P-glycoprotein as a Drug Target in the Treatment of Multidrug Resistant Cancer

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Abstract: Multidrug resistance (MDR) is a major obstacle to successful cancer chemotherapy. One important mechanism of MDR involves the multidrug transporter, P-glycoprotein (Pgp), which confers upon cancer cells the ability to resist lethal doses of certain cytotoxic drugs by pumping the drugs out of the cells and thus reducing their cytotoxicity. Pgp belongs to the ATP-binding cassette (ABC) family of transporter molecules which require hydrolysis of ATP to run the transport mechanism. The substrates of Pgp may be endogenous (steroid hormones, cytokines) or exogenous (cytostatic drugs). A number of studies have demonstrated a negative correlation between Pgp expression levels and chemosensitivity or survival in a range of human malignancies. In principle, Pgp mediated drug resistance can be circumvented by treatment regimens that either exclude Pgp substrate drugs or include Pgp inhibitory agents. Experimental studies have demonstrated that certain structural modifications of anthracyclines confer the ability to escape Pgp transport. The therapeutic benefit of Pgp inhibitors as chemosensitizers is currently being explored in phase III clinical trials, and the first promising results have already been reported. Another therapeutic option for Pgp inhibitors has recently evolved as several Pgp inhibitors, many of which are generally low-toxic substances, by themselves constrain proliferation and cause cell death by apoptosis in certain MDR cancer cell lines. The dual effect of Pgp inhibitors, targeting MDR cancer cells selectively, may translate into improved efficacy of cancer chemotherapy and perhaps new and less toxic drug treatment strategies in human MDR cancer.

INTRODUCTION

Modern chemotherapy has a limited potential for cancer cure due to acquired or intrinsic resistance of cancer cells to anticancer drugs. Resistance to one drug often implies resistance to a series of different drugs (multidrug resistance, MDR) which leaves the clinician with few therapeutic options and the patient with a sinister prognosis. Gastrointestinal, hepatobiliary and renal cancers are largely unresponsive to chemotherapy and thus, have a high degree of intrinsic MDR, whereas leukemias, lymphomas, ovarian and breast cancers often respond to treatment initially, but acquire resistance during the course of the disease. Growing tumours often develop physical obstacles to drug delivery due to deficient tumor blood flow and elevated interstitial pressure which opposes even distribution of agents. Furthermore, several biochemical mechanisms have been identified including increased intracellular inactivation of cytotoxic drugs [1,2], changes in the activity of target enzymes [3,4], changes in intracellular drug metabolism [5,6], and emergence of mechanisms that actively remove certain anticancer drugs from the interior of neoplastic cells [7,8].

One important mechanism of MDR involves the multidrug transporter, P-glycoprotein (Pgp), which confers upon cells, the ability to resist lethal doses of certain cytotoxic drugs by pumping the drugs out of the cells and thus, reducing their cytotoxicity [9,10]. Several clinically important anticancer drugs may be removed from neoplastic cells by Pgp-mediated transport, despite the diversity in chemical structures and mechanisms of action [7,11]. This ability is apparently reflected in a negative correlation between Pgp expression and chemosensitivity or survival in leukemias [12,13], lymphomas [14], osteogenic sarcoma [15], small-cell lung cancer [16], breast cancer [17,18] and pediatric solid tumours [19,20].

Despite the prominent role of Pgp in our current understanding of chemoresistance, there are still a series of questions to be addressed regarding diagnosis and treatment of Pgp-mediated drug resistant malignancies. How should Pgp be measured? Does structural modifications of cytostatic drugs change the selectivity of Pgp-mediated resistance? Does clinical application of Pgp inhibitory agents improve drug therapy in...
multidrug resistant cancer? Does Pgp inhibition modulate vital cancer cell functions? We have recently demonstrated the ability of different Pgp inhibitory agents to induce growth inhibition and cell death by apoptosis in leukemia cells with high expression levels of Pgp [21]. This intriguing finding represents a potential therapeutic option that is currently being studied in vivo, and should be considered during the ongoing development of strategies to overcome multidrug resistance.

MULTIDRUG TRANSPORTERS

Pgp belongs to the ATP binding cassette (ABC) family of transporter molecules [8,22], which also include the MDR-associated protein (MRP or MRP1) [23], the canalicular multispecific organic anion transporter (cMOAT, cMRP, or MRP2) [24], the ATP binding cassette transporters 1(ABC1) [25] and 2 (ABC2) [26], and the breast cancer resistant protein (BCRP) [27]. The substrate specificity of Pgp and MRP is partly overlapping despite being distant related with an amino acid sequence identity of only 15% [23]. Basically, MRP differs from Pgp in being a multiple organic anion transporter, which transports certain glutathione conjugates and is apparently dependent on intracellular glutathion levels for the transport of anthracyclines [28-30].

The predictive significance of MRP in cancer patients is not established. There are reports of MRP as a marker of poor prognosis in lung cancer [31] and neuroblastoma [32], whereas other studies have failed to demonstrate correlation between MRP expression and prognosis in breast cancer [33], colorectal carcinoma [34], and childhood leukemia [35]. Overexpression of cMOAT in cancer cells could potentially lead to drug resistance because of its proven ability to transport vinblastine [36]. However, no correlation have been established, thus far between cMOAT overexpression and MDR in cultured cells, but there was a positive association with cisplatin resistance, raising the possibility that cMOAT might contribute to cisplatin resistance by mediating excretion of cisplatin-glutathione complexes [36].

Another transporter, the lung resistance related protein (LRP) [37], has been identified as the major component of certain ribonucleoprotein particles (vaults) which possibly translocate cytotoxic drugs from nuclei via cytoplasmic vesicles to the cell surface [38]. This transport mechanism is distinct from that of the ABC transporters. Even though LRP appears to be a marker of poor prognosis in ovarian carcinoma and acute myeloid leukemia [39,40], there is to this date no evidence that LRP actually transport drugs and thereby contributes to the poor treatment outcome in these patients.

THE BIOLOGY OF P-GLYCOPROTEIN

The gene encoding Pgp belongs to the MDR multigene family [22], which consists of two highly homologous genes MDR1 and MDR2 (formerly designated as MDR3) situated on chromosome 7q21.1 in humans [41]. Transfection studies have demonstrated that MDR1 cDNA consistently transfers multidrug resistance to primarily drug sensitive cell lines [42,43]. Conversely, the human MDR2 gene does not confer drug resistance in transfection studies [44,45]. MDR gene homologues have been highly conserved through the phylogensis as demonstrated by the presence of MDR-like genes in marine sponges [46], in plants [47], in microorganisms [48,49], and in vertebrates [50,51]. The MDR gene products are subdivided into two different classes [52]. Class I consists of multidrug transporters such as the human MDR1 gene product, Pgp. Class 2 includes Pgp isoforms that are not engaged in drug transport such as the human MDR2 gene product which is engaged in the transport of phosphatidylcholine into the liver bile canaliculi and probably functions as a taurocholate-dependent lipid translocase [53-55].

Pgp is a phosphorylated and glycosylated protein 1280 amino acids long, which intersects the plasma membrane and consists of two homologous halves, each of which contains six hydrophobic domains and a hydrophilic nucleotide binding fold (Fig. 1) [52]. The hydrophobic regions represent putative transmembrane domains forming a pore-like structure [56]. The two adenosine triphosphate (ATP)-binding folds are located intracellularly and exhibits significant ATPase activity [57]. The drug binding sites are localized in the transmembrane domains close to the cytosolic surface [58], the drugs apparently beeing removed from within the membrane bilipid layer [59]. Activation may be regulated through phosphorylation by protein kinase C [60-62], and its efflux function may be affected by membrane lipid composition [63] and fluidity [64-66].

Pgp is naturally expressed in some normal tissues such as the apical surfaces of epithelial cells in the kidney, liver, gastrointestinal tract, the endothelial cells of the brain and testis, adrenal glands, bone marrow stem cells, and normal peripheral blood lymphocytes [67-69]. The
physiological function of Pgp is deduced from the tissue distribution indicating a role in renal and hepatic elimination of toxins and toxic metabolites, in protection from ingested exogenous toxins, in tissue protection at the blood-brain barrier and the blood-testis barrier, and in transport of certain cytokines [70,71]. A steroid-transporting role of Pgp has been suggested in the pregnant uterus [72], and the adrenal [73]. An active transport of cortisol, aldosterone and dexamethasone has been demonstrated in porcine cells transfected with human MDR1 cDNA isolated from the human adrenal gland [73]. Pgp situated on the luminal surface of intestinal mucosa may contribute to the elimination of certain drugs via feces and thus limit their bioavailability after oral administration [74]. A potential application of Pgp inhibitors would therefore be adjunctive treatment to improve the bioavailability of Pgp substrate drugs.

A cryptic feature of Pgp is the ability to recognize and transport a broad but well-defined spectrum of structurally unrelated anticancer drugs with dissimilar intracellular targets. These drugs are often termed MDR drugs. The binding of drugs to Pgp was first demonstrated using MDR drugs and later extended to include Pgp inhibitory agents. Structure-activity studies of tioxantens [75], and reserpin analogs [76] have described a general pharmacophore for Pgp inhibitors, which contains planar aromatic domains. Drugs that interact with Pgp are frequently large (MW > 400), hydrophobic or amphipatic molecules with a planar ring system and a basic nitrogen side chain [76]. They often carry a positive charge at physiological pH, but this is not true for colchicine and certain hydrophobic peptides indicating that these substrates engage alternative substrate binding sites [77-80]. To date there is no consensus on the number, nature and interrelationships of the drug and modulator binding sites because data derived from a series experimental studies addressing this issue have been ambiguous [81].

The spectrum of drugs that interact with Pgp includes natural product cytotoxics produced by fungi or plants (e.g. anthracyclines, vinca alkaloids, taxanes), peptide antibiotics (e.g. gramicidin D, valinomycin), steroid hormones (e.g. cortisol, aldosterone, dexamethasone), immunosuppressive agents (e.g. cyclosporin A, FK 506) and calcium channel blockers (e.g. verapamil, azidopine) [81]. The most important MDR drugs are listed together with endogenous Pgp substrates and Pgp inhibitory agents in (Table 1).

Table 1. Compounds which interact with Pgp.

<table>
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<tr>
<th>MDR Drugs</th>
<th>Pgp Inhibitory Agents</th>
<th>Endogenous Pgp Substrates</th>
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<tr>
<td>Daunorubicin</td>
<td>Verapamil</td>
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<td>Doxorubicin</td>
<td>Nifedipin</td>
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<td>Epirubicin</td>
<td>Azodipin</td>
<td>IL-2</td>
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<td>Mitoxantrone</td>
<td>Quinine</td>
<td>IL-4</td>
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Certain areas of substrate interaction within Pgp have been identified using photoaffinity analogs of various drugs that recognize Pgp, such as $^3$H-azidopine [82] and $^{125}$I-iodoaryl azidoprazosin [83]. These photoaffinity probes label two sites, one in the N-terminal half and the other in the C-terminal half of the protein. The former binding site have been mapped to the putative transmembrane domain (TM) 4-6 [58,83], and the latter to the TM 11-12 regions [83,84]. Correspondingly, mutational analyses have provided further evidence for the importance of TM 5-6 [85-87] and TM 11-12 [85,88,89] regions for substrate recognition and binding of Pgp. For example, mutations of phenylalanin to alanin in position 335 or 978 dramatically reduced the ability of Pgp to transport doxorubicin [85].

Since the substrates for Pgp are largely hydrophobic, access to the substrate binding site on the protein is likely to be from the lipid bilayer, rather than the aqueous phase [Fig. (2)]. Accordingly, transport and binding studies suggest that Pgp interacts with substrates in the inner leaflet of the lipid bilayer of the plasma membrane and acts by flipping substrates to the outer leaflet of the bilayer [81,90,91]. This could also explain the unusual broad specificity of Pgp as the main determinant of specificity would be the ability to intercalate into the lipid bilayer, for which the amphipathy of the substrate must be suitable. Thus, hydrophobicity or amphipathy within a certain range of the drug molecule is possibly crucial for drug-Pgp interaction.
DIAGNOSIS OF DRUG RESISTANCE INDUCED BY P-GLYCOPROTEIN

Expression of the MDR1 gene in cells or tissue samples may be determined by measurements of MDR1 mRNA [92-94], protein expression using monoclonal antibodies [95-98] and protein function [99].

Detection of MDR1 RNA

The high degree of base homology between the MDR1 and MDR2 genes requires highly specific mRNA assays to discern between the two genes [100]. MDR1 mRNA can be measured using nucleotide probes in Northern blot or slot blot analyses [101], reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ hybridization. The expression levels of MDR1 mRNA have been shown to correlate with the degree of drug resistance in MDR cell lines [102,103].

RT-PCR is a highly sensitive method for detection of small amounts of mRNA in cell samples based on the ability to sense and antisense DNA primers to MDR1 complementary DNA (cDNA) generated from mRNA by the enzyme reverse transcriptase (RT) [94,104]. A minute amount of cDNA can be amplified several thousand times by repeated cycles of heat-denaturation and subsequent annealing with the primers at a lower temperature. It is critical to use MDR1-specific primer sequences to avoid amplification of false positive gene sequences. RT-PCR assays are highly sensitive and provide a method for detection of MDR1 mRNA in samples with a low level of drug resistance and in small sample sizes. Because the assay utilizes bulk material; normal and neoplastic cells can not be separated.

Although MDR1 mRNA assays are sensitive, contamination of tumor samples by Pgp-expressing normal stroma cells represents a significant problem for the interpretation of the analysis results [105]. This is particularly true for the highly sensitive RT-PCR assays, and very few studies have shown a significant correlation between MDR1 mRNA by way of RT-PCR and response to chemotherapy [106-108]. One additional difficulty with these analyses is the instability of the single-stranded RNA due to readily degradation by ubiquitous RNases. The correspondence with functional studies have been reported to be inferior to protein detection due to relatively frequent appearance of false positives [109]. It is also important to keep in mind that mRNA assays does not take into account any posttranslational regulation of MDR1 expression and does not provide information regarding function of the gene product.

Detection of the MDR1 Gene Product

Pgp is an integral plasma membrane protein with extracellular glycosylation sites and intracellular ATP binding sites [52]. Antigenic epitopes are found on either side of the membrane. Numerous monoclonal antibodies (MAbs) have been raised against both internal (C219, C494, JSB-1) and external (Hyb-612, MRK16, MRK17, 265/F4, 4E3, UIC2) epitopes of Pgp [110]. We have demonstrated that anti-Pgp antibodies directed against surface epitopes were superior to those directed against cytoplasmic epitopes in determination of low and variable levels of Pgp [98]. Several of the antibodies used have well-defined cross-reactivity with proteins other than Pgp. Both JSB-1 [111] and C494 [112] cross-react with pyruvate carboxylase, whereas C219 recognises the MDR2 gene product [113], the heavy chain muscle myosin [114], and the c-erbB2 oncogene product, which is a factor of poor prognosis in breast cancer [115].

Several methods for protein determination are available. Western blot require extraction of cell membranes and denaturation of proteins which are size-fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with relevant antibodies. The denaturing and reducing conditions allows determination of the precise size of the target protein. On the other hand, the immunohistochemical detection of Pgp works at the single cell level which allows morphological characterization of Pgp expressing cells and segregation of tumor cells from stromal cells in a tumor sample. Flow cytometric immunofluorescence detection provides a quantitative method of Pgp expression analysis in suspended tumor cells, and allows identification of tumor subpopulations with diverse Pgp expression. Unlike the other methods, flow cytometry can be performed with viable cells by determination of external epitopes of Pgp.

In a multicenter comparative trial of Pgp determination in clinical specimens derived from patients with acute myelogenous leukemia (AML) using a panel of five antibodies, it was demonstrated that flow cytometry was clearly superior to immunocytochemistry in distinguishing specimens with low or intermediate levels of Pgp expression, and that the results obtained with MRK16 provided superior correlation with functional studies of Pgp [109].
Functional Assays

The function of Pgp as a transporter of known substrates may be utilized in functional assays of Pgp detection. The earliest assays were based on determination of transmembrane fluxes of radiolabeled anticancer drugs such as vincristine and doxorubicin [116-118]. Later, less laborious methods were developed using flow cytometry and fluorochromes as Pgp substrates. These assays were more suitable for routine testing of clinical samples, and the introduction of anthracyclines as fluorescent probes have provided close congruity with the clinical situation [119,120]. The anthracyclines are able to discriminate between drug resistant and sensitive cells because they are retained at low levels in cells enduring Pgp-mediated resistance. Certain vital dyes like rhodamine-123, fluor-3 and hydroethidine appear to have the same properties as fluorescent Pgp substrates, and have been widely used in functional assays of Pgp [121-123].

Flow cytometric methods based on daunorubicin fluorescence to measure drug accumulation in cancer cells correspond closely with 
$^3$H-daunorubicin and 
$^3$H-vincristine radioactive methods [99]. However, diversities in fluorescence can not always be ascribed to different levels of Pgp expression, because other transport mechanisms may contribute to the drug or dye efflux, and binding of the fluorochrome to intracellular structures such as DNA may reduce the fluorescence intensity. Combined incubation in the presence or absence of selective inhibitors of Pgp function provides a simple way of increasing the specificity of the functional Pgp assays. To discern between Pgp and MRP, rhodamine-123 appears to be a favourable probe because it is selective for Pgp [124], whereas the transport kinetics of anthracyclines by MRP and Pgp are very similar [30]. Dual parameter flow cytometry combining determination of Pgp generated immuno-fluorescence and a fluorescent probe provides the most specific functional assay which may demonstrate directly the inverse relationship between Pgp expression and intracellular drug level [98,121,125]. With this approach it is also possible to detect drug efflux in the absence of Pgp expression as an indicator of drug resistance involving other drug efflux mechanisms.

Choice of Diagnostic Procedure

The plurality of methods for diagnosis of Pgp expression reflects the imperfection of currently available diagnostic procedures. In particular, the lack of standardization has been a major concern, because conflicting definitions of assay end points, scarcity or diversity in control samples, incommensurable assay reagents, and variable methods of sample preparation, data acquisition, analysis, and quality control contribute to notable inconsistencies in Pgp detection. To address these problems the St. Jude MDR Workshop on Methods to Detect P-Glycoprotein-associated Multidrug Resistance in 1996 reached a set of consensus recommendations for standardization of Pgp assays [109]. This was a timely and appropriate initiative to improve the reliability of the most common Pgp assays in clinical samples (immunocytochemistry, flow cytometry, functional test, RT-PCR). The recommendations specified sample handling, assay procedures, data analyses, and quality control in detail, but no advice were given with respect to choice of diagnostic procedure. Whether the assay parameter should be MDR1 mRNA, MDR1 gene product, or Pgp substrate transport ability remains unresolved. However, multiparameter assays provide an adequate compromise which should be considered whenever possible. Dual parameter flow cytometry is a less cumbersome and still attractive approach due to the combination of protein detection and functional test in one single assay.

CIRCUMVENTION OF DRUG RESISTANCE INDUCED BY P-GLYCOPROTEIN

Attempts to overcome Pgp-mediated MDR can be made from different angels. First, one could possibly avoid the problem by using cytotoxic drugs that are not substrates of Pgp and thus retain activity in cells with high Pgp expression levels, or develop non-cross-resistant analogs of MDR drugs. Second, the use of drug sensitizers that interfere with the drug efflux driven by Pgp may restore drug sensitivity in MDR cells. Third, the MDR phenotype associated with high expression levels of Pgp is apparently susceptible to interruption of the Pgp function per se, and thus separate use of Pgp inhibitors may add a novel treatment strategy to the already established approaches to overcome Pgp-mediated MDR.

Modified MDR Drugs

Unfortunately, MDR drugs are not easily substituted by non-MDR drugs such as antimitabolites, alkylating agents and platinum compounds, though these are highly efficient in selected malignancies. Both anthracyclines and
taxanes are currently irreplaceable in a range of chemotherapy regimens because of their unique antineoplastic activity. Therefore, it would be attractive to chemically modify MDR drugs in order to delete their affinity for Pgp. In anthracyclines the 9-alkyl substitution of the anthracene A ring and certain sugar modifications have been associated with reduced affinity for Pgp and maintenance of cytotoxic activity in certain MDR tumor cell lines [126,127]. Accordingly, the 9-alkyl trisaccharide aclarubicin has structural features different from those of typical Pgp substrates such as the classic anthracyclines epirubicin, doxorubicin and daunorubicin (Fig. 3). In a comparative experimental study we showed that aclarubicin accumulates freely in MDR cells, which is reflected in retained cytotoxic activity and deficient response to the Pgp inhibitor PSC 833 with respect to drug accumulation, drug efflux and drug cytotoxicity [128]. Morpholinyl substitution of the sugar moiety is also associated with circumvention of Pgp [127,129] although it appears that morpholinyl anthracyclines are not entirely unresponsive to Pgp inhibition [130,131].

The ability of anthracyclines to circumvent MDR is shown to improve with increasing lipophilicity [132]. Thus, the highly lipophilic anthracyclines annamycin and idarubicin should be attractive substitutes for conventional anthracyclines in MDR cancers. In fact, it has been reported that annamycin is totally unaffected by Pgp expression in cultured cells, and not susceptible to manipulation by the Pgp inhibitor verapamil [133,134]. Furthermore, idarubicin has been shown to preserve substantial cytotoxicity in various selected MDR cell lines [135], in cells transfected with the MDR1 gene [136], and in Pgp-positive blasts retrieved from leukemia patients treated with the drug [137]. Comparisons of anthracycline intercalation into
DNA of Pgp-positive and Pgp-negative cells by fluorescence resonance energy transfer (FRET) analysis have revealed that intercalation of idarubicin, in contrast to daunorubicin, is unaffected by Pgp [138]. However, other studies have shown that idarubicin efflux by Pgp occurs at similar rates as daunorubicin [11,139], and that the favourable effect of idarubicin in MDR cells is rather due to an enhanced influx rate compared to the classic anthracyclines [140]. Consequently, idarubicin cytotoxicity may possibly be further enhanced in the MDR phenotype by modulators such as PSC 833 [141].

It should be kept in mind that the activity of a drug depends largely upon its intracellular concentration which is basically determined by the kinetics of influx and efflux of the drug across the cell membrane. Rapid uptake by cells characterizes the highly lipophilic anthracyclines idarubicin and annamycin, and suggests that the influx rate may be the more important contributor to the intracellular concentration. This would mean that the activity of Pgp is overridden by the influx mechanism governed by drug lipophilicity, and thus explain the apparent circumvention of MDR by these drugs. Correspondingly, Marbeuf-Gueye and co-workers have recently demonstrated that the relative drug resistance poorly correlates with drug efflux kinetics of both Pgp- and MRP-expressing cells [142]. Therefore, both modulation of the parameters that will lead to an increase of the uptake kinetics and those which could lead to a lowering of the efflux kinetics by Pgp can contribute to MDR circumvention in cancer cells.

**Chemosensitization by Pgp Inhibitors**

Pgp has a broad specificity for substrates, and several non-cytotoxic drugs may competitively inhibit efflux of cytotoxic drugs by Pgp and thereby downmodulate MDR [143]. Several classes of modulators have been identified among drugs that were originally developed for other therapeutic indications, including calcium channel blockers, calmodulin antagonists, steroid hormonal agents and immunosuppressive agents [144]. The calcium channel blocker verapamil was the first agent that was shown to modify MDR in vivo and in vitro [145], but unfortunately the MDR modulating activity required concentrations that are associated with severe cardiac toxicity in patients [146,147]. The stereospecificity of some of these drugs may affect their toxic potential. Thus, dexverapamil, the R-enantiomer of verapamil, is one such derivative that has retained the drug-sensitizing activity of verapamil but is markedly less cardiotoxic [148,149]. Despite improved therapeutic index, cardiovascular side-effects still remain dose-limiting for dexverapamil and prohibit its use in many patients [150]. The immunosuppressive agent cyclosporin A (CsA) has been shown to be a highly potent inhibitor of Pgp both in cell lines [151,152] and in animal models [153,154]. Although CsA inhibits Pgp at clinically tolerable concentrations in these experiments, the immunosuppression restricts its utility in clinical oncology.

In the recent years a new generation of modulators with substantially improved potency for inhibition of Pgp in vitro has evolved. These agents are highly specific and effective at low nM concentrations. The toxic potential is regarded as generally low in these agents. They are usually a product of specific drug discovery programs and include the non-immunosuppressive cyclosporin PSC 833 [155,156], the cyclopeptolide 280-446 [157], and the cyclopropyldibenzosuberane LY 335979 [158].

The clinical experience with Pgp modulators have so far been limited. Early clinical modulation trials have mostly included calcium channel blockers and cyclosporins [159]. The tolerability of combined treatment with chemotherapeutic agents and modulators have been evaluated in phase I and II trials. These trials have shown that the early modulators frequently exercise unacceptable toxicity at plasma concentrations equal to the dose level required to circumvent MDR in vitro [159]. Furthermore, these trials have demonstrated certain pharmacokinetic interactions with anticancer drugs. The modulators tend to increase the serum half-life and thus the AUC of cytotoxic agents such as etoposide, doxorubicin, and paclitaxel, presumably by inhibiting their renal and hepatic elimination [160-163]. The highly potent modulator PSC 833 may require 30-60% dose reductions of the anticancer drugs to maintain AUC unchanged, and thus avoid excessive toxicity [164]. These dose reductions will not compromise the therapeutic efficacy as long as an equivalent AUC is maintained. On the contrary, the modulator may affect the tissue distribution and selectively enhance the antitumor efficacy against Pgp expressing cancer cells. Interestingly, Pgp modulation does not increase toxicities to normal tissues with constitutive Pgp expression presumably due to involvement of alternative resistance mechanisms in these tissues and possibly a low proliferation rate compared to cancer cells [164].

To date, only five randomized phase III studies of Pgp modulators have been published. No
beneficial effect was observed from the addition of oral verapamil to the combination chemotherapy employing vincristine, doxorubicin and dexamethasone for the treatment of drug-resistant myeloma patients [165]. The addition of megestrol acetate to standard first-line cytotoxic therapy including cyclophosphamide, doxorubicin, vincristine, etoposide and cisplatin in small-cell lung carcinoma did not improve treatment outcome [166], nor did the addition of quinine to mitoxantrone and cytarabine combination chemotherapy of poor-risk acute myeloid leukemias [167]. Two recent studies investigated the effect of CsA as adjunct to combination chemotherapy including arabinosyl cytosine (Ara-C) and daunorubicin in patients with relapsed or refractory myelogenous leukemia. One of the studies showed that there was no therapeutic gain from low doses of CsA (5-10 mg/kg/day) [168]. However, the other study which used higher doses of CsA (16 mg/kg/day), demonstrated for the first time a beneficial effect of a modulator in a randomized clinical trial [169]. In this study by the Southwest Oncology Group (SWOG) CsA treatment improved the remission duration and survival by statistical significance. Apparently, the positive effect of CsA was unrelated to the pharmacokinetic interaction with daunorubicin ensuing impaired elimination of the cytotoxic drug. Thus, the study indicates that Pgp modulation is feasible in clinical practice, and provides promising perspectives for further clinical investigation using more potent and less toxic modulators. The highly potent and efficacious modulator PSC 833 is currently undergoing phase III clinical trials in acute myeloid leukemias and multiple myeloma [164], and the results from these studies are awaited with considerabel interest.

Inhibition of P-glycoprotein May Trigger Apoptosis

The classical approach to circumvent Pgp-mediated MDR by using modulators as chemosensitizers is apparently not the only way to assault chemoresistant cancer. An alternative approach has recently emerged since modulators separately inhibit cell growth and induce apoptosis in leukemia, epidermoid carcinoma, and breast carcinoma cell lines [21,170-173]. Moreover, we recently demonstrated the ability of PSC 833, 280-446 and LY335979 to provoke cytokinesis failure and G2/M cell cycle arrest in leukemia cells with elevated expression levels of Pgp [21]. The exact mechanism for this activity is presently unknown. We have hypothesized that interruption of the ability of Pgp to act as an efflux pump of endogenous substrates conferring survival signals may play a role, but separate mechanisms detached from the efflux activity may also be involved.

Chaudary and Roninson have proposed that Pgp in hematopoietic stem cells may participate in the export of a growth regulatory molecule [174]. Interruption of a possible autocrine pathway supporting cell growth by endogenously produced and exogenously secreted cytokines may thus inhibit cell proliferation. Interleukin-2 (IL-2) shears the features of a growth regulatory molecule [175,176] and a substrate of Pgp-mediated transport [70,71], but no study has so far shown that IL-2 is actually involved in execution of apoptosis by way of Pgp inhibition. On the other hand, List and colleges recently showed that inhibition by PSC 833 of the ABC1 transporter interrupts the efflux of IL-1β form leukemia cells and causes apoptosis which is avoided by exogenous substitution of IL-1β [171]. It is also known that both IL-3 and IL-6 may act as endogenous growth factors in autonomously growing leukemic cell lines [177,178], and endogenously produced GM-CSF may promote growth of malignant hematopoietic cells by secretion and binding to surface GM-CSF receptors [179]. Therefore, there are several candidates for a growth regulatory molecule working in an autocrine loop maintained by Pgp, but at present no study has provided proof of such a mechanism or identified the effector molecule.

An alternative explanation for the antiproliferative effect of Pgp inhibitors has evolved from recent experiments that have disclosed increased intracellular content of ceramide in MDR cancer cells exposed to the Pgp inhibitors CsA, PSC 833 and tamoxifen [172,173]. Ceramide is a potentially toxic sphingolipid which is involved in signaling events that result in G0/G1 cell cycle arrest and apoptosis [180]. The metabolism of ceramide is quite important for cellular homeostasis because the membrane composition of sphingolipids plays a role in regulation of cell growth, differentiation, senescence and apoptosis [181]. For example, ceramide glycosylation opposes the intracellular accumulation of ceramide, and the glycosylation product, glucosylceramide, lacks the ability of ceramide to promote execution of the cell death program. The net effect of ceramide glycosylation is thus avoidance of apoptosis.

The formation of ceramide can be enhanced by chemotherapeutic agents, which may contribute to the cytotoxic activity of these agents and possibly explain their ability to induce apoptosis [182,183]
Interestingly, the capacity to glucosylate ceramide is enhanced in MDR cancer cells resulting in elevated levels of glucosylceramide and corresponding decline in the level of ceramide [184]. The intracellular availability of ceramide in MDR cancer cells may be additionally reduced by defective generation of ceramide in response to chemotherapy [185]. Thus, deficient intracellular recruitment of ceramide may contribute to the MDR phenotype.

The effect of Pgp-inhibitors on sphingolipid membrane composition may not necessarily involve Pgp, because the apparently Pgp-deficient MCF-7 breast carcinoma cell line also augments ceramide formation in response to PSC 833 exposure [186]. On the other hand, the applied doses were approximately 10-fold higher than those which were required to induce apoptosis in the MDR phenotype of leukemia cell lines [21]. Furthermore, van Helvoort and coworkers have provided evidence that Pgp may translocate sphingolipids across the plasma membrane to the outer surface of MDR cells [187]. Thus it is possible that toxic sphingolipids are excessively removed from MDR cancer cells by Pgp-mediated transport, and that Pgp-inhibitors enhance the intracellular availability of ceramide and thus promote the apoptotic process. However, no firm conclusions can yet be drawn with respect to the role of ceramide in apoptosis, because it is not entirely clear whether drug-mediated generation of ceramide is essential for execution of the cell death program or simply represents a non-specific stress response.

FUTURE PERSPECTIVES

Multidrug resistance is a complex issue with profound impact on treatment outcome in cancer patients. Pgp is one out of many resistance mechanisms at work, but represents undoubtedly the best studied target for MDR circumvention at present. Although a series of clinical studies with modulators of Pgp are currently being performed, some controversy still exist with respect to diagnostic measurements of Pgp expression. A therapeutic success of the modulators is likely to rely on the expression level of Pgp in targeted tumours. Therefore, more studies on development of reliable diagnosis of both Pgp and other resistance factors are warranted. High quality diagnostic procedures will not only unveil the clinical significance of the different mechanisms of chemoresistance, but also provide guidance to clinical trials addressing circumvention of chemoresistance with novel therapeutic approaches.

Besides being a target for drug sensitization, Pgp appears to be a target for execution of apoptosis in certain cancer cells with elevated Pgp expression levels. The Pgp inhibitors may thus provide a mode for selective cell kill, which is an attractive feature suggesting a potential for therapeutic exploitation. Further studies are needed to identify responsive phenotypes and to better understand the mechanisms behind the cytotoxic effect. Most important, however, is the question whether the selective cytotoxicity of Pgp inhibitors may translate into clinical practice. The first step to answer this intriguing question has already been taken in our laboratory as we have initiated experiments with PSC 833 monotherapy in KG1a/200 leukemia xenotransplanted NOD-SCID mice (non-obese mice with severe combined immunodeficiency) for response evaluation in vivo.

REFERENCES


P-Glycoprotein as a Drug Target in the Treatment


