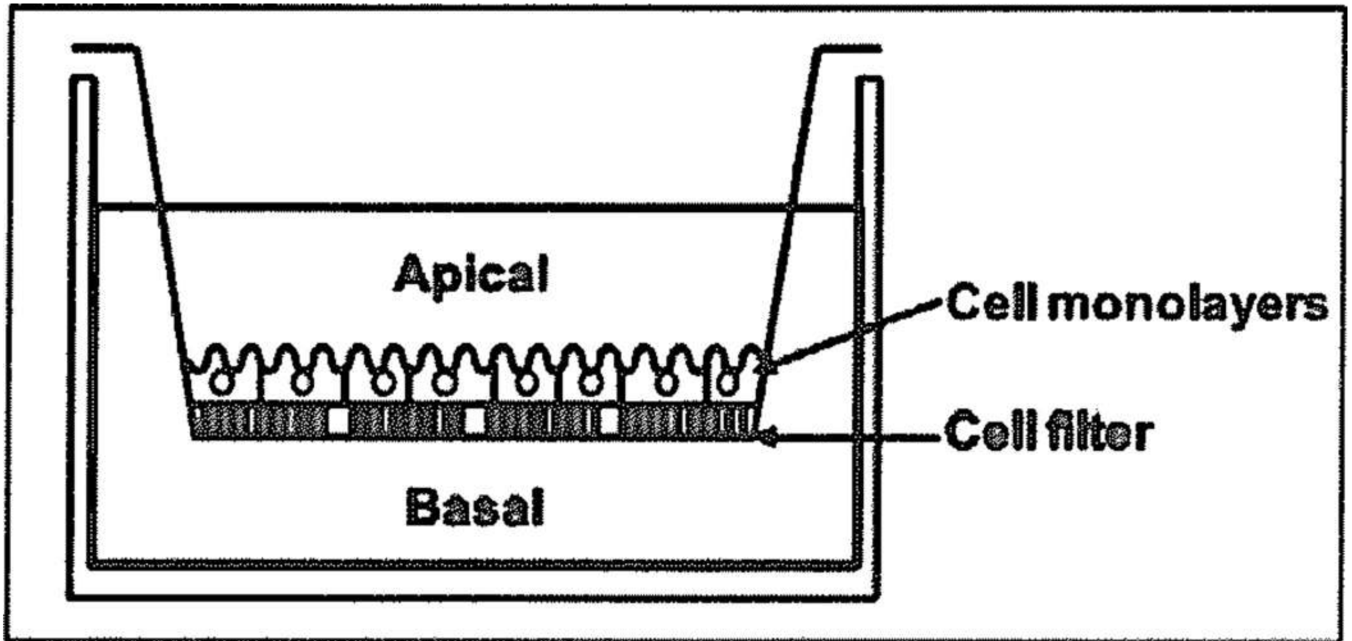

Fig. (2).
Cell monolayer grown on permeable support for drug transport assay. Apical-to-basal or basal-to-apical drug translocation can be evaluated separately.

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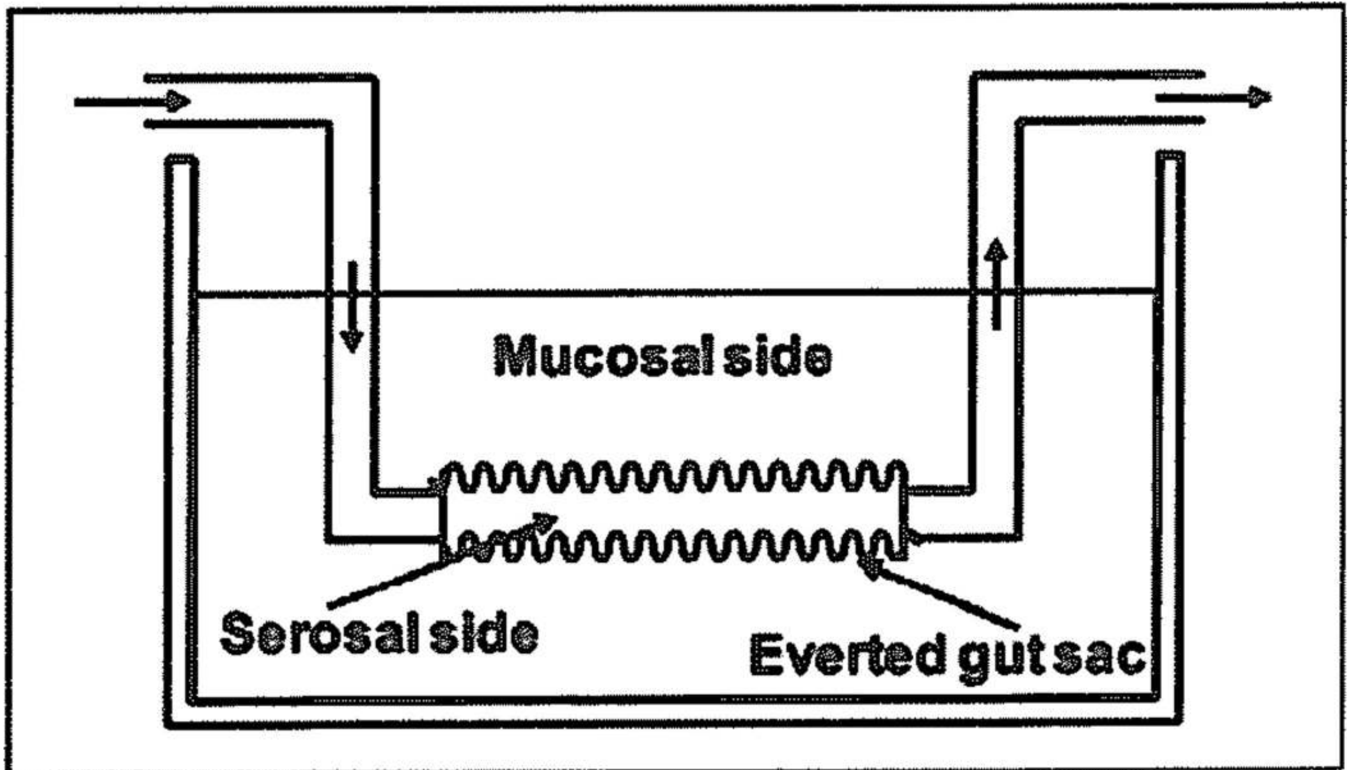

Fig. (3).

Everted intestinal sac for the assessment of directional drug transport (e.g., serosal-to-mucosal).

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Table 1.

Major classes of anticancer drugs.

Pharmacotherapy	Agents
Chemotherapy	Antimetabolites
	Genotoxic drugs
	Mitosis inhibitors
Endocrine therapy	Steroids
	Inhibitors of hormone synthesis
	Hormone receptor antagonists
Immunotherapy	Antibodies
	Vaccines

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Table 2.

Common small-molecule cytotoxic drugs, protein kinase inhibitors and hormonal agents used for cancer treatment.

Group	Sub-group	Example drugs
Anti-metabolites (inhibit the syntheses of building blocks required for DNA replication)	Pyrimidine antagonists	5-Fluorouracil
		Gemcitabine
		Arabinosylcytosine
	Purine antagonists	6-Mercaptopurine
		6-Thioguanine
		Fludarabine
	Folate antagonists	Acyclovir
		Methotrexate
		Trimethoprim
		Pyrimethamine
		Hydroxyurea
Genotoxic agents (directly or indirectly damage DNA in the nucleus of the cell)	Alkylating agents	Busulfan
		CisplatinCyclophosphamide
		Mechlorethamine
		Ifosfamide
		Chlorambucil
	Intercalating agents	Actinomycin D Doxorubicin
		Daunorubicin
		Epirubicin
	Topoisomerase inhibitors	Irinotecan
		Topotecan
		Etoposide
		Teniposide
		Mitoxantrone
Mitotic spindle inhibitors (inhibit **mitosis** bv disruDtins **microtubules**)	Vinca alkaloids	Vinblastine
		Vincristine
	Taxanes	Paclitaxell

Group	Sub-group	Example drugs
		Docetaxel
	Others	Colchicine
Protein kinase inhibitors (block the actions of protein kinase enzymes or receptors)	Bcr-Abl inhibitors	Imatinib
		Nilotinib
	EGFR inhibitors	Gefitinib
		Afatinib
		Erlotinib
		Lanatinib
	Others	**Dasatinib**
		Flavopiridol
		Sorafenib
		Axitinib
Hormones (modulate endocrine system)	SERMs	Tamoxifen
		Raloxifene
	Aromatase inhibitors	Anastrozole Letrozole
		Letrozole
		Aminoglutethimide
		Exemestone
	Androgen antagonists	Flutamide
		Bicalutamide
		Cyproterone
		Niltamide
	GnRH analogs	**Leunrolide**
		Goserelin
	Others	Abiraterone
		Dutasteride
		Finasteride
		Megestrol

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Table 3.

Common drugs transported by P-gp, MRP1 and BCRP.

P-gp (ABCB1)	Anticancer drues: doxorubicin, daunorubicin. epirubicin. colchicine, antinomvcin D. etoposide. teniposide. methotrexate, mitocvcin C, paclitaxel, mitoxantrone, docetaxel, vinblastine, vincristine
	Antihvnertensives: losartan. celiorolol. reseroine. talinolol. Nicardipine
	Antiarrhvthimics: dieoxin. propafenone, auinidine. verapamil, amiodarone
	Antibiotics: ervthromvcin. rifampin, levofloxacin. clarithromycin, tetracycline
	Antivirals: amorenavir. indinavir, nelfinair. ritonavir, saauinavir
	Antidepressants: amitrintvline. fluoxetine, paroxetine, sertraline
	Immunosuppressants: cyclosporine A. sirolimus. tacrolimus, valspodar
	Opioids: methadone, morphine
	Lipid lowerine drues: atorvastatin. lovastatin
	Glucocorticoids: aldosterone, cortisol, dexamethasone. Methvlprednisolone
	Antihistamines: fexofenadine, terfenadine
	Others: oroeesterone. itraconazole, phenobarbital. phenvtoin. cimetidine
MRP1 (ABCC1)	Anticancer drues: doxorubicin, daunorubicin. colchicine, toootecan. irinotecan. SN-38. methotrexate, etoposide. teniposide. vincristine. vinblastine, imatinib, gefitinib
	Antivirals: indinavir, ritonavir, saauinavir
	Antibiotics: ciprofloxacin, difloxacin. ereoafloxacin. pirarbicin
BCRP (ABCG2)	Anticancer drues: doxorubicin, daunorubicin. epirubucin. methotrexate, toootecan. irinotecan, SN-38. etoposide. teniposide. imatinib, gefitinib
	Antibiotics: ciprofloxacin, norfloxacin, ofloxacin
	Lipid lowerine drues: cerivastatin. pravastatin, rosuvastatin
	Antivirals: lamivudine. Zidovudine
	Antihvoertensives: reseroine
	Others: azidothvmidine. lamivudine

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Table 4.

ABC transporter inhibitors as chemosensitizers.

MDR1	Amlodipine, cyclosporine, quinidine, quinine, verapamil, nifedipine, dexniguldipine, PSC-833, VX-710, GF120918, LY475776, LY335979, XR-9576, V-104, R101933, disulfiram, pluronic L61
MRP1	Cyclosporine, quinidine, quinine, verapamil, VX-710, LY475776, V-104, disulfiram, MK571, tricyclic isoxazoles
BCRP	Cyclosporine, VX-710, GF120918, XR-9576, ftimi-tremorgin C