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Review Article

Disrupting P-glycoprotein function in clinical settings: what can we learn from the fundamental aspects of this transporter?

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Abstract: P-glycoprotein is one of the most well-studied drug transporters, significant for its role in cancer multiple drug resistance. However, using P-gp inhibitors with the aim of enhancing the therapeutic efficacy of anti-cancer drugs has led to disappointing outcomes. Furthermore, several lead compounds suggested by *in vitro* and pre-clinical studies have shown variable pharmacokinetics and therapeutic efficacies when applied in the clinical setting. This review will highlight the need to revisit a sound approach to better design and apply P-gp inhibitors in light of safety and efficacy. Challenges confronting the issue hinge upon myriad studies that do not necessarily represent the heterogeneous target population of this therapeutic approach. The application of P-gp modulators has also been complicated by the promiscuous substrate-binding behaviour of P-gp, as well as toxicities related to its intrinsic presence in healthy tissue. This review capitalizes on information spanning genetics, energetics, and pharmacology, bringing to light some fundamental aspects that ought to be reconsidered in order to improve upon and design the next generation of P-gp inhibitors.

Keywords: Cancer therapeutics, drug resistance, energetics, P-glycoprotein, pharmacokinetics, transporter

Introduction

The ubiquitous nature of P-glycoprotein (P-gp) across mammalian species strongly suggests a critical role in survival by its ability to expel xenobiotics, toxic compounds, and metabolites [1]. In humans, P-gp is coded by the multiple drug resistance *MDR1* gene, whose expression in different tissues indicates its essential function. P-gp belongs to the ATP-binding cassette (ABC) transporter family, which utilizes ATP hydrolysis to transport various substrates, anti-cancer agents, and macromolecules such as peptides and lipids across the plasma membrane [2]. Great interest in P-gp was sparked by its connection to multiple drug resistance (MDR) in human cancers. As shown in hematologic malignancies and solid tumors, these transporters become overexpressed following chemotherapy, leading to therapeutic failure as a result of insufficient intracellular drug concentration and penetration.

Inhibiting ABC transporter activity is therefore a logical strategy to reverse the associated impact of P-gp overexpression in human malignancies demonstrating MDR. Many *in vitro* studies paved the way for the development of various P-gp inhibitors [3]. In the clinic, however, several challenges have been encountered. First-generation inhibitors such as verapamil resulted in cardiotoxicity due to the high doses required to effectively block P-gp activity [4]. Other inhibitors were also not successful, even though major toxicities were not observed [5-9]. The next line of inhibitors was developed to improve drug potency and reduce toxicity associated with inhibition of transporters in normal tissues. Despite these efforts, interactions between P-gp inhibitors and cytochrome P450 have complicated the issue further with regards to altered pharmacokinetics of anti-cancer drugs. Third-generation inhibitors have aimed to address these critical issues, but still resulted in disappointing clinical trial outcomes (**Table 1**).

Insights from previous empirical undertakings are crucial in redirecting our strategies. In particular, an integrated perspective utilizing infor-

Table 1. Summary of clinical studies utilizing 3rd generation P-gp inhibitors

P-gp Inhibitor	Common Name	ATPase Activator	Efflux	Study Intervention	Study Disease	Study Subjects	Intervention Response (without inhibitor vs with inhibitor)	Survival Rates (without inhibitor vs with inhibitor)	Conclusion	Ref.
CBT-1	CBT-1	No	?	Paclitaxel ± CBT-1	solid tumors	10	Not determined	Not determined	Warrants further studies	[96]
PSC-883	Valspodar	No	Yes	Valspodar Cytosine Arabinoside, Daunorubicin, & Etoposide ± Valspodar	acute myeloid leukemia (AML)	302	75% for both regimens	1.34 vs 1.09 years (median disease free survival)	No improved clinical outcomes	[97]
				Carboplatin & Paclitaxel ± Valspodar	advanced ovarian or primary peritoneal cancer	762	41.5% vs 33.6% (ORR)	13.5% vs 13.2% (TTP)	No improvement	[98]
				Vincristine, Doxorubicin, & Dexamethasone ± Valspodar	recurring or refractory multiple myeloma	94	29% (PR) vs 44%	7 vs 4.9 months (median disease free survival)	No improvement	[99]
				Mitoxantrone, Etoposide, & Cytarabine ± Valspodar	aml & myelodysplastic syndrome	129	25% vs 17% (CR)	9.3 vs 10 months (median disease free survival)	No improvement	[100]
MS-209	Dofequidar	No	?	Cyclophosphamide, Doxorubicin, & Fluorouracil ± Dofequidar	advanced or recurrent breast cancer	221	42.6% vs 53.1% (ORR)	241 vs 366 days (PFS)	Well tolerated; effective for patients without prior therapy	[101]
DPPE	Tesmilifene	Yes	?	Doxorubicin ± Tesmilifene	metastatic or recurrent breast cancer	305	2% vs 3% (CR) 27% vs 26% (PR) 45% vs 44% (SD)	6.0 vs 5.9 months (median disease free survival)	No difference	[102]
LY 335979	Zosuquidar	No	No	Cytarabine, Daunorubicin ± Zosuqidar	AML	433	43.4% vs 46.2% (CR)	2.0 vs 3.0 months (median disease free survival)	No improvement	[103]
XR9576	Tariquidar	Yes	?	Docetaxel ± Tariquidar	solid tumors	48	8% (PR) w/inhibitor	Not determined	No difference	[16]

ORR (overall response rate); CR (complete response); PR (partial response); SD (stable disease); TPP (time to progression); PFS (progression-free survival); ? (unknown).

mation about P-gp expression and activity in cancer cells, its connection to cellular energetics, and its ability to respond to metabolic demands has not yet been explored in great detail. This review is an initial attempt to provide perspectives on the problem.

P-gp expression in the clinical setting

Based on recent clinical reports, the expression of ABC transporters plays a significant role in clinical drug resistance in certain hematological malignancies and solid tumors. A study by Burger and co-workers was conducted on 59 primary breast tumor specimens of patients who received chemotherapy as first-line systemic treatment for advanced disease. They noted an inverse relation of *MDR1* expression and the efficacy of first-line chemotherapy: a high level of expression was noted to be a predictor of poor prognosis for patients with advanced disease [10]. A meta-analysis on 31 breast cancer studies reported that the fraction of breast tumors expressing the *MDR1* gene in all studies was approximately 41.2% [11]. Patients with tumors expressing *MDR1* were three times more likely to be refractory to chemotherapy than those who were tumor-*MDR1* negative. A similar observation was reported in a study of 62 osteosarcoma patients. Tumors that did not express P-gp had significantly better relapse-free rates (87% vs. 0%) and improved survival rates of 5 to 14 years (97% vs. 35%) [12].

Increased expression of P-gp following chemotherapy has been reported for both hematologic malignancies and solid tumors. For instance, an increase in P-gp expression from 15% to 43% in fine needle aspiration breast tumor biopsies treated with conventional chemotherapy was reported [13]. In an acute myelogenous leukemia (AML) study, P-gp expression was 24% at diagnosis, which increased to 67% during relapse [14]. In addition, a shorter duration of overall survival or disease-free survival in patients with AML was associated with elevated P-gp expression. Likewise, this trend is similar for multiple myeloma, wherein 6% of patients were reported to express P-gp at diagnosis, and more than 43% overexpress P-gp after treatment [15].

It is possible that the MDR phenotype is due to: 1) upregulation influenced by chemotherapy-induced stress, which is supported by studies on the control of *MDR1* expression (see *Transcriptional regulation of P-glycoprotein*); 2) culling of cells expressing P-gp below a certain clinically-relevant threshold. To establish a clinically-relevant threshold on the protein, transcript, or copy number level is a significant challenge for patient profiling. The next sections will further examine how P-gp expression and activity can impact the next generation of P-gp inhibitors.

The role of P-gp activity in drug resistance development

Variable or altered drug pharmacokinetics as a result of P-gp activity has presented a significant drawback in clinical studies on cancer patients. In this section, attention is focused on the application of 3rd-generation P-gp inhibitors and some difficulties encountered in such studies.

Kelly and co-workers used tariquidar (XR9576) in combination with docetaxel for a phase 2 trial of patients with lung, ovarian, or cervical cancer [16]. Non-hematologic grade 3/4 toxicities were noted to be minimal (2-8% out of 48 patients enrolled), with 4 partial responses.

One parameter used in the study to measure inhibition of P-gp activity by tariquidar was rhodamine efflux from circulating $CD56^+$ cells; however, this may not directly mirror P-gp behavior in the tumor cells of cancer patients. Also, rhodamine efflux as a marker of P-gp activity has certain disadvantages: 1) non-specificity for P-gp, and 2) inter-individual variation when applied to circulating lymphocytes (unpublished data).

Imaging is a more direct way to examine P-gp activity. The group utilized single-photon emission computed tomography (SPECT) imaging, wherein the reduction in ^{99m}Tc-sestamibi clearance from the liver was used as a marker for P-gp inhibition. One disadvantage of the tracer is that it is a known substrate of another transporter, MRP [17]. Confounding factors such varying MRP activity and liver blood flow in cancer patients need to be considered as well. Hence, a wide variation of 6-250% ^{99m}Tc-sestamibi accumulation in the liver, as a surrogate marker for P-gp activity, was obtained pre-treatment of tariquidar [16].

Positron emission tomography (PET) is another imaging platform, which holds certain advan-

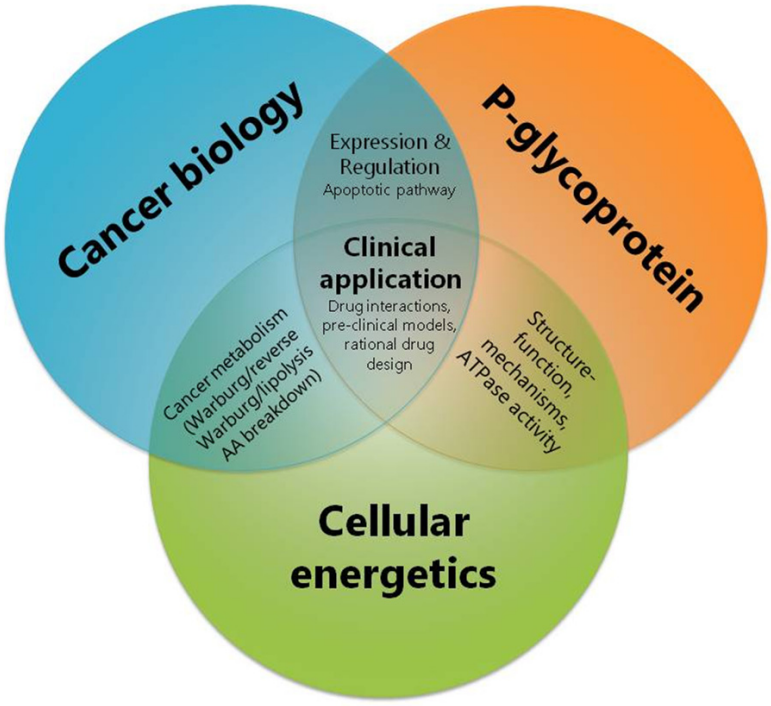

Figure 1. Expanded framework for the application of P-gp inhibitors in the clinical setting. There is an elevated energy demand in cancer cells, presenting novel opportunities for targeting metabolic pathways. The excellent coping mechanism of cancer cells given metabolic stress may be a contributor to drug resistance development, possibly in concert with P-gp regulation and its reliance on the overall energetic status of the cell. P-gp has also been associated with the suppression of the apoptotic signaling pathway, favoring cell survival over cell death. The outcome of cancer therapy may have strong correlations with *MDR1* expression, P-gp activity, and cellular energetics, warranting a more comprehensive foundation for pre-clinical studies and rational drug design. Thus, a better understanding of the profound roles of P-gp in cancer would be necessary to improve current treatment programs.

tages over SPECT: spatial sensitivity resolution, superior quantitation, and the tracer ^{11}C being easily incorporated into organic compounds such as drugs or P-gp inhibitors. PET, however, requires rapid specimen processing due to the tracer having a 20.4-minute half-life (^{11}C). For example, ^{11}C-verapamil may serve as the basis for clinical studies of ^{11}C-labeled anti-cancer drugs or Pgp-inhibitors [17], particularly to accurately determine pharmacokinetic parameters and drug accessibility *in vivo*. The first study to measure P-gp activity and inhibition in the human brain using PET was done using ^{11}C-verapamil and cyclosporin-A (CsA) [18]. AUC_{brain}/AUC_{blood} was found to be increased by about 88% during CsA treatment, demonstrating P-gp inhibition. This study was later extended to interrogate P-gp in the blood-placental barrier of non-human primates, the closest pre-clinical model to humans [19, 20]. Pregnant non-human primates underwent ^{11}C-verapamil PET scans before and after CsA administration (12 or 24 mg/kg/h) to measure placental P-gp activity and inhibition during mid-and late-gestational age. The change in $AUC_{fetal\ liver}/AUC_{maternal\ plasma}$ after CsA administration was used as a surrogate marker of placental P-gp activity; a significant increase in P-gp activity from mid-(+35%) to late gestation (+125%) was found. Further analysis reported considerations of blood flow changes during the different phases of pregnancy [21]. These PET studies may have an impact in pregnant women who are receiving drugs that are P-gp substrates or inhibitors, as well as demonstrate the applicability of this approach to determine more accurate pharmacokinetic parameters *in vivo*.

A recent report has suggested that the concentration of tariquidar used in the study of Kelly and co-workers may have been insufficient [30]. Understandably, this is debatable at this point-translation of optimal P-gp inhibition from pre-clinical studies to cancer patients may lead to unexpected outcomes. Another important factor to consider in clinical trials is the variable P-gp phenotypic characteristics, which may impact the intratumoral accumulation of drugs such as doxorubicin [22-24].

Previous chemotherapy may induce variable expression and functionality of P-gp in the individual. Thus, chemotherapy-naive patients would more accurately parallel pre-clinical models. P-gp-overexpressing cell lines or *in vitro* correlations on patient-derived tumor cultures may be a suitable system to validate various drug candidates for cancer patients who have undergone chemotherapy.

Other clinical studies have used dosing regimens higher than the US FDA recommenda-

tions. For example, a phase 1 trial of tariquidar in non-small cell lung cancer patients used a higher dose of vinorelbine, an anti-cancer agent, at 25 mg/m^2 compared to a previous study that reported a dose of 22.5 mg/m^2 as the maximum tolerated dose when co-administered with tariquidar [25]. This is apparently contrary to the principle of P-gp inhibition as an intervention, wherein the ultimate goal is to reduce the doses of anti-cancer agents to achieve clinically-relevant results.

It is a significant challenge to attribute drug resistance to a single transporter protein like P-gp due to inter-individual differences in transporters [26]. Based on several reports, it is clear that drug pharmacokinetics is highly variable because of differential P-gp expression and activity.

An interesting approach is to employ anti-cancer drugs that can simultaneously inhibit P-gp activity. This opens the possibility of increasing the potency of a co-administered chemotherapeutic agent. One preliminary study demonstrated that crizotinib, an anti-cancer drug that targets ALK and ROS1, also inhibits P-gp activity and consequently leads to intracellular accumulation of co-administered doxorubicin [27]. This may open a discussion on the merits of a dual- or even multiple-target therapy approach that includes P-gp inhibition as a mechanism. One hypothesis worth investigating is if the potency of crizotinib can also be attributed to an ability of the drug to "overwork" the ATPase domains of P-gp, creating an unsustainable energy demand in cancer cells. "Druggability" of P-gp has been presented in the context of its ATPase activity [28], warranting investigation into the roles energetics and metabolism play in the therapeutic approach of P-gp inhibition.

The role of energetics in P-gp-mediated drug resistance

Cancer energy balance (or lack thereof) is multi-faceted, sometimes contentious in literature, and has not been given primacy in the discussion of P-gp-mediated drug resistance. Because P-gp is an ATP-driven efflux pump that is highly implicated in MDR cancers, forging a wider perspective that marries P-gp's role in cancer biology with cancer metabolism and energetics becomes a significant clinical and scientific goal (**Figure 1**). Here we describe a short history of studies that lead to a rational foundation for probing such connections.

Early work by Broxterman and co-workers established a link between P-gp stimulation and ATP metabolism in P-gp-overexpressing tumor cells. They determined that different P-gp substrates induce differential energetic demands: for example, while verapamil starkly increased ATP production by glycolysis [29], cyclosporine A did not [30]. These findings would be highly significant in the exploration of mechanistic-energetic connections in subsequent studies.

Work by Ambudkar and co-workers in 1997 built on early attempts to determine the stoichiometry of P-gp ATPase activity [31], quantifying activity in terms of vinblastine turnover rates in the presence or absence of verapamil. While limited by several assumptions and uncontrolled ATP levels in the living cells, a strong correlation between drug resistance and pumping rate was suggested. Shapiro & Ling then determined the coupling ratio for transport activity (as moles of substrate transported/moles of ATP hydrolyzed) [32]. Their figures suggested that mechanistic coupling was negatively correlated with ATP concentration-that is, P-gp function is "sub-optimal" at ATP concentrations in the cell. They noted the possibility of this phenomenon as an additional form of control (on top of membrane composition and phosphorylation), although they also recognized the possibility of experimental conditions causing this behavior. Interestingly, while they determined a coupling ratio of 1 substrate/ATP at "optimal" and 0.57 at "sub-optimal" conditions, later studies and current mechanistic knowledge support the "sub-optimal" ratio of 1 substrate/2 ATP. It could be argued that these early studies supported a view of the cellular energetic state as a key factor in P-gp activity, albeit not explicitly.

Hrycyna and co-workers applied the orthovanadate (VO_4^{3-}, Vi)-induced ADP trapping technique to P-gp in 1998, the first application of its kind on an ABC transporter [33]. In vanadate-ADP trapping, ATP and Vi are co-incubated with the protein of interest, one round of hydrolysis occurs, and Vi stabilizes (traps) what is believed to be the catalytic transition state of P-gp: P-gp-MgADP-Vi. UV irradiation then cleaves the pro-

tein at the 3rd residue of its Walker A nucleotide-binding domain consensus sequences (GXXXXGKT/S). Expected cleavage products were found in treated P-gp; immunoblots of these demonstrated that the ATP hydrolysis sites cannot be used simultaneously and are functionally interdependent. Since then, many mechanistic studies have been performed using this system.

Sauna & Ambudkar then determined an additional role of ATP in the catalytic cycle [34]. In their proposed model, 2 ATPs are hydrolyzed per pump instead of 1: the first ATP is required to attain the transition state and effect transport, while another is used to return to the substrate-binding state. Using vanadate-ADP trapping, they found that substrate binding (of a radioactive prazosin analogue) is reduced significantly upon ATP hydrolysis (and not simply binding of the nucleotide). They also convincingly demonstrated that release of ADP+Pi is necessary for recovery of the substrate-binding characteristics, and that this phenomenon requires an additional round of ATP hydrolysis. The mechanistic model was thus updated to consist of discrete steps involving 2 ATP hydrolyses (similar to the phosphorylated CFTR chloride channel), although the exact timing of events (i.e. substrate/nucleotide binding and release) remained elusive. Their group then went on to demonstrate that the central transition state of the catalytic cycle, P-gp-MgADP-Pi/Vi, could form through 1 of 2 pathways: via ATP hydrolysis within the protein (hydrolytic) or via direct insertion of ADP and Pi/Vi into the protein [35]. However, they noted two factors that cause P-gp to favor the hydrolytic ("forward") reaction: 1) the energy barrier for non-hydrolytic trapping is intrinsically high (~2.5 times higher than the hydrolytic pathway); and 2) substrates known to stimulate catalytic activity under hydrolytic conditions (such as veramapil) instead severely hamper the non-hydrolytic pathway. Their findings suggested a functional coupling of ATP hydrolysis with substrate binding and transport, indicative of an underlying vectorial metabolism. This has consequences for cancers that overexpress P-gp, warranting investigations into the energetic state of cancer cells and how their ATP supply is funneled to P-gp.

By 2007, crystallographic information on nucleotide-binding domains (NBDs) of P-gp and other ABCs, combined with mechanistic and site-directed mutation studies, clarified much about the catalytic cycle and structure-function relationships of P-gp NBDs [36]. It is now known that the general structure of P-gp comprises 2 transmembrane/drug-binding domains (TMDs), each with 6 transmembrane helices that form a promiscuous (and poorly-conserved) drug-binding pocket, and 2 nucleotide-binding domains (NBDs), each with a very distinct (and well-conserved) structure. The prevailing model describes ATP-mediated NBD dimerization and formation of an ATP "sandwich". The alternating catalysis suggested by earlier studies is rationalized as alternating occlusion and hydrolysis of bound ATP molecules by the NBD dimer. Fine details about the ATP hydrolysis step for restarting the pump are still not certain. However, the level of mechanistic clarity by this point, together with the well-documented coupling of drug transport to ATP hydrolysis, point to a connection between the energy economy of the cell and P-gp activity/control.

By designing a novel assay based on extracellular acidification rates (ECARs), Landwojtowicz and co-workers resolved a disputed issue in literature about pH-mediation by P-gp [37]. Highly-sensitive microphysiometer measurements allowed them to determine a ratio of ~2 H^+/verapamil transported (independent of other regulatory proton pumps), which they suggested could correlate with the established value of 2 ATP/catalytic cycle and thus P-gp activity. They proposed three possible means by which proton transport could be caused by P-gp: 1) direct transport; 2) co-transport by exogenous substrates; and 3) co-transport by endogenous substrates. TMD residue analysis would clarify the first possibility, while structural analysis of known substrates would clarify the second. Co-transport by endogenous substrates is a feasible means to shuttle H^+, as P-gp has been shown to possess flippase activity for a broad spectrum of endogenous lipids: phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, etc. [38] as well as glycosphingolipids [39]. This flippase activity has additional significance: 1) it has been used to rationalize the strange observation of constitutive ATPase activity (basal ATP "burning") in the absence of drugs/substrates of P-gp; and 2) it also suggests membrane composition as a controlling factor for P-gp activity, which had

been suggested at in prior years. This apparent "proton pumping" by P-gp (whether through the above mechanisms or coupled with a transporter such as the lactate transporter), combined with the knowledge that many human tumors overexpress P-gp and that extracellular acidification is linked to cancer drug resistance and metastatic invasion [40], hints to an expanded role of P-gp in cancer biology and invasion *in vivo*. Studies evaluating putative P-gp inhibitors for cancer therapy that make use of this information are warranted.

A 2004 study by Gatlik-Landwojtowicz and coworkers utilized the ECAR method to measure P-gp activation and inhibition in the presence of metabolic stressors. Notably, they explicitly worked under the framework that extracellular acidification, which correlates strongly with P-gp activity, could be indicative of the overall metabolic state of the cell [41]. They addressed two significant concerns: 1) the connection between cellular energetic state (reflected in ATP concentration) and P-gp activity; and 2) the connection of metabolic state (starvation, etc.) to substrate/drug activity. This second point in particular has far-reaching implications in the clinical application of P-gp inhibitors, and deserves to be explored in pre-clinical models. The group observed that verapamil could still stimulate P-gp even under conditions of metabolic stress (pyruvate perfusion, reduction of glucose supply, or complete removal of carbon sources). They interpreted this carbonless stimulation of P-gp as upregulation of glycolysis to meet the energy demand by P-gp activation, even without exogenous carbon sources. Perhaps, more detailed pathway analysis could elucidate whether lipolysis or even amino acid catabolism could also contribute to alleviate P-gp-mediated spikes in energy demand; negligible contributions from beta-oxidation [42] or amino acid catabolism would support glycolysis and the Warburg effect [43] as pathways of choice. Studies on this phenomenon occurring in P-gp-overexpressing cancer cells would corroborate the early reports on P-gp activity and ATP demand.

Thus, in order to consider a connection between the metabolic state of cancer cells with P-gp activity and regulation (and thus MDR), one is forced to confront mechanistic considerations. This becomes imperative given that multiple ATP hydrolyses are required in the catalytic pathway and P-gp-mediated transport is associated with proton shuttling. Tracing energetic pathways affected by P-gp inhibition and stimulation would provide a broader perspective on the clinical problem of overcoming the problem of P-gp-mediated MDR.

Clinical applications in cancer are complicated further by the possibility of energetic contributions from the tumor microenvironment. For example: in solid tumors, cancer persistence and growth are mediated by hypoxia effects and tumor-associated fibroblasts (the reverse Warburg effect) [43]. In ovarian cancers, associated adipocytes promote tumor growth via donation of fatty acids for beta-oxidation [44]; similar associations with adipocytes have been documented for prostate cancer [45]. Still other possibilities exist, such as altered gluconeogenesis or novel pathways. These considerations present a significant hurdle in the clinical application of P-gp inhibitors, and warrant more appropriate basic research and pre-clinical studies in the investigation of P-gp as a therapeutic target.

Genetic polymorphisms of P-glycoprotein

Determining differences between normal and cancer-expressed P-gp on the genetic, epigenetic, or transcriptomic levels may yield new insights into developing a holistic approach to targeting P-gp for MDR cancers.

Due to P-gp being a primary hallmark of the MDR phenotype in cancer, there have been numerous studies about its genetic polymorphisms. There are currently over 20 polymorphisms documented for the *MDR1* gene. At least 9 of these mutations alter the amino acid sequence of the P-gp such as: A61G SNP located near the N-terminus of P-gp, which causes an Asn to Asp substitution; and the G1199A mutation located in the cytoplasmic loop close to the first ATP binding domain, which causes a Ser to Asn substitution [46]. Many polymorphisms do not seem to have an effect on P-gp activity, and therefore do not significantly alter the susceptibility of the cell to cytotoxic drugs.

The best-studied polymorphism is the silent C3435T polymorphism at exon 26, first described by Hoffmeyer and co-workers. It was associated with P-gp expression in intestinal epithelial tissue [47]: people with the C/C genotype have a substantially higher MDR expres-

sion level in the small intestine, approximately twice of those with the T/T genotype. This trend was corroborated by a later study using peripheral blood mononuclear cells [48]. However, a study by Nakamura and co-workers found non-significantly increased *MDR1* levels in Japanese with the T/T genotype compared to the C/C and C/T [49]. Further investigations into ethnic variability determined the allelic frequency of the C allele to be 73-84% in the African lineage, compared to 34-59% in European or Asian lineages [50]. This could signify a need for drug design tailored to different ethnicities based on susceptibility or resistance conferred by significant polymorphisms.

The C3435T polymorphism also affects drug disposition. C3435T was found to be linked to plasma concentrations of digoxin, phenytoin, and fexofenadine [47, 49-51]. Similar significant trends have yet to be established for other drugs.

It should also be noted that the G2677T polymorphism and C1236T polymorphism have been linked to the C3435T polymorphism [46]. This may signify that haplotypes, rather than individual polymorphisms, may be more relevant clinically.

Expression of P-glycoprotein

Currently, clinical studies are focused on small molecule inhibitors delivered extracellularly in order to inhibit the activity of P-gp. An alternative approach would be to control the expression of P-gp at the transcriptional level.

Since P-gp is strongly linked with the MDR phenotype, it would be expected that *MDR1* mRNA transcripts and P-gp protein levels are abundant in MDR cancer cells. However, this is not exactly the case. In a study by Roy and co-workers, the levels of *MDR1* mRNA transcripts and P-gp protein level were determined in non-small cell lung cancer. Surface P-gp was observed in 26/30 (86.7%) samples, interestingly, only 17% exhibited the presence of *MDR1* transcripts [52].

In addition to interrogating the expression levels of *MDR1* in cancer cells with the MDR phenotype, it would also be prudent to consider downstream pathways of *MDR1* in order to understand the consequences of altering its expression. *MDR1* gene expression is activated when cells are exposed to environmental (particularly chemical) stress, as is expected for cancer cells faced with a drug challenge. To determine the effect of an increase in *MDR1* expression on other pathways, one study determined the expression profile of U-2 OS osteosarcoma cells after transfecting and overexpressing the *MDR1* gene [53]. Several cellular pathways were affected, including: drug influx/efflux, metabolic enzymes, cell adhesion, apoptotic signalling, senescence, tumor suppression, and immune receptor signaling. Therefore, *MDR1* also plays a role in the apoptosis signalling pathway (and many others) in addition to its well-examined drug efflux activity. This shows that *MDR1* may be contributing to the MDR phenotype of cancer cells in more ways than clinical studies have elucidated.

Transcriptional regulation of P-glycoprotein

Transcription of P-gp is not a straightforward process, as it is affected by many factors: various response elements, variability of transcription factors, accessibility of the *MDR1* gene, chromatin structure, and associated protein complexes [54]. By understanding the molecular mechanisms of *MDR1* transcription, it may be possible to regulate the expression and/or activity of P-gp.

To ensure that there is a rapid emergence of the MDR phenotype in cells undergoing chemical stress, *MDR1* has a redundant network of regulators from different signalling pathways that can trigger upregulation (reviewed in detail in [55]). Thus, targeting these pathways may present novel cancer therapies. Some examples are discussed below.

Inhibitors of the MAPK pathway significantly reduce the survival of P-gp-expressing MDR cancer cells [56-58]; the MAPK protein c-Jun NH_2-terminal kinase (JNK) has also been associated with the development of MDR and is present in P-gp-associated MDR variants of cervical cancer [59-63]. p38 MAPK downstream signalling, which is implicated in apoptosis and cellular response to drug treatment, has been associated in the MDR phenotype of murine leukemia cell lines L1210/VCR and SB203580 [64-66].

The roles of cAMP and cAMP-dependent protein kinase A (PKA) in P-gp-induced MDR are also well studied. Various extracellular signals

that stimulate PKA-related proteins result in activation of *MDR1* [67, 68]. The related PI3K signalling pathway is upstream of Rac activation, which is associated with the induction of *MDR1* expression and may contribute to the evolution of drug resistance in liver cancer [69].

Nuclear factor κB (NF-κB) is a transcription factor that binds to the *MDR1* promoter (-167 to -158 bp) [70]. Because expression of *MDR1* appears to be NF-κB-dependent, inhibiting NF-κB may harbour therapeutic potential. However, its role seems to be modulatory, as it both represses and activates *MDR1* transcription. It activates *MDR1* transcription in response to acute stress, but represses it to promote apoptosis upon chronic stress [71-73].

A study involving the transcription factor E2F-1 found that downregulation of this factor could lead to reversal of MDR in gastric cancer, both *in vitro* and *in vivo*. Silencing E2F-1 via shRNA led to cell cycle arrest, apoptosis, and increased susceptibility to doxorubicin, cisplatin, and fluorouracil in the SGC7901/DDP cell line. E2F-1 downregulation was also recorded to decrease *MDR1* as well as other genes associated with MDR such as *MRP, Bcl-2/Bax, c-Myc, Skp2, Survivin,* and *Cyclin* D1 [74]. The mechanism of E2F-1-mediated regulation of *MDR1* may be through its promoter sequence, despite not having direct interaction. A study by Andorfer and Rotheneder found that E2F-1 and a downstream protein, EAPP, can both independently stimulate the *MDR1* promoter [75]. Its strong link to *MDR1* makes E2F-1 a prime candidate for a drug target against MDR.

Another transcription factor that affects MDR is RhoGD12. Studies have shown that the MDR phenotype in ovarian, gastroenterologic, and breast cancer could be induced by RhoGD12 activation [76-81]. Chemoresistant fibrosarcoma cells and paclitaxel-resistant ovarian cancers were shown to have upregulated RhoGD12 as well [82, 83]. It was also reported that RhoGDI2 confers resistance against multiple drugs (cisplatin, etoposide, and staurosporin) in gastric cancer cells [77]; upregulation in the gastric cancer cell line MKN-45 led to an increase in transcription of *MDR1*, synthesis of P-gp, and P-gp activity. RhoGD12 thus appears to be a potent regulator of *MDR1* [84, 85], and may present a potential target for MDR cancers.

A novel compound called RY10-4, discovered by Xue and co-workers in 2013, has been shown to restore drug susceptibility in MCF-7/ADR breast cancer cells by inhibiting cell growth, inducing apoptosis, downregulating *MDR1* expression, and reducing intracellular ATP level [86]. This is a prime example of a multiple-target approach to MDR cancers via simultaneous effects on P-gp activity and energetics, similar to crizotinib discussed earlier.

RNA interference

RNA interference (RNAi) presents a promising approach to address P-gp at the post-transcriptional level, particularly in patients whose mRNA transcript levels concord well with observed drug resistance. The use of siRNAs stimulates the RNA-induced silencing complex (RISC), which degrades target mRNA transcripts [87]. The advantages of RNAi include reduced toxicity against non-target tissues and a higher degree of specificity.

Clinical trials for RNAi to target *MDR1* have not yet materialized; most studies utilizing RNAi are currently conducted on cancer cell lines or animal models (reviewed in [88]). Some examples of pre-clinical approaches are discussed below.

Two RNAi-delivery systems have shown promise for reversing the MDR phenotype in cancer cell lines. *MDR1* siRNA-loaded dextran nanoparticles efficiently suppressed P-gp expression on two MDR osteosarcoma cell lines (KHOSR2 and U-2OSR2). Furthermore, combination treatment with doxorubicin determined a 100-fold reduction in the IC_{50} of doxorubicin (10 to 0.1 uM for KHOSR2, 6 to 0.06 uM for U-2OSR2) [89]. This particular delivery system for anti-*MDR1* RNAi was successful in restoring susceptibility of osteosarcoma cells to doxorubicin through P-gp inhibition. Another study utilizing cationic liposomes coated with PEGylated hyaluronic acid (PEG-HA-NP) for the delivery of anti-P-gp siRNA provided comparable cellular uptake and P-gp downregulation efficacy (85% knockdown) in MCF-7/ADR cells compared with Lipofectamine RNAiMAX (90%) and naked NP (78% knockdown). When applied to mouse models, PEG-HA-NP had the highest intratumor accumulation, cellular uptake, and P-gp-silencing capability (34% P-gp downregulation) [90]. These preliminary findings suggest that PEG-HA-NP is a promising vehicle that may be considered for future clinical testing.

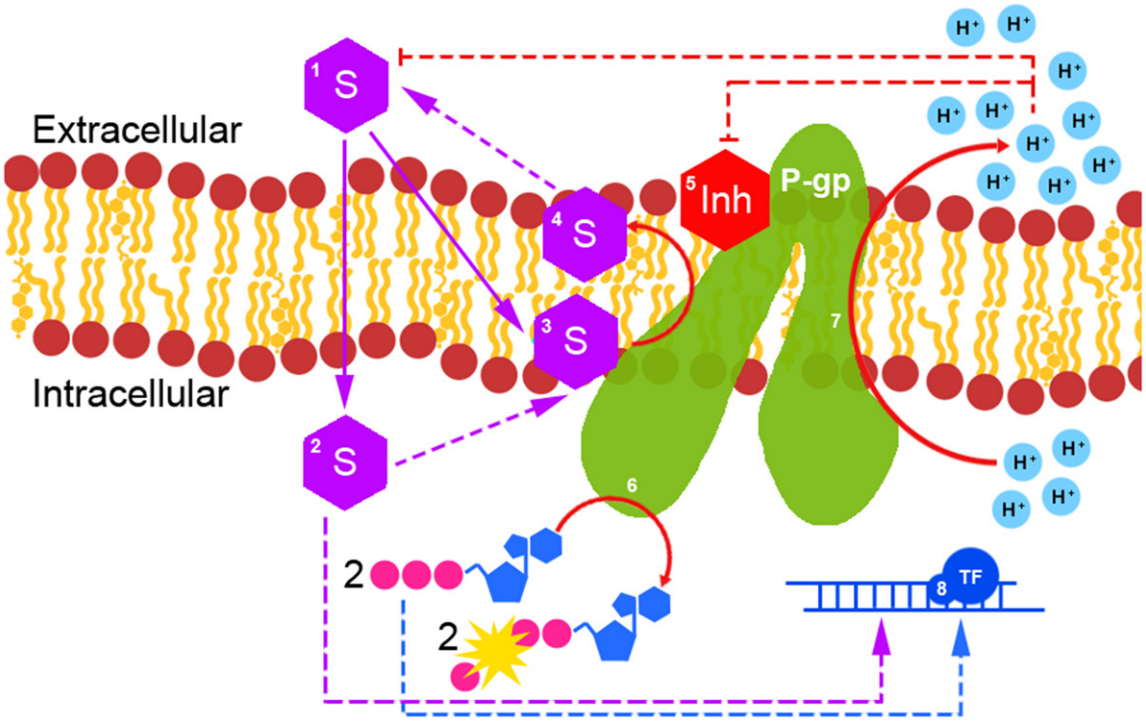

Figure 2. Graphical representation of various interactions of P-gp with the cancer cell. (1) The anti-cancer drug (substrate, S) is initially present extracellularly, and must enter the plasma membrane to exert its therapeutic activity (2). Here, it is subject to transport and partitioning at the lipid bilayer. At the inner leaflet (3), it encounters the transmembrane domain of P-gp and is effluxed (4) with concomitant hydrolysis of ATP. A P-gp inhibitor (Inh), upon binding to P-gp (5), may trigger changes in ATP demand (6). An associated effect of P-gp stimulation is extracellular acidification (7), although the precise mechanism of this symport-like activity is still unknown. Acidification of the extracellular compartment may alter the chemistry of the inhibitor or drug substrate, changing their distribution patterns across the extracellular and intracellular compartments, as well as within the lipid bilayer. This event may further support drug resistance development. The substrate and inhibitor may also activate transcription factors (TF) that modulate P-gp expression (8). The broken lines represent speculative relationships.

The use of short hairpin RNA interference (shRNAi) has been tested in multiple models. For example, two shRNAi constructs targeted against human *MDR1* inhibited expression of P-gp by >90%, correlated with increased sensitivity of *MDR1*-transfected cells toward vincristine, paclitaxel, and doxorubicin. These results were corroborated in tumor implants and a mammalian liver [91]. This is yet another interesting approach to P-gp control.

Certain roadblocks to the RNAi approach in clinical studies include the difficulty of delivery and reduction of downregulation in collateral tissues [92-95]. There are at least 22 RNAi-based drugs against other genes that are currently being tested in clinical trials (mostly in phase 1, some phase 2 underway), but there is currently none for *MDR1*. The data from these trials will be critical in paving the way for yet another generation of P-gp inhibitors. Thus, it remains to be seen which platform for exerting control over P-gp expression and activity, particularly in concert with the other possible mechanisms discussed earlier, will achieve widespread clinical application.

Perspective

After several decades of studying P-gp, there are still many questions left unanswered regarding the clinical application of P-gp inhibitors. If we are to deliver a high-value therapeutic approach, we must consider efficient penetration into tumor tissue, specificity, retention time, and potent induction of cell death (**Figure 2**). As we embrace a more rational approach to drug design, investigators should also consider P-gp genetics, expression, metabolism/energetics, pharmacokinetics, interactions with other drugs, and the cellular (tumor) microenvironment. This review has presented insights that, taken together, emphasize the need for an expanded approach to overcome the problem

of P-glycoprotein-mediated multiple drug resistance in cancers.

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Disclosure of conflict of interest

None.

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