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Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein

Abstract Intrinsic and acquired multidrug resistance (MDR) in many human cancers may be due to expression of the multidrug transporter P-glycoprotein (Pgp), which is encoded by the *mdr1* gene. There is substantial evidence that Pgp is expressed both as an acquired mechanism (e.g., in leukemias, lymphomas, myeloma, and breast and ovarian carcinomas) and constitutively (e.g., in colorectal and renal cancers) and that its expression is of prognostic significance in many types of cancer. Clinical trials of MDR modulation are complicated by the presence of multiple-drug-resistance mechanisms in human cancers, the pharmacokinetic interactions that result from the inhibition of Pgp in normal tissues, and, until recently, the lack of potent and specific inhibitors of Pgp. A large number of clinical trials of reversal of MDR have been undertaken with drugs that are relatively weak inhibitors and produce limiting toxicities at doses below those necessary to inhibit Pgp significantly. The advent of newer drugs such as the cyclosporin PSC 833 (PSC) provides clinicians with more potent and specific inhibitors for MDR modulation trials. Understanding how modulators of Pgp such as PSC 833 affect the toxicity and pharmacokinetics of cytotoxic agents is fundamental for the design of therapeutic trials of MDR modulation. Our studies of combinations of high-dose cyclosporin (CsA) or PSC 833 with etoposide, doxorubicin, or paclitaxel have produced data regarding the role of Pgp in the clinical pharmacology of these agents. Major pharmacokinetic interactions result from the coadministration of CsA or PSC 833 with MDR-related anticancer agents (e.g., doxorubicin, daunorubicin, etoposide, paclitaxel, and vinblastine). These include increases in the plasma area under the curve and half-life and decreases in the clearance of these cytotoxic drugs, consistent with Pgp modulation at the biliary lumen and renal tubule, blocking excretion of drugs into the bile and urine. The biological and medical implications of our studies include the following. First, Pgp is a major organic cation transporter in tissues responsible for the excretion of xenobiotics (both drugs and toxins) by the biliary tract and proximal tubule of the kidney. Our clinical data are supported by recent studies in *mdr*-gene-knockout mice. Second, modulation of Pgp in tumors is likely to be accompanied by altered Pgp function in normal tissues, with pharmacokinetic interactions manifesting as inhibition of the disposition of MDR-related cytotoxins (which are transport substrates for Pgp). Third, these pharmacokinetic interactions of Pgp modulation are predictable if one defines the pharmacology of the modulating agent and the combination. The interactions lead to increased toxicities such as myelosuppression unless doses are modified to compensate for the altered disposition of MDR-related cytotoxins. Fourth, in serial studies where patients are their own controls and clinical resistance is established, remissions are observed when CsA or PSC 833 is added to therapy, even when doses of the cytotoxin are reduced by as much as 3-fold. This reversal of clinical drug resistance occurs particularly when the tumor cells express the *mdr1* gene. Thus, tumor regression can be obtained without apparent increases in normal tissue toxicities. In parallel with these trials, we have recently demonstrated in the laboratory that PSC 833 decreases the mutation rate for resistance to doxorubicin and suppresses activation of *mdr1* and the appearance of MDR mutants. These findings suggest that MDR modulation may delay the emergence of clinical drug resistance and support the concept of prevention of drug resistance in the earlier stages of disease and the utilization of time to progression as an important endpoint in clinical trials. Pivotal phase III trials to test

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these concepts with PSC 833 as an MDR modulator are under way or planned for patients with acute myeloid leukemias, multiple myeloma, and ovarian carcinoma.

Key words Drug interactions · Modulation · Multidrug resistance · PSC 833

Introduction

More than two decades have passed since the initial description of a multidrug resistance (MDR) phenotype in cells and the discovery of a membrane protein, the multidrug transporter P-glycoprotein (Pgp), associated with this phenotype. Knowledge of the biology of Pgp and the human gene that encodes it, *mdr1*, has grown exponentially in the meantime [2, 33, 40, 56].

Clinical trials attempting to reverse or "modulate" MDR have been inspired by a large body of information indicating that:

1. *Mdr1* is highly expressed in many clinically resistant tumors. In some cases its expression at diagnosis has been proven to be an adverse prognostic factor [1, 7, 12–14, 27, 28, 30–34, 47, 54, 57]. In other cases, *mdr1* is not expressed at diagnosis but appears after relapse from remission, suggesting that Pgp was a survival mechanism in a subclone of cancer cells that eventually regrew [2, 21, 25].
2. Pgp is a membrane transporter with broad specificity for many different hydrophobic drugs, most of which are cationic or amphipathic (Table 1). Several noncytotoxic drugs (e.g., verapamil, cyclosporin, nifedipine, quinidine, and reserpine) are transport substrates for Pgp and competitively inhibit its function. This inhibition can sensitize MDR cells to chemotherapeutic drugs in vitro [25, 62, 66].
3. Combined therapy with MDR-related cytotoxins and inhibitors shrinks tumors and prolongs the life span in some animal models [9, 36, 38, 56, 66]. This beneficial effect can be achieved without an increase in toxicity, but for potent modulators like cyclosporin and its analogue PSC 833 the optimal therapeutic effect requires a reduction in the dose of the cytotoxin due to the effect of Pgp inhibition on drug disposition.

New drugs developed specifically to inhibit Pgp and modulate MDR are currently in late preclinical or early clinical trials, and data regarding clinical efficacy are not yet available. Prior to the availability of these agents, several clinical studies utilized "off-the-shelf" drugs as modulators of MDR [20–22, 24–26, 48, 51, 62, 67]. Although these studies have varied in their objectives, experimental methods, and results, in the process we have learned much about the complexity of the biological and clinical issues involved in modulating MDR. However, the therapeutic results have been generally negative or only modestly positive and have not fulfilled the promise of the preclinical data.

Table 1 Anticancer drugs that are substrates for the multidrug transporter Pgp and to which MDR cells are cross-resistant

Vinca alkaloids	Anthracyclines	Taxanes	Epipodophyllotoxins	Others
Vinblastine	Doxorubicin	Paclitaxel	Etoposide	Mitoxantrone
Vincristine	Daunorubicin	Docetaxel	Teniposide	Dactinomycin
Vinorelbine	Epirubicin			Amsacrine
	Idarubicin			Trimetrexate
				Topotecan
				Mithramycin
				Mitomycin C

An appreciation of the variables within and limitations of these studies is essential to the formulation of optimal approaches to understanding clinical drug resistance and to designing future trials of MDR modulation. There are probably two general reasons for the failure of many of these trials to show beneficial effects: (1) the use of weak and nonspecific modulators; and (2) multiple, redundant mechanisms of resistance. Nonspecific and weak inhibitors of Pgp were used, because more potent and specific inhibitors were not available, in an attempt to exploit their binding to and transport by Pgp. Even the most active of these agents in preclinical models, verapamil and cyclosporin, cannot be used at optimal doses to reverse MDR. For example, >10 μM verapamil is required to reverse MDR in most cell models; this is 10-fold the dose that is readily achievable in clinical trials. The problem of multiple, redundant mechanisms of resistance is only beginning to be appreciated as we gain a more complete understanding of these mechanisms and are capable of assessing them.

MDR modulation and type of cancer

The most encouraging results for MDR modulation, generally in the form of anecdotal reports of reversal of clinical resistance, have been noted among the hematolymphoid malignancies: acute myeloid leukemia [20, 25, 41, 63], lymphomas [21, 22, 39, 48], and multiple myeloma [21, 22, 24, 63]. The majority of these cancers are *mdr1*-expression-negative at diagnosis and sensitive to MDR-related chemotherapy drugs. The high incidence of tumor *mdr1* expression in patients who relapse or are resistant (50–80% at relapse as compared to 10–30% at diagnosis) suggests that MDR may be an important clinical mechanism in these cancers. *Mdr1* expression at diagnosis has been shown to be a negative prognostic factor in acute leukemias [12, 31, 45, 54, 57] and non-Hodgkin's lymphomas [53]. These cancers should be considered prime candidates for randomized phase III trials of MDR modulation. They also offer the additional advantage of accessibility of tumor cells in the peripheral blood, bone marrow, or lymph nodes for correlative studies of *mdr1* expression and function.

A second category of cancers is moderately responsive to MDR-related drugs and does not usually express *mdr1* at diagnosis, although some relapsed or refractory tumors (20–50%, depending upon the prior therapy) express the

gene. These include breast, ovarian, and small-cell lung cancers, sarcomas, and some pediatric cancers [1, 13, 14, 28, 34]. Since many of these do not express *mdr1*, assays of *mdr1* and Pgp in biopsy specimens should be considered for phase II studies. The inclusion of patients whose tumors are clinically resistant but *mdr1*-negative may underestimate the ability of an MDR modulator to reverse resistance. Difficulties with the approach of selecting patients for study on the basis of Pgp status include the reproducibility, sensitivity, and specificity of current *mdr1* and Pgp assays and the accessibility of tumors for biopsy.

A third group of tumors are those derived from tissues that constitutively express *mdr1*, e.g., colorectal and renal cancers [27, 28, 32]. These are generally refractory to MDR-related cytotoxins, and the experience with MDR modulation has been largely negative to date [25, 26]. These tumors are also resistant to most other classes of cytotoxic drugs, which may be an indication that they express many different mechanisms of resistance. Even with more effective modulators, colorectal and renal carcinomas may exhibit clinical resistance to most, but hopefully not all, MDR-related cytotoxins.

Mdr1 expression in normal tissues

Pgp is expressed at significant levels in the biliary canaliculi of the liver, the proximal tubules of the kidneys, and the small intestine, colon, and adrenal cortex [28, 56, 65]. In most tissues, Pgp is expressed on the luminal surface, thus transporting substances into the bile or urine and functioning as a barrier (small bowel and colon). The endothelial cells of the central nervous system, testes, and placenta also express Pgp; in these systems, Pgp is thought to contribute to the blood-brain, blood-testicular, and blood-placental barriers [19, 65]. In our initial phase I studies with high-dose cyclosporin and various cytotoxic drugs, two potential consequences of MDR modulation were observed in the central nervous system: an increase in nausea and vomiting (possibly due to increased etoposide distribution to the vomiting centers of the brain stem) and greater than expected obtundation when lorazepam was used as an antiemetic [4, 25, 26, 63, 67]. Some subsets of hematolymphoid cells express *mdr1*, including bone marrow stem cells, lymphocytes (particularly CD-56$^+$, natural killer cells), and activated macrophages [15, 16, 23, 46].

Lessons from *mdr*-gene-knockout mice

The possibility of normal physiological roles for Pgp (other than detoxification and excretion of xenobiotics) has been a subject of speculation. Recently, researchers at the Netherlands Cancer Institute have succeeded in knocking out both of the *mdr* genes in a mouse strain (mice have two *mdr* genes that function in drug resistance as compared to the one human gene). Interestingly, these mice grow and develop into adulthood normally, are fertile, and have no obvious abnormality. Thus, it appears that Pgp has no essential physiological function. However, these mice are very sensitive to MDR-related toxic chemicals, including chemotherapeutic drugs, and are deficient in the excretion of these agents [59–61]. A particular feature of these mice is that they accumulate and retain more of these toxins in the central nervous system, consistent with a major role for Pgp in forming the blood-brain barrier.

Toxicities related to MDR modulation

Although there is a theoretical concern that MDR modulation may reveal new anticancer drug toxicities due to the inhibition of Pgp in normal tissues, this has not been realized to date: no excess gastrointestinal toxicity (diarrhea, bowel perforation, or mucositis) has been observed in any study. It is likely that there are redundant mechanisms of resistance other than MDR in tissues such as the gastrointestinal tract. However, this remains a concern for future clinical trials exploring the broad spectrum of MDR-related agents and the development of more potent MDR modulators. The potential for toxicity to the central nervous system, as evident in the *mdr*-knockout mice, has also not been seen in clinical trials. This may also be because even the newer modulators are not sufficiently potent to "knock out" Pgp function pharmacologically or, perhaps, because the blood-brain barrier is more complex in humans.

The hyperbilirubinemia produced by high-dose cyclosporin [4, 41, 62, 67] is apparently not related to Pgp inhibition but may be due to inhibition of another newly discovered protein, the canalicular multiple organic anion transporter (cMOAT). This transporter is highly expressed in the liver and is responsible for excretion of bilirubin and, probably, that of other organic anions [52]. Interestingly, cyclosporin is a more potent inhibitor of cMOAT than is its analogue PSC 833 and produces higher bilirubin levels, whereas PSC 833 is a more potent inhibitor of Pgp. This hyperbilirubinemia, which is not produced by most Pgp inhibitors, illustrates the capacity of drugs to affect multiple targets.

Although novel toxicities due to inhibition of Pgp have not been problematic, the pharmacokinetic interactions associated with decreased drug excretion and increased drug exposure will increase the toxicity of anticancer drugs unless doses are reduced to compensate for the interaction.

Pharmacokinetic interactions and drug dosing

Modulation of MDR should be considered a programmed drug interaction that is targeted at resistant tumor cells but has consequences for drug disposition and toxicity. Such consequences were predicted from animal studies that examined the interactions of high-dose verapamil combined with vincristine [35] or doxorubicin [64]. Plasma verapamil concentrations of $>10\ \mu M$ in combination with vincristine

could be maintained in mice, indicating greater tolerance for the modulator in mice than in humans. Verapamil administration produced increased retention of vincristine in the small intestine, liver, and kidneys and a tripling of the plasma half-life of the cytotoxic drug. There is one clinical report of excessive vincristine neuropathy associated with the use of cyclosporin to modulate MDR [8].

Major effects on the pharmacokinetics and toxicity of cytotoxins have not been observed in the clinical trials of MDR modulation utilizing verapamil, probably due to the higher concentrations of verapamil achievable in mice. Nevertheless, the preclinical data (and particularly the work in *mdr*-knockout mice described above) illustrate the potential for pharmacokinetic drug interactions inherent in trials of MDR modulation. Increased toxicity of the MDR-related anticancer drugs may occur due to both pharmacokinetic effects and inhibition of a protective function of Pgp in healthy tissues. Conversely, it is unlikely that adequate concentrations of an MDR modulator would have been achieved in any clinical trial that did not reveal a pharmacokinetic interaction or in which there was no alteration in toxicity for healthy tissues.

Kerr et al. [39] demonstrated a modest pharmacokinetic interaction between verapamil and doxorubicin in humans. The cross-over design of this study, with patients serving as their own controls, enabled significant observations to be made with only five patients. Oral verapamil increased the peak level, terminal half-life, and volume of distribution of doxorubicin and decreased plasma drug clearance.

Our work defining pharmacokinetic interactions of MDR cytotoxins with cyclosporin and PSC 833 has sought to define the interactions of MDR modulators with a variety of cytotoxic drugs [4, 20, 25, 26, 42–44, 67]. For etoposide, doxorubicin, and paclitaxel, in vivo Pgp inhibition produces reproducible increases in the half-life and normalized dose exposure of these drugs. The dose modification for myelosuppression is 2- to 2.5-fold, with corresponding myelosuppression and other toxicities leading to the recommendation of a 50–60% reduction in the dose of the MDR-related cytotoxin when the latter is given with PSC 833.

The question arises as to whether these pharmacokinetic interactions can be separated from reversal of MDR in tumors and whether it will be difficult to distinguish increased dose intensity from inhibition of tumor drug resistance [47]. The clearest way to answer this question is to design controlled phase III trials with equivalent dose exposures and potential toxicities in the two arms by reducing the dose of MDR-related cytotoxins in the experimental arm as compared to the standard arm. The optimal dosage of MDR modulators should also be explored further. The first generation of clinical trials in this field used agents that were limited by their toxicity at doses below those required to produce significant inhibition of Pgp in vivo. An ideal modulator would have no target other than Pgp and would be used at doses that would saturate effects such as pharmacokinetic interactions and surrogate markers of Pgp inhibition in most patients.

Measurement of *mdr1* and other drug-resistance-gene expression in tumor cells

Measurements of the expression of *mdr1* and other drug-resistance genes in tumors are an important aspect of some types of clinical trials of MDR modulation; an analogy can be made with estrogen receptor status and hormonal therapy of breast cancer. Resistant tumors that express non-MDR resistance mechanisms should not respond to attempted MDR modulation, and some *mdr1*-positive tumors may not respond due to the presence of redundant mechanisms of resistance. The high level of *mdr1* expression in some healthy cells may lead to false-positive results. The two most commonly used assays are flow cytometry or immunoassays to detect heterogeneity in cellular Pgp expression and reverse transcriptase-polymerase chain reaction detection of *mdr1* mRNA [11, 46].

Functional assays for Pgp in patients' specimens utilize the efflux and retention of rhodamine 123 and other fluorescent substrates of Pgp; such assays have been used by many groups to examine both tumor cells (particularly leukemia and myeloma cells, which are readily assayed by flow cytometry) and $CD56^+$ lymphocytes, which have relatively high Pgp levels. Finally, pilot experiments using technetium 99-sestamibi to image Pgp expression in patients by gamma scintillation scanning are being conducted by several investigators. This radiopharmaceutical is a good transport substrate for Pgp and accumulates to low levels only in Pgp-negative tumors in animals, and its uptake and retention can be increased by MDR modulators. These surrogate markers of Pgp function and MDR modulation may be useful in defining the clinical activity of new MDR modulators.

Other drug-resistance genes

MDR due to Pgp expression is only one of many mechanisms of resistance to MDR-related chemotherapy drugs, and it is likely that resistant cancers express more than one mechanism of resistance to any particular drug. Decreased or altered topoisomerase II has been found in many cells resistant to epipodophyllotoxins, mitoxantrone, and anthracyclines [5]. A non-Pgp membrane transporter termed the multidrug-resistance-associated protein (MRP) has been identified, and its gene sequenced in a doxorubicin-selected lung cancer cell line [18]. A 110-kDa vesicular protein recognized by the LRP-56 antibody is differentially expressed in several cell lines cross-resistant for MDR-related agents and has recently been reported to be identical to the major vault protein [58]. A 95-kDa membrane protein is highly expressed in cells coselected with doxorubicin and verapamil and has recently been found to be associated with drug-resistant acute myeloid leukemia [17]. The *bcl*-2 gene and related proteins regulate chemotherapy-induced apoptosis and have the capability of conferring broad cross-resistance [49, 50]. Doubtless there are many other genes

regulating apoptosis and other mechanisms of drug resistance that have not yet been discovered.

Data are increasing on the expression and prognostic importance of these non-Pgp-related mechanisms of resistance in various cancers. Their role relative to *mdr1* will require prospective assessment, and it is likely that some of these other resistance mechanisms will also be amenable to modulation. The ability to reverse these other mechanisms may lead to trials of combined modulation with Pgp inhibitors to address the problem of redundant mechanisms of resistance.

New modulators of MDR

Two potent new inhibitors of Pgp are currently in clinical trials, the cyclosporin analogue PSC 833 [3, 9, 29, 37, 38, 55, 58] and an acridonecarboxamide compound with high affinity for Pgp [36]. A report on the first phase I clinical trial of MDR modulation using PSC 833, using an intravenous formulation together with etoposide, has recently been published [10]. The dose-limiting toxicity of this agent is cerebellar ataxia, which is related to the peak level of the parent drug and is reversible. Several other clinical trials using an oral formulation have been completed but the results have not yet been published, and phase II studies are ongoing in leukemias, lymphomas, myeloma, and carcinomas of the breast, ovary, colon, and kidney. Pivotal phase III trials are planned for acute myeloid leukemias, multiple myeloma, and ovarian carcinoma.

Prevention of drug resistance

We have recently reported evidence that MDR modulators may inhibit the development of Pgp-related drug resistance in vitro [6]. In experiments with populations of a human sarcoma cell line, on the basis of Luria-Delbruck fluctuation analysis, PSC 833 reduced the mutation rate for doxorubicin resistance by a factor of 6, from 1.6×10^{-6} to 2.5×10^{-7}/cell generation. Moreover, with doxorubicin alone the predominant mechanism of resistance was activation of the *mdr1* gene, leading to expression of Pgp and MDR. Mutants selected with doxorubicin plus PSC 833 did not express Pgp but showed decreased expression of topoisomerase IIα.

The clinical implication of these findings is that treatment of drug-sensitive Pgp-negative cancers with effective MDR modulators might suppress the appearance of resistant MDR subclones and result in a prolonged time to progression and, perhaps, even in the cure of an additional subset of patients. Thus, controlled trials of MDR modulation should be considered early in the course of the disease in populations of patients whose tumors are potentially drug-sensitive and do not express Pgp (e.g., in newly diagnosed patients with leukemias, lymphomas, or myeloma or as first-line metastatic breast cancer therapy). The major clinical endpoint of such trials would be duration of remission or time to progression.

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