Inhibit or Evade Multidrug Resistance P-glycoprotein in Cancer Treatment

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Abstract

Multidrug resistance (MDR) is a major cause of failure in cancer chemotherapy. P-glycoprotein (P-gp), a promiscuous drug efflux pump, has been extensively studied for its association with MDR due to overexpression in cancer cells. Several P-gp inhibitors or modulators have been investigated in clinical trials in hope of circumventing MDR, with only limited success. Alternative strategies are actively pursued, such as the modification of existing drugs, development of new drugs, or combining novel drug delivery agents to evade P-gp-dependent efflux. Despite the importance and numerous studies, these efforts have mostly been undertaken without *a priori* knowledge of how drugs interact with P-gp at the molecular level. This perspective highlights and discusses progress toward and challenges impeding drug development for inhibiting or evading P-gp in the context of our improved understanding of the structural basis and mechanism of P-gp-mediated MDR.

Graphical Abstract
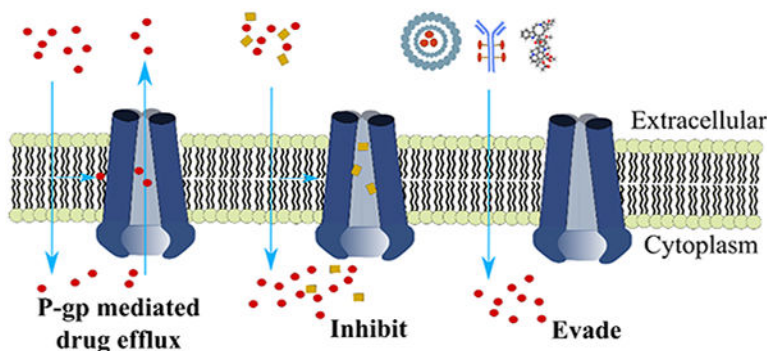
Introduction

Many cancer cells acquire resistance to a broad spectrum of structurally and mechanistically distinct anticancer drugs by a phenomenon called multidrug resistance (MDR).[1, 2] The

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Author Contributions

D.W. and Q.Z. both contributed to the writing of this manuscript.

The authors declare no competing financial interest.

development of MDR presents major challenges to cancer chemotherapy, especially in managing patients with metastatic cancers that are resistant to traditional therapies.[3, 4] MDR occurs intrinsically in some cancers due to genetic and epigenetic alterations that affect drug sensitivity without previous exposure to chemotherapy agents. MDR can also be acquired during the course of chemotherapeutic treatment of cancers that were initially drug-sensitive but later recur in drug-resistant form. It is also recognized that cancers usually consist of a heterogeneous population of drug-sensitive and drug-resistant cells.[5, 6] During the course of treatment, drug-sensitive cells are selectively removed, and resistant cells come to dominate the cancer cell population. The huge impact of chemotherapeutic drug resistance has led to extensive studies of the mechanistic aspects and strategies to understand, modulate or evade MDR.

A prevalent mechanism of cancer MDR is the expression of a class of energy-dependent efflux pumps called adenosine triphosphate (ATP)-binding cassette (ABC) transporters.[7, 8] In humans, there are 48 members in this protein family, which are involved in diverse physiological functions such as transporting lipids, sterols, peptides, toxins, and ions.[9] Among these members, at least three transporters, including P-glycoprotein (P-gp in short, also called multidrug resistance protein 1, MDR1, or ABCB1), MDR-associated protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (BCRP or ABCG2), have been characterized in relation to multidrug resistance.[10–12] These three transporters have broad drug specificity and transport an array of structurally diverse compounds, causing lowered drug accumulation inside cells and consequently diminished drug efficacy.

Discovered over 40 years ago, P-gp is the first identified and most characterized MDR transporter and has long been recognized as a viable target to overcome MDR in cancer.[10] Expression of P-gp was detected in more than 50% of the NCI-60 tumor cell lines including all melanomas and central nervous system tumors and with high levels in renal and colon carcinomas.[13, 14] The elevated P-gp expression in cancer cells has been linked to reduced chemotherapeutic responses and poor clinical outcome in various cancer types including both blood cancers and solid tumors. Some tumors with low levels of P-gp expression at baseline, such as leukemia and breast cancer, have shown upregulation of P-gp after disease progression following chemotherapy.[15] A plethora of anticancer drugs that are central to many chemotherapeutic regimes are susceptible to P-gp-mediated efflux (Figure 1), such as the microtubule-targeting vinca alkaloids (e.g. vinblastine and vincristine) and taxanes (paclitaxel and docetaxel), the DNA-chelating anthracyclines (doxorubicin and daunorubicin), the topoisomerase inhibitors (topotecan and etoposide), and the tyrosine kinase inhibitors (dasatinib and gefitinib), among many others.[2, 16–18] To overcome P-gp-mediated MDR, many small molecule drugs have been tested to modulate or inhibit the activity of P-gp.[19, 20] However, these programs have been halted in recent years due mostly to failures in clinical trials. A major challenge with this strategy is the lack of potent and non-toxic inhibitors among other limiting factors, on which we will make further comments in the next sections of this perspective. Alternatively, many drug development programs now place an emphasis on the discovery of new compounds or strategies to bypass the activity of P-gp.

In addition to the role of P-gp in cancer which is a focus of this article, P-gp plays a pivotal role in normal physiological detoxification and host protection processes by transporting numerous exogenous and endogenous substrates.[2] P-gp is distributed in the epithelial cell surfaces where it is expressed at high levels, such as in the mucosa of the gastrointestinal tract, in biliary epithelium of the liver, in proximal tubules of the kidney, in the adrenal cortex and in blood-tissue barriers.[21–23] The latter include the placenta, endometrium, testicular tissue and the endothelial component of the blood-brain barrier. Subject to imperfections in P-gp detection methods and quantification errors, the amount of P-gp expression in adrenal and kidney has been shown as high as or higher than levels detected in some MDR tumor cell lines.[22, 24] The presence of P-gp protects hematopoietic progenitor cells of bone marrow against the cytotoxic effects of chemotherapeutic agents.[25] Additionally, P-gp expressed in the intestinal epithelia has a major impact on the bioavailability of orally administered drugs due to its capacity to alter tissue absorption and elimination of the drugs into bile and urine.[26] As such, the evaluation of drug candidates for their P-gp susceptibility has become an important step in the development of novel therapeutics in the pharmaceutical industry.[2, 27] The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) now mandate documentation of drug interactions with P-gp and several other transporters for approval of any new drug.[28] Thus, studies of P-gp and drug interactions are exceedingly important not only for cancer treatment but also in a broad range of drug discovery programs.

Conceivably, these medicinal chemistry efforts involving P-gp can greatly benefit from a detailed understanding of the structure and mechanism of the underlying transporter. Significant progress has been made in recent years in characterizing the structure of P-gp at near-atomic resolution, and these structures have provided a first glimpse of the polyspecific drug binding sites of P-gp. In this perspective, we will present the state of knowledge of P-gp structures, and in that context we will review and discuss the progress and impeding challenges in the drug development to inhibit or evade P-gp in cancer. As understanding of P-gp and its drug interactions continues to improve, we can anticipate that a rational approach may be undertaken in future drug designs.

Structural insights into P-gp-mediated MDR

Overall architecture and conformations of P-gp

P-gp has been a highly sought-after target for structural determinations, yet with formidable challenges due to its conformational flexibility and inherent water insolubility as an integral membrane protein. The first high-resolution structure of mouse P-gp was reported in 2009, determined by X-ray crystallography at a resolution of 3.8 Å.[29] This success was achieved after decades of community efforts for biochemical characterizations and for establishment of large-scale expression and purification of quality protein materials. Mouse P-gp shares 87% amino acid identity with human P-gp, yet is more stable and can be expressed in yeast with good yield.[30] Several X-ray structures of mouse P-gp have been determined in recent years at marginally improved resolutions (up to 3.3 Å) in addition to a P-gp structure from *Caenorhabditis elegans*, showing overall similar structural features (Figure 2).[31–34] These

recent structures resolved the issue of transmembrane (TM) registry in previous lower resolution structures.[35]

The structure of P-gp displays the canonical ABC transporter fold consisting of two pseudo-symmetric halves, each containing a long transmembrane domain (TMD) and a cytosolic ABC or nucleotide binding domain (NBD). The two NBD domains, which are largely conserved in ABC proteins, dimerize to bind and hydrolyze ATP at the interface. To date all X-ray structures of P-gp have been solved in inward-facing conformations with inverted V-shaped architecture and the NBDs separated to varying degrees (Figure 2).[29–35] Two bundles of TM helices, formed with a characteristic domain swapping (TM4/TM5 in domain I and TM10/TM11 in domain II cross over and associate with the opposing monomer, respectively) within the B-subfamily of ABC transporters, enclose a large cavity of > 6000 $Å^3$ in the inward-facing structures.[29] Drugs are thought to enter this cavity for binding through portals open to the cytoplasm and the inner leaflet of the membrane. Not represented in the crystal structures is a flexible linker of ~60–70 amino acids which connects the two halves of P-gp in a single polypeptide chain. This linker region contains several sites for phosphorylation but with no defined role in the expression and drug transport of P-gp.[36]

P-gp undergoes dynamic conformational changes through which the drug binding sites alternately access the two sides of the membrane, binding the cargo on one side and releasing it on the other. Distinct outward facing conformations, with the TM-enclosed drug-binding cavity open to the extracellular side, have been determined for several homologous bacterial ABC transporters in the presence of bound nucleotide.[37, 38] These structures are consistent with an alternating-access model in which ATP binding and hydrolysis drive the conformational change via NBD dimerization. In keeping with biochemistry, substrate transport by P-gp is often accompanied with stimulated ATP hydrolysis while the basal rate of ATPase hydrolysis is low in the absence of substrate. For P-gp, however, no outward-facing structures have been determined by X-ray crystallography to date. An ATPase-catalytically deficient mutant of mouse P-gp has been co-crystallized with ATP, but showed an inward facing conformation similar to other apo structures (Figure 2).[33] Divergent from consensus views of general ABC transporter mechanisms, a recent study using double electron-electron resonance spectroscopy suggests that P-gp only attains the outward-facing state upon ATP hydrolysis.[39] The difficulty of capturing this outward facing state under steady-state ATP hydrolysis conditions was indicated in previous single particle analyses of P-gp conformations by electron microscopy (EM) imaging.[40] Thus the outward-facing P-gp may exist as a transient high-energy state during the transport cycle. Of note, the outward-facing P-gp were indeed enriched in the presence of substrates that stimulate the basal rate of ATP hydrolysis, as well as by the combination of ADP and vanadate which represents an artificially stabilized nucleotide-bound (post-ATP hydrolysis) condition.[39, 40] However, in another interesting EM study of human P-gp with bound antibody fragment (UIC2, which binds to the extracellular region of human P-gp), the transporter displayed a significant proportion (> 50% of particles analyzed) of NBD-closed conformations even in the absence of bound nucleotide.[41] Of note, there has been some controversy in the field as to whether the NBD-open, inward-facing P-gp crystal structures are physiologically relevant, as this conformation could arise from crystallographic constraints, the use of detergents, or the

absence of nucleotides and transport substrates.[42, 43] It can be argued that the two NBDs in P-gp, or likely in any ABC transporter, should always have nucleotide bound since the cellular concentration of ATP (~3–5 mM) far exceeds the binding constant. Despite remaining controversies and lack of a clear picture of the entire conformational pathway of P-gp, the majority of structural evidence presently suggests that the inward facing state likely represents a high affinity drug-binding conformation, which is highly relevant for the modeling of inhibitor and drug binding.

Polyspecific drug binding sites of P-gp

A significant aspect of P-gp structural studies is to disclose how this single transporter is able to recognize a wide variety of structurally and chemically diverse substrates. In early work using photoaffinity labeling, cysteine-scanning mutagenesis, and derivatization with thiol-reactive reagents, the drug binding sites of P-gp appeared to reside within the membrane-embedded region at the interface between the two TM halves.[44, 45] Multiple substrate binding sites in P-gp have been documented based on drug binding and competition studies, for example the H site for binding to Hoechst-33342 and colchicine, the R site for rhodamine-123 and anthracyclines, and the P site for prazosin and progesterone. [46–48] These functional characterizations establish neither exact nor relative locations of binding for these substrates. In this regard, the successful co-crystallization of P-gp with several cyclic peptide ligands revealed unprecedented insights into the polyspecific drug-binding sites.[29, 34]

The first compounds to be precisely localized in the drug-binding region surrounded by the TM helix bundles of inward-facing P-gp were a pair of cyclic peptide enantiomers (*tris*-(*R*)-valineselenazole: QZ59-*RRR*; *tris*-(*S*)-valineselenazole: QZ59-*SSS*), which by definition possess the same chemical composition but opposite amino acid chirality (Figure 3).[29] These cyclic peptides were derived from marine natural products[49–52] displaying MDR reversal activities, with several features implemented to facilitate their co-crystallization within the flexible drug binding region of P-gp, including extreme structural rigidity, and a homotrimeric structural symmetry.[53] Also, labeling of the peptides with three symmetrically distributed selenium atoms as X-ray anomalous scattering markers was particularly helpful to the positioning and modeling of these molecules in the P-gp structures solved at moderate resolutions. The co-crystal structures revealed one molecule of QZ59-*RRR* roughly at the center of an internal cavity of the drug-binding region. Interestingly, the enantiomer QZ59-*SSS* bound two sites per P-gp molecule, which are distinct from but overlapped with the binding site of QZ59-*RRR*. These structures essentially established that P-gp can distinguish between the ligand stereoisomers with distinct binding sites, orientations, and stoichiometry.

Based on the rationale from the first structural view of P-gp ligand binding, a detailed structure activity relationship (SAR) for cyclic peptide QZ59-*SSS* was undertaken to determine the impact of ligand side chain variation on P-gp interactions.[34] Homotrimers were designed with identical cyclic peptide backbone and systematic increases in size and hydrophobicity of the side chain groups to generate the four compounds QZ-Ala, QZ-Val (QZ59-SSS), QZ-Leu and QZ-Phe (Figure 3). All these cyclic peptides inhibit substrate transport and sensitize cancer cells,[34] likely through competition for P-gp binding by

substrates or anticancer drugs. Measurements of P-gp's ATPase activity revealed that the smaller and less hydrophobic QZ-Ala conferred the highest degree of stimulation followed by QZ-Val, while QZ-Leu and QZ-Phe had less effect. Consistent with these substrate-like interactions, P-gp mediated mild resistance to QZ-Ala in cells.[34]

Additional novel structural insights into P-gp's ligand binding sites were gained by the newer set of P-gp co-crystal structures solved with all four cyclic peptides (Figure 4). These structures were determined at improved resolutions (3.6–3.8 Å vs 4.5 Å) and in a more open conformation than the first set of co-crystal structures with QZ59-*RRR* and QZ59-*SSS*.[29, 34] Within the TMD-enclosed binding region, the smaller ligands QZ-Ala and QZ-Val shared an upper and a lower binding site, whereas the larger and more hydrophobic ligands QZ-Leu and QZ-Phe shared a different upper binding site, with QZ-Phe also binding to a second, unique lower site (Figure 4B and 4C). A remarkable finding was that P-gp binding of the substrate-like ATPase stimulators QZ-Ala and QZ-Val induces a large conformational change, a kink in helix TM4 (Figure 4B). This conformational change in TM4 is structurally linked to a short intracellular helix, which couples the movement between NBD2 and the associated TM helices. This structural insight provides a clue to the mechanism of how substrate binding in the TMD region in P-gp can stimulate ATP hydrolysis at the ATP binding sites ~40 Å away. Of note, a recent cryo-EM structure of MRP1 shows that substrate binding induces a narrower separation distance between the NBDs compared to the substrate-free structure.[54] It is possible that the unchanged spacing of the two NBDs in QZ-Ala or QZ-Val-bound P-gp co-crystal structures relative to the apo structure is restricted by crystallographic constraints.

Molecular basis of P-gp-mediated MDR

It is evident that P-gp possesses a large and versatile drug-binding region encompassing multiple and overlapping binding sites, which can accommodate smaller or larger molecules or several molecules simultaneously. Recently described mouse P-gp structures also correlate the opening and closing of the two halves of P-gp with rotation and translation of individual helices in the TM domains, thereby causing continuous change in surface topology.[33] P-gp substrates or inhibitors are typically lipid-soluble and amphipathic molecules.[55] Consistent with this observation, the drug-binding region of Pgp is made up mostly of hydrophobic and aromatic residues that bind substrates via hydrophobic and van der Waals interactions (Figure 4A). We note that there are a few polar side chains (e.g. Gln343, Gln721, Gln942, Gln986, and Ser 975; residue numbers referring to mouse P-gp) in addition to the aromatic tyrosine and tryptophan residues also facing the drug binding pocket in the inward-facing P-gp structures, and these polar residues may mediate additional H-bond interactions with certain ligands. Additionally, the drug binding residues are mostly conserved between mouse and human P-gp, so that the conclusions drawn from the current mouse model can for the most part be extended to human P-gp, though with some caution in light of a few minor differences in drug binding between human and mouse P-gp.[56, 57]

P-gp has been proposed to function as a "hydrophobic vacuum cleaner" by extruding drugs from the inner leaflet of plasma membranes.[58] An additional binding site for QZ-Val was also observed in the co-crystal structure on the surface of P-gp facing the inner leaflet of the

membrane bilayer and just above the elbow helix EH2 (Figure 3).[34] This site lies close to the predicted membrane-water interface and was proposed to represent a substrate entry point. The structural data lends strong credence to the proposal of an initial lower-affinity "ON-site" for ligands near the inner leaflet of the membrane preceding the higher-affinity "ON-site(s)' within the central binding cavity.[59, 60]

Overall, a molecular understanding of how P-gp mediates MDR has started to emerge from the recent structural determinations, although P-gp structures in complex with actual anticancer drugs have not yet been determined, and a detailed drug transport pathway has yet to be fully elucidated in the context of a dynamic conformational landscape for P-gp. The observed binding positions of QZ cyclic peptides in P-gp appear to correlate with the structure and activity of ligands, and this structure-activity correlation also needs to be scrutinized with a greater number of drugs and drug-bound structures.

Development of P-gp inhibitors and impeding challenges

There has been significant interest in the past in the development and discovery of small molecule P-gp inhibitors or modulators, with a hope to overcome P-gp-mediated MDR in cancer treatment. Figure 5 shows a number of such compounds that have been tested in clinical trials in conjunction with prescribed anticancer drugs. However, excitement has largely dissipated due to limited success with these compounds. The reasons for the failures of these clinical trials are complex and not completely clear, despite quite extensive discussion and analyses of these results in the literature.[61, 62] Here we comment on some limiting factors related to available inhibitors. One obvious challenge arises from their toxicity to normal tissues, owing to the wide distribution of P-gp and its role in the protection of normal cells and tissues. To minimize the undesired toxicity of P-gp modulators against normal tissues, targeted delivery of these drugs to cancer cells may be developed. A second major concern with existing P-gp inhibitors is their generally low potency and specificity. Early generation P-gp inhibitors were not specifically developed to inhibit MDR and they exert other pharmacological activities. For example, verapamil, one of the first P-gp inhibitors tested in clinical trials, is a calcium channel blocker and its P-gp binding affinity is only about 10 μM.[63] Cyclosporine A, another P-gp inhibitor with low μM affinity that was used in early clinical trials, is also prescribed as an immunosuppressant.[64] The low affinity of these inhibitors necessitated the use of high doses, resulting in serious side effects and toxicity. In addition, co-administration of these MDR inhibitors elevated plasma concentrations of anticancer drugs by interfering with their metabolism and excretion. Relatively newer MDR drugs such as tariquidar and zosuquidar, were optimized for increased potency (EC_{50} < 100 nM) and higher specificities toward P-gp.[65, 66] Some of these new compounds show little to no pharmacokinetic interactions with current anticancer drugs. However, contradicting data exist on whether tariquidar is an ATPase stimulator or inhibitor and whether it also acts as a substrate of P-gp.[67, 68] Additionally, tariquidar is reported as a substrate and inhibitor of BCRP.[69] Overall, the potency and specificity of known P-gp inhibitors are low compared to most drugs in clinical use.

From a structural perspective (Figures 2 and 4), P-gp does not have a well-defined drug-binding pocket as compared to many receptors or enzymes, making it intrinsically

challenging to design highly potent P-gp-specific inhibitors. That identical or different ligands occupy proximal binding sites within P-gp may be utilized as a basis for fragment-based drug design to create potent compounds. Some bivalent inhibitors have been designed in literature to exploit the multiple binding sites of P-gp, showing greatly increased potency compared to monomers.[70, 71] For P-gp inhibition, another related question is whether particular drug binding conformations of P-gp can be targeted by high-affinity inhibitors, an area which remains to be explored and requires additional Pgp conformations and drug binding modes/sites to be revealed. Most inhibitors block Pgp efflux likely by high-affinity binding to the open inward-facing conformations. However, a recent cross-linking study has shown that tariquidar inhibits P-gp transition to the open state during the catalytic cycle.[72] As MDR remains a major obstacle in cancer treatment even with the advent of new therapies, the discovery of new and effective P-gp modulators that will overcome the drawbacks of current compounds may still be of great value despite these significant challenges.

Overcoming P-gp-mediated MDR in cancer chemotherapy - alternative strategies

As no drugs directly targeting or inhibiting P-gp have been brought to clinical use, alternative approaches have been extensively explored to overcome MDR in cancer treatment. In this section we will review two strategies that remain the major focus in today's drug discovery: one is the development of new or modified anticancer drugs, and the other the use of delivery systems to evade transporter-mediated efflux. Other approaches, including antisense strategies (e.g. antisense oligonucleotides, ribozymes, and small interfering RNAs) that down regulate ABCB1 gene expression at the mRNA level, or transcriptional regulators that inhibit the transcriptional activation of the ABCB1 gene or by exploitation of the collateral sensitivity of MDR cells to certain drugs,[14, 73] are relatively less explored, and a discussion of these strategies can be found in the provided references.

Designing new drugs or optimizing existing drugs to evade P-gp-mediated efflux

Development of anticancer drugs that are not or poor substrates of P-gp and therefore that are less susceptible to efflux by P-gp overexpressing tumor cells is an important strategy to improve the drug efficacy. This can be achieved either by identification of new lead compounds or the chemical modification of existing anticancer drugs. Notable successes have been reported where strategic modifications of known anticancer drugs or natural products significantly reduced P-gp-mediated efflux (Figure 6).[74] A class of naturally occurring microtubule-targeting compounds, the epothilones, with structure and mechanism of action similar to taxanes, are shown to be poor substrates for P-gp.[75]

Interestingly, structural modification at specific sites lead to altered substrate affinity that allows them to bypass resistance associated with P-gp overexpression.[75–77] The use of Ixabepilone (also known as azaepothilone B and BMS-247550) (Figure 6A), a semisynthetic analogue of epothilone B, in combination with capecitabine, showed effectiveness in the treatment of metastatic breast cancer, where known anthracycline and taxane chemotherapeutics failed.[78] Ixabepilone showed high cytotoxicity, comparable to epothilone

B, with IC_{50} of 2.60 and 0.41 nM, respectively, against the drug-sensitive cell line HCT116 (Figure 6A).[79] Furthermore, the IC_{50} values for Ixabepilone were shown in a relatively narrow range of 1.4–24.5 nM against a panel of over 20 tumor cell lines including the sensitive and P-gp overexpressing resistant cells.[80]

Semisynthetic chemical modifications of the framework of taxanes have given rise to several compounds with significantly improved activity against paclitaxel and docetaxel-resistant tumor cells. Promising examples include cabazitaxel and ortataxel (IDN5109) (Figure 6B), with the former received FDA approval for patients with hormone-refractory metastatic prostate cancer showing significant overall survival and higher response rate,[81–83] and the latter currently in phase II clinical trials for the treatment of several solid tumors[84]. Both taxane derivatives significantly decreased susceptibility to Pgp efflux, giving about 7–14 (for cabazitaxel)[85] and 30–50 (ortataxel)[84] fold of increase in activity relative to paclitaxel, against various P-gp overexpressing tumor cell lines. Of note, several modified taxanes, including ortataxel,[84] SB-T-1213 and SB-T-1250,[86] have been studied not only as potent anticancer agents but also shown as good P-gp inhibitors. These compounds are less susceptible to P-gp-mediated efflux possibly by a novel mechanism of self-inhibition.

Extensive research has been carried out on the modification of vinca alkaloids as well.[87, 88] Vinflunine (Figure 6C), a derivative of vinblastine which contains a fluorinated modification at the C20' position, was shown to have decreased susceptibility to P-gp-mediated efflux compared to vincristine and vinorelbine by factors of 2.5 to 13-fold, with lower in vitro neurotoxicity and enhanced bioavailability.[89] Vinflunine was approved for the second-line treatment of urothelial cancer by EMA in 2009, and remains under investigation in several clinical trials for different cancer treatment.[90] As also demonstrated by Boger *et al.*, this C20' position of vinblastine represents a site with great potential for functionalization leading to substantial enhancement in potency with simultaneous decrease in P-gp binding and transport.[91] The C20' isoindoline urea derivative (1) was first shown to display a reduced differential in activity against the vinblastine-sensitive HCT116 and vinblastine-resistant HCT116/VM46 cell lines of 1020 fold (vs ca. 100-fold for vinblastine). Further modifications of the isoindoline moiety on **2** afforded ultrapotent compound **2** (IC_{50} values of 50–75 pM and 830–880 pM against HCT116 and HCT116/VM46 cells, respectively) (Figure 6C), which gave a stunning ~700 fold increase in activity relative to vinblastine against the same P-g overexpressing HCT116/VM46 cell line.[92] A more recent and detailed study of several vinblastine C20' aryl amide derivatives (such as compound **3**, identified from the screening of 180 compounds) have confirmed little or no P-gp transport by directly assaying the P-gp transport and membrane permeability using Caco-2 cells. Compound **3** was >10-fold more potent than vinblastine against the sensitive cell lines (IC_{50} = 400–500 pM), and >300-fold more active against the resistant HCT116 cell line (IC_{50} = 1.8 nM).[93]

Given the structural complexity seen in many natural product-derived anticancer drugs, it is difficult to predict the exact position and/or groups to optimize these drug molecules in order to suppress P-gp-mediated efflux while maintaining drug potency. As P-gp typically recognizes hydrophobic compounds for export,[55] adding polarity to existing drug entities is often perceived as a feasible strategy to overcome P-gp-medicated efflux. However, this empirical rule is not manifested in the modifications exemplified in Figure 6, specifically

with the added structural complexity and hydrophobicity in the vinblastine modifications. Thus, a fundamental question still remains as to how P-gp reacts upon different chemical structural modifications on transport substrates. Nevertheless, extensive modifications on several classical anticancer drugs such as taxanes and vinca alkaloids, as illustrated in above examples through decades of research in many laboratories, have provided valuable information with regards to the optimal sites for further modification and for detailed SAR studies to elude P-gp efflux.

Complexing or conjugating with drug delivery agents to counteract the activity of P-gp

Complexation or attachment of drugs to a delivery agent has offered the possibility to outcompete P-gp mediated efflux by enhancing drug uptake or potentially modifying the mechanism of cellular entry. Various nano-sized carriers have been exploited in drug formulations,[94, 95] including polymeric conjugates or nanoparticles, lipid-based carriers such as liposomes and micelles, dendrimers, carbon nanotubes, and gold nanoparticles (Figure 7A).[96–98] Some of these nanocarriers have been investigated to address challenges associated with P-gp-mediated MDR within cell lines or in mouse tumor models.[99–102] Among them, liposomes have received the most attention in clinical applications, with the first nano-drug, doxorubicin, approved by FDA in 1995. Many other liposome-based drugs are currently in use and in clinical development.[103] Polymer nanoparticles have also been widely studied. For example, SP1049C is a P-gp targeting non-ionic block-copolymer formulation containing doxorubicin as the cytotoxic payload.[104] SP1049C has been shown to circumvent P-gp-mediated doxorubicin resistance in a mouse model of leukemia,[105] and the formulation with doxorubicin has gone through phase II clinical trials against advanced esophageal cancer.[106] Abraxane, designed to address insolubility problems associated with paclitaxel, is an albumin-bound paclitaxel nanoparticle formulation.[107] It was approved by the FDA as a second-line treatment for metastatic breast cancer.[108] Other drug-polymer conjugates with cytotoxic drugs have also been extensively used to overcome MDR in highly resistant cells *in vitro* and *in vivo*; these include drugs like doxorubicin, camptothecin, paclitaxel, and platinate with polymers like N-(2-hydroxylpropyl)methacrylamide (HPMA), poly-L-glutamic acid, poly(ethylene glycol) (PEG), and dextran.[94, 109] Opaxio (formerly Xyotax: PG-TXL-(poly (L-glutamic acid-paclitaxel) has advanced to Phase III clinical trials as the first polymer-drug conjugate for the treatment of breast, ovarian, lung and other types of cancers.[110] The rationale behind the use of polymer-drug conjugates lies in the improved aqueous solubility of the chemotherapeutic drug by conjugation to a water-soluble polymer, which could help bypass P-gp by an endocytic internalization pathway.[111]

The best examples of covalent delivery systems include the use of cell-penetrating molecular transporters and antibody-drug conjugates (ADCs). The former strategy, pioneered by Wender *et al.*, employs a cationic, guanidinium-rich molecular scaffold to tag the conjugated drug so as to drastically alter drug recognition by P-gp (Figure 7B).[112, 113] Several versions of these guanidinium-rich molecular transporters have been devised, based on the scaffolds of peptides, peptoids, oligocarbamates, dendrimers, oligocarbonates, carbohydrates, or oligophosphoesters.[114, 115] These small molecule transporter drug conjugates are highly water-soluble, show high cellular uptake, and are not substrates for P-gp-mediated efflux.[112]

The uptake of these molecular transporters was proposed to be the attribute of the number and the spatial array of the quanidinium groups, which associate with anionic lipids to form reversed-micelle-like complexes.[113] Release of free drug from these conjugates can be controlled by linker design and target cell characteristics. The efficacy of this strategy was demonstrated with taxol-octaarginine conjugates to overcome resistance *in vitro, in vivo* and *ex vivo* in malignant ascites.[116, 117] Taxol-octaarginine conjugates were shown to be very effective (4–100 fold over unconjugated taxol) in a variety of ovarian cancer cell lines including taxol-sensitive and taxol-resistant variants. Other examples include conjugates with doxorubicin and methotrexate.[118, 119]

Antibody-drug conjugates (ADCs) are designed with a goal of targeted drug delivery together with reduced systemic toxicity.[120] Upon binding to tumor cell surface antigens, ADCs are internalized by receptor-mediated endocytosis, which leads to drug release inside the target cell (Figure 7C). In developing ADCs, much attention has been focused on the development of highly potent cytotoxic payloads, the linker, and the antibody components as well as conjugation technologies. Similar to other types of drug delivery systems, the use of ADC for drug internalization also reduces P-gp-mediated efflux. However, cytosolic drug release by these various delivery systems may not completely evade P-gp-mediated efflux, since the drugs can repartition into the plasma membrane. Additionally, inward facing structures suggest that drug substrates may enter P-gp from the cytosol as well as from the plasma membrane (Figure 2). As a matter of fact, sensitivity of the cytotoxic drugs to P-gp-mediated efflux still plays a crucial role in limiting efficiency of ADCs. Mylotarg[121] (gemtuzumab ozogamicin) was the first ADC to gain FDA approval but later withdrawn due to lack of improvement in overall patient survival. *In vitro* cytotoxicity assays in AML cell lines showed that P-gp expression altered the potency of the cytotoxic payload (Calicheamicin), and potency of the drug was restored by the use of a P-gp inhibitor.[122, 123]

Another example related to MDR mechanisms involves AVE9633, which comprises an anti-CD33 antibody linked through a disulfide bond to the maytansine analog DM4. *In vitro* data demonstrated that P-gp activity was a critical factor in resistance against AVE9633 and DM4 cytotoxicity in leukemia cell lines[124] Kovtun et al. have shown that modification of maytansine DM1 in an ADC with a hydrophilic oligo(ethylene oxide) linker, which is retained after intracellular processing, converts DM1 to a poor P-gp substrate, and this modification markedly improves the therapeutic index of the ADC in eradicating P-gp-expressing human xenograft tumors.[125] Extensive chemical modifications have also been conducted on the potent cytotoxic drug monomethyl auristatin E (MMAE),[126] which is used in many ADCs including the FDA-approved brentuximab vedotin (BV) for treatment of refractory Hodgkin lymphoma and systemic anaplastic large cell lymphoma. MMAE is susceptible to P-gp-mediated efflux, and structural optimization of its ADCs to help the drug escape efflux may thus improve the therapeutic index.[127, 128]

Future Perspectives and Concluding Remarks

Despite extensive efforts, MDR is as much of a problem in cancer treatment now as it ever was, even with the advent of new therapies. The strategy of using multiple anticancer drugs is helpful but cancer cells have proven to be able to adapt and develop MDR. Given the

significant and abundant clinical data supporting the role of P-gp in cancer MDR, development of selective P-gp inhibitors with further assessment of safety and efficacy remains relevant. In addition to P-gp, studies toward the inhibition of other MDR transporters such as BCRP and MRP1 are also important, as these transporters may confer resistance to the same or different sets of drugs. It is thus important to analyze the cause of clinical MDR and develop tailored therapeutics to target these transporters.

Suppressing drug efflux is an important goal in many drug development programs. Chemically modifying or redesigning an anticancer drug to completely bypass P-gp is challenging, and there are no clear rules, as P-gp is able to recognize diverse structures that permeate cellular membranes. At the same time, modifications of an anticancer drug without diminution of drug potency presents another significant challenge. In comparison, drug delivery systems offer seemingly innumerable possibilities and provide the potential for safer and high-dose delivery of anticancer drugs, while using noninvasive tracking techniques that are target specific. In these cases, controlling drug release inside the cells could be key since the drug residence and accumulation are counter-balanced by the efflux via membrane transporters.

The recently solved X-ray structures of P-gp in complex with several ligands have provided a molecular basis of how P-gp mediates MDR. The presence of overlapping sites inside the large and flexible binding pocket lined with aromatic residues explains the extraordinary array of chemical structures that interact with Pgp. Despite the progress, many profound questions pertinent to the mechanism of P-gp and drug interactions remain to be addressed. So far the X-ray structures of P-gp have been captured only in inward-facing states, at limited resolutions of above 3.3 Å and with very few solved in complex with cyclopeptide inhibitors. Controversies remain about the range of P-gp conformations, and a complete pathway of drug transport coupled to such conformational changes is not known. No P-gp structure with an actual drug bound has been determined to date, but would presumably guide efforts to chemically alter the drug so as to evade efflux. How P-gp distinguishes a substrate for transport vs. an inhibitor is also highly relevant to drug design. Addressing all these questions should be among the next steps for the community effort to study P-gp and drug interactions.

Compared to P-gp, structural and mechanistic studies on other MDR transporters are quite limited.[54, 129] Future structural biology studies along with chemistry and biochemical characterizations on all these MDR transporters are crucial not just for understanding the biology, but potentially also for structure-based drug design. The field of membrane protein structural biology has seen a boost over the last few years due to technological advances in modern X-ray crystallography and cryo-EM. Expedited progress in high-resolution structural determinations of these MDR pumps and their drug complexes should be anticipated.

Acknowledgments

Biographies:

Deepali Waghray obtained her Ph.D. degree in organic chemistry from Katholieke Universiteit Leuven (KU Leuven), Belgium. From 2009 to 2013 she worked in the laboratory of Professor Wim Dehaen on the design, synthesis and optical property studies of hetera[*n*]helicenes. During a short postdoctoral experience from 2014–2015 she worked on the design and synthesis of shape persistent macrocycles, synthesis of imine-linked COFs for the formation of extended 2D networks and characterization of 2D-networks using Scanning Tunneling Microscopy (STM) in the laboratory of Professor Steven De Feyter. She moved to USA in 2016 and joined the laboratory of Professor Qinghai Zhang at The Scripps Research Institute (TSRI) as a Research Associate in 2017, and her current project centers on studies of P-glycoprotein and drug interactions.

Qinghai Zhang received his Ph.D. degree in organic chemistry in 2001 under the supervision of Professor Xiyan Lu from Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. From 2001 to 2004, he conducted postdoctoral research with Professor Jeffery Kelly at TSRI, while studying the mechanism of protein amyloidosis using chemical, biochemical, and biophysical approaches. He is now an Associate Professor in the Department of Integrative Structural and Computational Biology at TSRI. His research interests focus on chemical and structural biology of membrane proteins aiming at drug development and mechanistic studies.

Abbreviations Used

MDR	multidrug resistance
P-gp	P-glycoprotein
ABC	ATP-binding cassette
MRP1	MDR-associated protein 1
BCRP	breast cancer resistance protein
TM	transmembrane
TMD	transmembrane domain
NBD	nucleotide binding domain
EM	electron microscopy
ADC	antibody-drug conjugate

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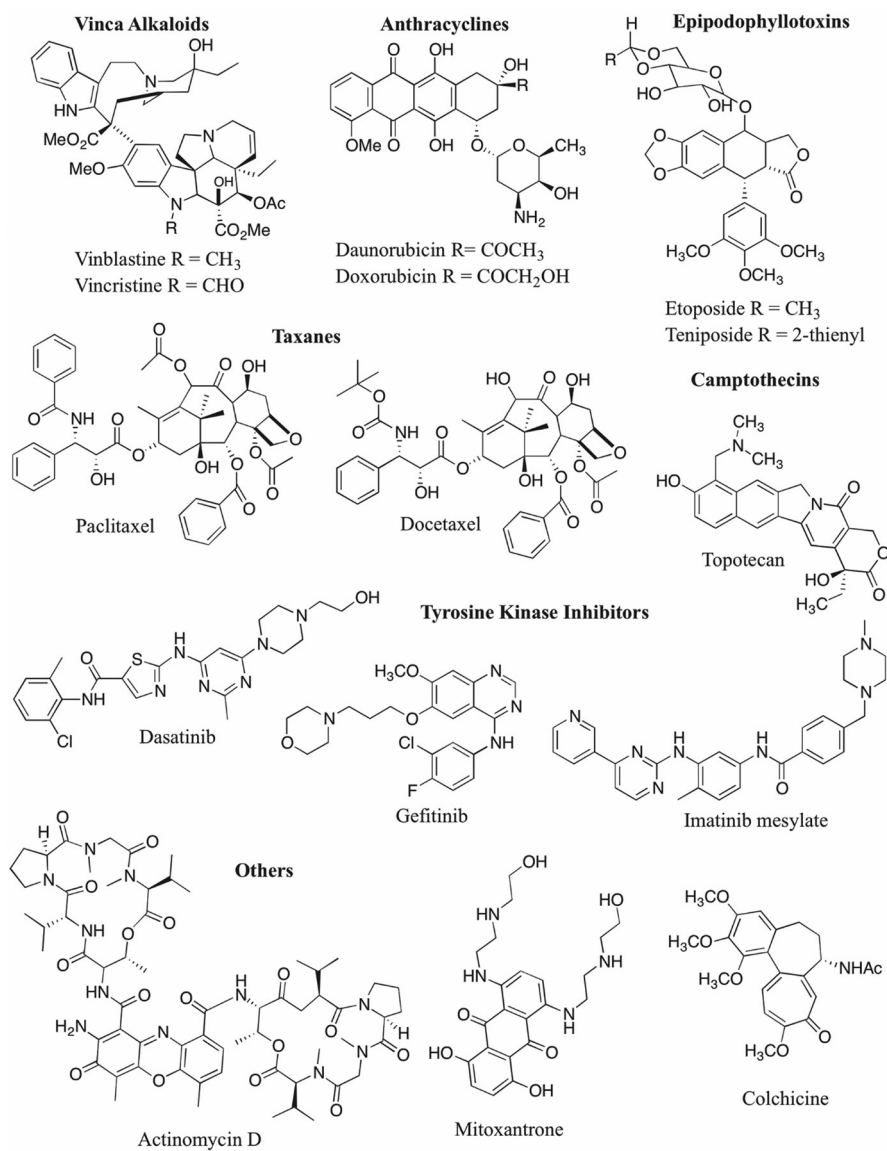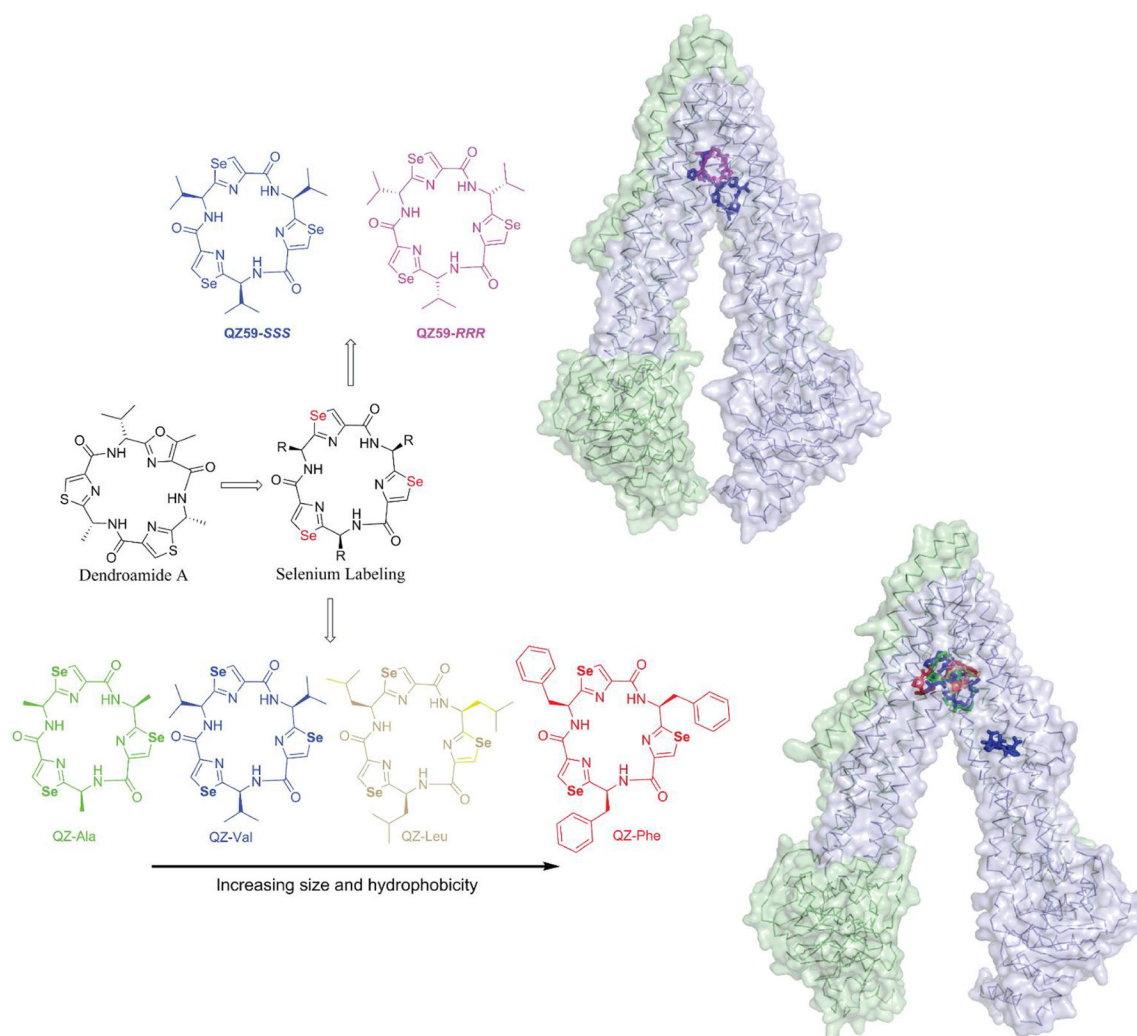
Figure 1.
Examples of frontline anticancer drugs that are susceptible to P-gp-mediated efflux.
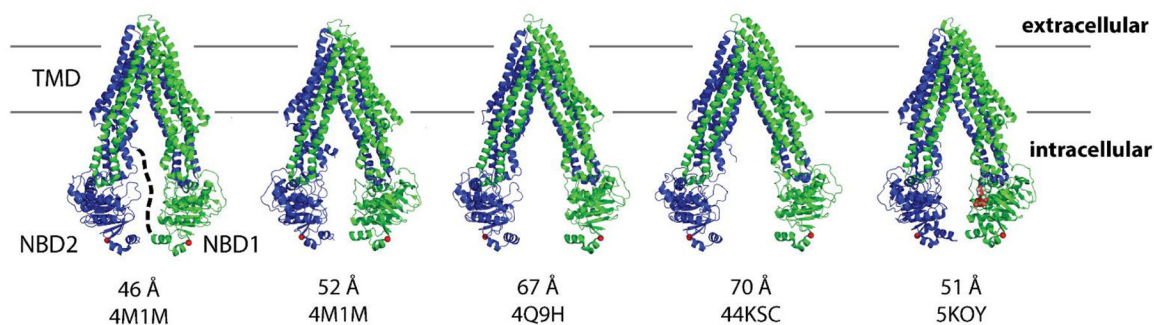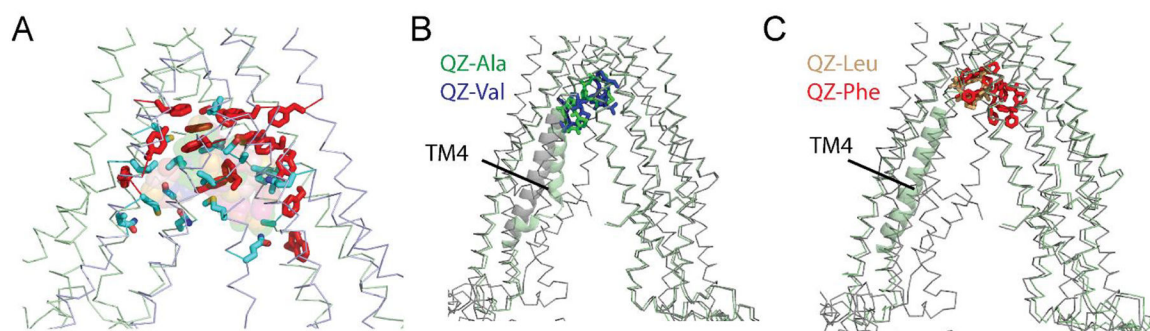
Figure 2.
X-ray structures of mouse P-gp solved in various inward-facing conformations. Shown below the structure cartoons are the PDB codes together with the distances measured between the N607 and T1262 $C\alpha$ positions (marked by red dots) in the separated NBDs. The N- and C-terminal TMD-NBD halves are depicted in blue and green, respectively. A short flexible linker, unresolved in these X-ray structures, is depicted by black dashes in the first structure that connect the end of NBD1 and the start of TMD2. Shown on far right is a structure (5KOY) with ATP (rendered in spheres) bound in NBD1. Estimated lipid bilayer positions are indicated in grey lines.
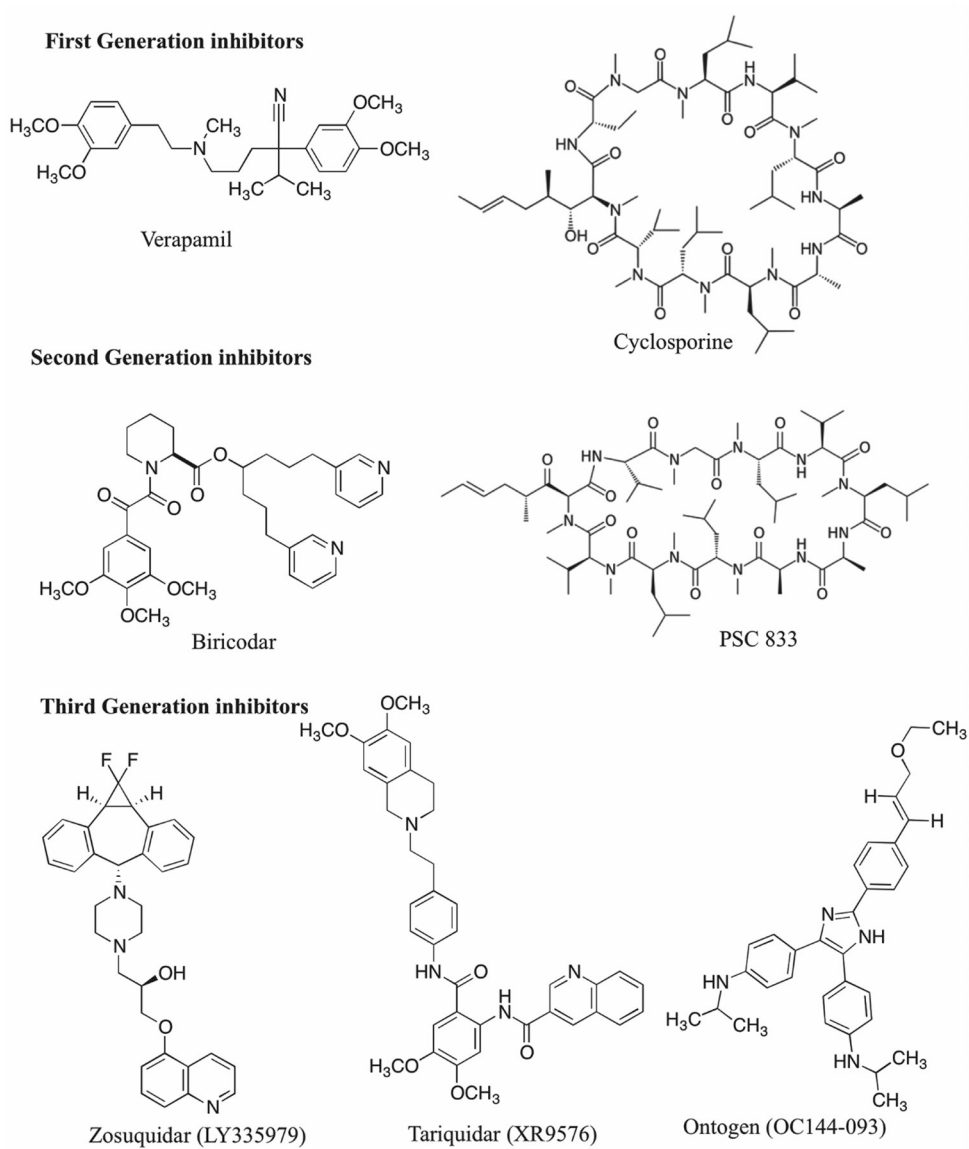
Figure 3.
Cyclic peptide mimetics co-crystallized with P-gp. Homotrimeric cyclic peptides were designed based on natural products (e.g. Dendroamide A) and labeled with selenium atoms to facilitate co-crystallization and subsequent structural determinations. Shown on right are the two sets of co-crystal structures, resolved in different inward-facing conformations, including with the enantiomeric QZ59-*RRR* and QZ59-*SSS* (top), and with four cyclic peptides integrating systematic side chain variations (bottom). Distinct binding sites were revealed in P-gp for each ligand (rendered in sticks with the same color schemes as for the respective 2D structures) and are further described in Figure 4.
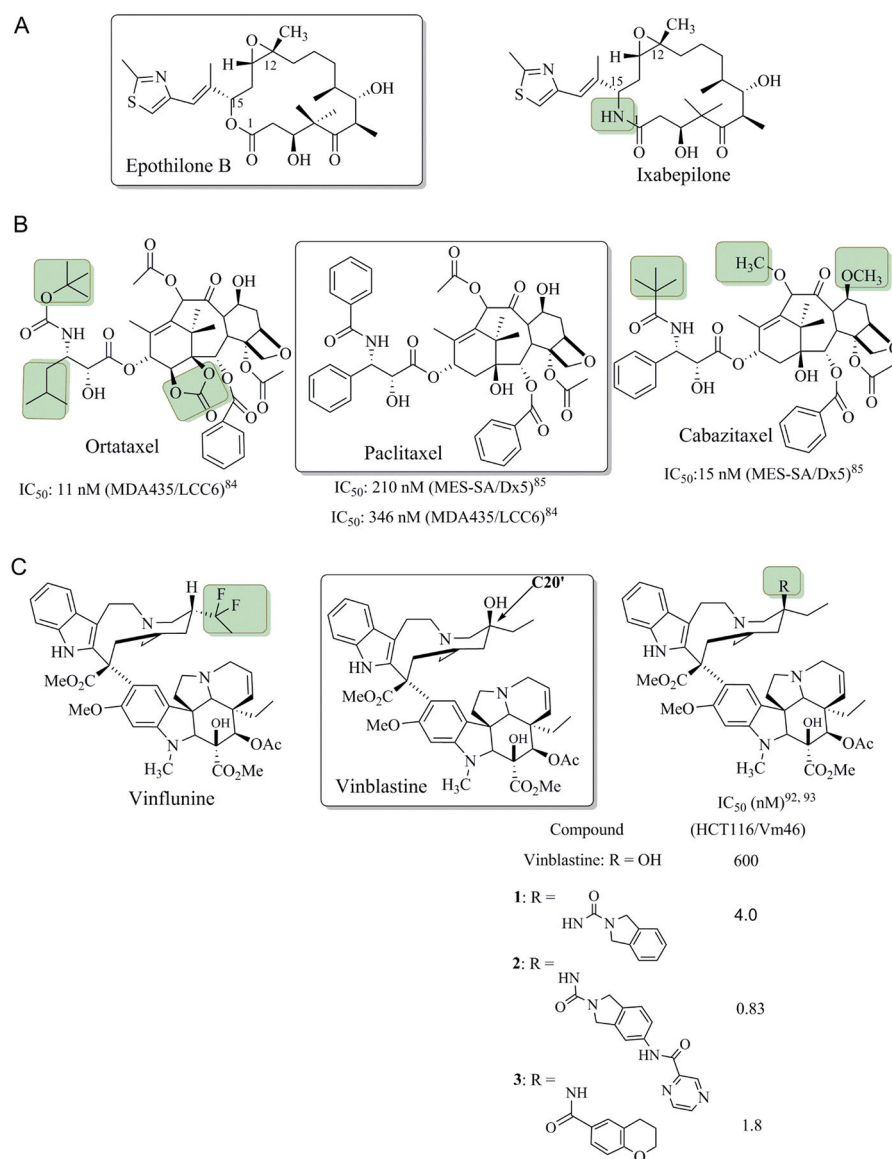
Figure 4.
Polyspecific drug binding sites of P-gp. (**A**) Drug binding residues identified near the binding sites of QZ-Ala, QZ-Val, QZ-Leu and QZ-Phe. Aromatic residues are shown in red sticks and non-aromatics in cyan. (**B**) QZ-Ala (green) and QZ-Val (blue) share similar binding sites and their binding induces a conformational change in the TM4 region compared to the apo structure (grey). (**C**) Location of the binding sites for QZ-Leu (wheat) and QZ-Phe (red). For clarity, only the N-terminal half of P-gp (pale green) cocrystal structures was superimposed with the apo structure in (**B**) and (**C**).

First Generation inhibitors

Verapamil

Cyclosporine

Second Generation inhibitors

Biricodar

PSC 833

Third Generation inhibitors

Zosuquidar (LY335979)

Tariquidar (XR9576)

Ontogen (OC144-093)

Figure 5.
Examples of three generations of P-gp inhibitors.

Figure 6.
Chemical modifications of several microtubule-targeting anticancer drugs to reduce P-gp-medicated efflux. The positions of chemical modifications on Epothilone B (**A**), paclitaxel (**B**), and vinblastine (**C**) are indicated in small shadowed boxes. Decreased susceptibility to P-gp efflux after modifications in (**B**) and (**C**) is indicated by the lower IC_{50} values against various drug-resistant, P-gp overexpressing cell lines.
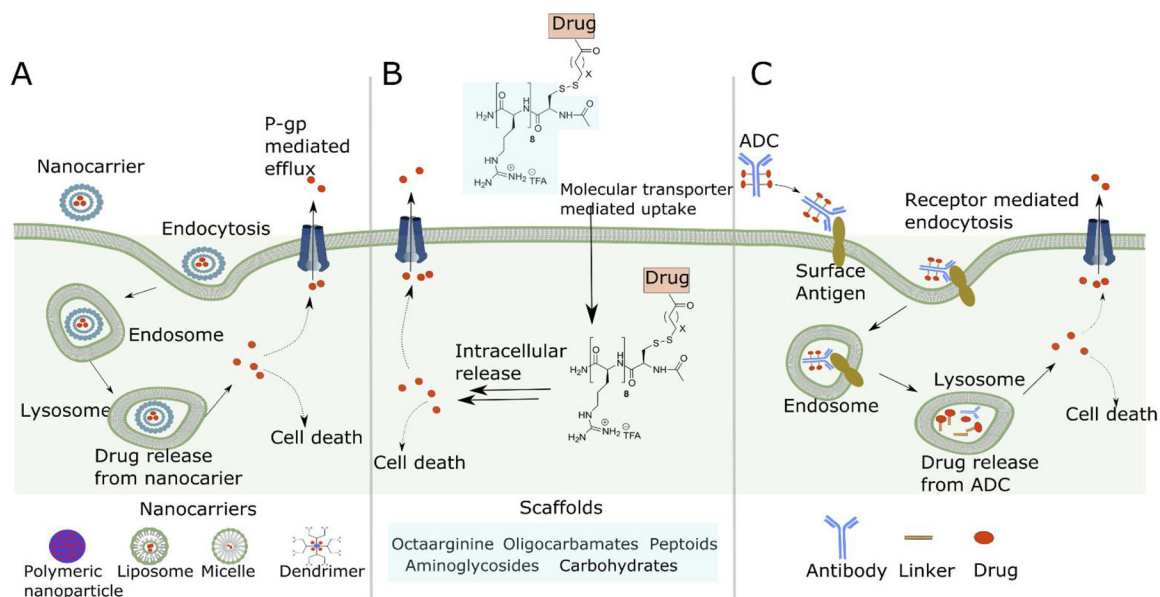
Figure 7.
Schematic representation of drug delivery systems to combat P-gp transport. Anticancer drugs are complexed or conjugated with these delivery agents and internalized for intracellular processing to release the free drugs into the cytoplasm, reducing their uptake from the plasma membrane and export by P-gp. (**A**) Nanoparticle carriers; (**B**) Guanidinium-rich molecular transporters; and (**C**) Antibody-drug conjugates (ADCs).