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Review

From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance

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Abstract. The ATP binding cassette (ABC) superfamily of membrane transporters is one of the largest protein classes known, and counts numerous proteins involved in the trafficking of biological molecules across cell membranes. The first known human ABC transporter was P-glycoprotein (P-gp), which confers multidrug resistance (MDR) to anticancer drugs. In recent years, we have obtained an increased understanding of the mechanism of action of P-gp as its ATPase activity, substrate specificity and pharmacokinetic interactions have been investigated. This review focuses on the functional characterization of P-gp, as well as other ABC transporters involved in MDR: the family of multidrug-resistance-associated proteins (MRP1-7), and the recently discovered ABC half-transporter MXR (also known as BCRP, ABCP and ABCG2). We describe recent progress in the analysis of protein structure-function relationships, and consider the conceptual problem of defining and identifying substrates and inhibitors of MDR. An in-depth discussion follows of how coupling of nucleotide hydrolysis to substrate transport takes place, and we propose a scheme for the mechanism of P-gp function. Finally, the clinical correlations, both for reversal of MDR in cancer and for drug delivery, are discussed.

Key words. Multidrug resistance; P-glycoprotein (MDR1); multidrug-resistance-associated protein (MRP); mitoxantrone resistance protein (MXR, BCRP, ABCP, ABCG2); ABC transporter; ATPase.

Introduction

Over 40 years ago it was recognized that certain proteins were able to transport substances across cellular membranes and against a concentration gradient (reviewed in [1]). It was early realized that such processes are characterized by the necessity for an input of energy, which requires the hydrolysis of ATP, directly or indirectly. Many actively transporting systems have been described [2]. A large subclass of these proteins subsequently became known as ABC proteins [3], named for their distinctive domains that bind ATP. These sequences, known as Walker A and Walker B, reside in the nucleotide binding domain (NBD), along with the ABC signature sequence, and are highly conserved across many species [4].

The phenomenon of MDR was originally described by Keld Danø as the active outward transport of anthracyclines and vinca alkaloids from murine Ehrlich ascites tumor cells [5, 6]. This transport could be blocked, and multidrug resistance reversed, by the addition of substrate analogs, the so-called chemosensitizers or modulators

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[7]. This phenotype was generalized and characterized in detail in separate studies by Biedler [8], Beck [9] and Ling [10]. They defined the phenomenon as resistance which arose from selection of Chinese hamster ovary cells in colchicine or actinomycin D, and human leukemia cells in vinblastine, yet rendered the cells resistant to a series of structurally unrelated compounds. The phenotype was linked to overexpression of a 170-kDa membrane glycoprotein, which was named P-glycoprotein (P-gp, P for permeability) [11]. This was the first known human ABC protein, shown to be a large protein found in the cell membrane, and capable of transporting drugs and other xenobiotic compounds out of the cell. Subsequently, *MDR-1*, the gene encoding P-gp, was cloned by Roninson and others in 1986 [12, 13]. The Tsuruo laboratory demonstrated that verapamil [14], trifluoperazin or zinc [15], other calcium channel blockers [16] and quinidine [17] could act as chemosensitizers, inhibiting MDR conferred by P-gp. Thus, the clinically important field of MDR was launched.

Today, a large body of literature now exists regarding substrate and antagonist specificity for P-gp. The regulation of P-gp, its normal tissue expression, and the incidence and frequency of expression in cancer are all problems that have been addressed. Further, the protein has been purified and reconstituted in lipid bilayers in order to answer specific questions about structure and function. This review will briefly summarize several areas of recent understanding and debate relating to P-gp structure and function, and then will review the evidence for its importance in clinical oncology. From that point, the evidence for the involvement of other transporters in the MDR phenotype will be reviewed.

The MDR Protein (P-gp)

P-gp as an ATPase

In order to fully appreciate how P-gp functions, an understanding of how P-gp couples ATP hydrolysis to drug transport is mandatory. It is widely believed that the coupling of these processes involves a transient conformational change in the protein that facilitates an interaction between the drug-binding domain and nucleotide-binding sites of P-gp. The underlying mechanism of this putative interaction has been the topic of intense scrutiny.

The connection between drug binding and ATP hydrolysis is evident when measuring the ATPase activity of P-gp in the presence of a variety of P-gp transport substrates and chemosensitizers. In his early studies using multidrug-resistant cells, Danø demonstrated that ATP was required to maintain a low intracellular daunorubicin concentration [6]. Many studies have characterized the stimulation of P-gp ATPase activity above basal levels (which are intrinsic to the enzyme, perhaps linked to the transport of a so-called 'endogenous substrate', which may be a membrane lipid) in the presence of transport substrates. These studies have used membranes derived from MDR1-expressing Sf9 cells [18], and from Ehrlich ascites tumor cells [19] and CHO cells [20], and also membrane vesicles containing reconstituted P-gp [21, 22]. The effects of drugs that interact with P-gp fall into three main categories: (i) compounds that stimulate ATPase activity at low concentrations but inhibit activity at high concentrations (such as vinblastine and verapamil), (ii) drugs that stimulate ATPase activity without inhibition (such as valinomycin) and (iii) agents that inhibit both the basal and drug-stimulated ATPase activity (such as cyclosporin A and gramicidin D) [23]. It should be noted that MDR blockers are not found specifically within one category. For example, verapamil consistently stimulates ATPase activity to a high degree [24], whereas cyclosporin A blocks all P-gp ATPase activity [25].

Conformational changes in P-gp

A large body of evidence indicates that conformational changes in the structure of P-gp are involved in the mechanism of substrate efflux. Several methods have been used to study the putative conformational changes of P-gp. Loo and Clarke showed that movement between transmembrane (TM)6 and TM12 is essential for the drug-stimulated ATPase activity of P-gp [26]. Subsequently, in several comprehensive studies, they applied cysteine-scanning mutagenesis of all the TM segments of P-gp to identify specific residues within the drug-binding domain, which they concluded consists of TMs 4, 5, 6, 10, 11 and 12 [27–29].

Liu and Sharom labeled cysteine residues located within the Walker A motifs with 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS). When located within an aqueous environment, MIANS is intrinsically fluorescent, and this fluorescence is quenched when MIANS is located within a lipophilic environment. These investigators studied the quenching of MIANS fluorescence in the presence of various P-gp ligands as an indication of P-gp conformational changes. Their findings implied that in the presence of these compounds, the NBD had changed position relative to the cell membrane [30].

Sonveaux and others studied the effect of the water-soluble quenching agent acrylamide on the quenching of the intrinsic fluorescence of P-gp tryptophan residues [31;32]. The acrylamide is introduced in vitro at the cytoplasmic face of P-gp that is embedded in membrane vesicles derived from the plasma membrane. The acrylamide is most effective in quenching tryptophan fluorescence when in the presence of different nucleotides present at the cytoplasmic face. This suggests that these ligands were inducing a change in the conformation of

P-gp in which the average location of tryptophan residues had been shifted from a hydrophobic environment to a hydrophilic environment. In contrast, the presence of anthracycline substrates inhibited the ability of acrylamide to quench tryptophan fluorescence, indicating that the same residues were then not located within a hydrophilic environment [32].

Another method, used by Wang and others, compares the profiles of P-gp fragments obtained by limited trypsin proteolysis, and subsequent Western blot analysis, in the absence or presence of a variety of nucleotides. This study found that the proteolytic profile of P-gp was altered in the presence of different nucleotides [33]. This suggested that the regions of P-gp outside of the lipid bilayer, which are sensitive to trypsin digestion, are differently conformed in the presence of nucleotides, and therefore the tertiary structure of P-gp has been altered by the presence of these nucleotides. The isolation of various proteolytic profiles suggested that the protein exists in up to four different conformational states [33, 34].

Finally, FACS (fluorescence-activated cell sorter) analysis has been employed to study changes in the reactivity of P-gp-expressing cells towards the monoclonal antibody UIC2 in the presence of various P-gp ligands. UIC2 recognizes a combinatorial, extracellular epitope of P-gp [35]. The combinatorial nature of the UIC2 epitope was determined when UIC2, similar to another P-gp-specific antibody, MRK16 [36], failed to react with denatured P-gp on a Western blot.

Mechetner and others observed that when incubated at 37 °C in the presence of various P-gp transport substrates, chemosensitizers and ATP-depleting agents, the reactivity of P-gp-expressing cells to UIC2 was markedly increased [37]. In contrast, the inherently high level of reactivity to MRK16 is unaffected by the presence of these compounds [37]. These increases in UIC2 reactivity did not occur at 4 °C, and therefore led to the hypothesis that during P-gp function, the protein was undergoing a conformational change in the presence of its transport substrates, chemosensitizers or ATP-depleting agents. This conformational change apparently exposed a paratope of UIC2 that was not accessible to the antibody in their absence [37]. Taken together with the strong inhibitory effect of UIC2 on P-gp function, the epitope for UIC2 apparently derives from a transient conformation of the protein that is involved in protein function. While the precise epitope for UIC2 has not been determined, it has been demonstrated that UIC2 reactivity to P-gp is dependent upon residues located within the first [38] and third [39] extracellular loops of P-gp. In comparison, the epitope for MRK16, which does not demonstrate altered reactivity in the presence of P-gp substrates or ATP-depleting agents [37], has been mapped to residues located within the first and fourth extracellular loops of P-gp [40].

Substrates and inhibitors of P-gp

One of the salient features of P-gp is its broad substrate recognition pattern. Over the past decade the substrate list expanded from the original description of P-gp as conferring resistance to the vinca alkaloids and anthracyclines, to the current very large list of compounds which includes structurally unrelated anticancer agents, anti-human immunodeficiency virus (HIV) agents and flurophores (table 1).

Table 1. P-glycoprotein substrates (A) and drugs which act as chemosensitizers (B).

Drug	Ref.
A. Substrates of P-glycoprotein	
Anthracyclines	[41, 42]
Daunorubicin	
Doxorubicin	[43]
Epirubicin	
Anthracenes	
Bisantrene	[44]
Mitoxantrone	[45]
Vinca alkaloids	
Vinblastine	[46]
Vincristine	[47]
Vinorelbine	
Vindesine	
Camptothecin derivates	[48]
CPT-11	
Topotecan	
Epipodophyllotoxins	[49]
VP-16 (Etoposide)	
VM-26 (Teniposide)	
Tubulin polymerizing drugs	
Colchicine	[50]
Paclitaxel	[51]
Docetaxel	
Chromopeptide antibiotic	
Actinomycin D	[52]
HIV-1 protease inhibitors	[53]
Ritonavir	
Saquinavir	
Indinavir	
Fluorophores	
Calcein-AM	[54]
Fluo-3 AM	[55]
Fura-2 AM	[56]
Rhodamine 123	[57]
Hoechst 33342/33258	[58]
B. Drugs which act as P-glycoprotein antagonists	
Calcium channel blockers	
Verapamil	[14]
Diltiazem	[16]
Calmodulin antagonists	
Trifluoperazine	[15]
Chlorpromazine	[59]
Triflupromazine	[60]
Fluphenazine	[61]
Pimozide	[62]

Table 1 (continued)

Drug	Ref.
Antiarrythmics	
Quinidine	[17]
Amiodarone	[63]
Propafenone	[64]
Antihypertensives	
Reserpine	[65]
Propranolol	[66]
Hormones	
Progesterone	[67]
Spironolactone	[68]
Tamoxifen	[69]
Hydrophobic peptides	
Cyclosporin A	[70]
SDZ PSC 833	[71]
Valinomycin	[72]
Gramicidin D	[73]
Antihistaminic drugs	
Promethazine	[74]
Terfenadine	[75]
Antimalarial drugs	
Mefloquine	[76]
Quinine	[77]
Other drugs	
Amitriptyline (antidepressant)	[78]
Dipyridamole (anticoagulant)	[79]
Fucidin (antibiotic)	[80]
GF120918 (synthetic)	[81]
N-acetyl daunorubicin (anthracycline analog)	[82]
Staurosporin (protein kinase C antagonist)	[83]

Experiments with the NCI drug screen suggested that the list of substrates could be extended by several hundreds [84–86]. In an effort to understand this broad pattern of substrate recognition, investigators have analyzed the protein for specific binding sites, and have searched for mutations and polymorphisms capable of altering drug affinity. Taken together, the results suggest that the binding sites are broad regions of recognition since mutations in almost any transmembrane region will affect the function of P-gp. However, it may also be argued that rather than acting directly through a substrate-binding site, mutations to the transmembrane domains could affect P-gp function by perturbation of the conformational change that occurs during the transport cycle.

Currently, evidence suggests that a variety of factors come together to determine if and how P-gp will interact and/or transport a given substrate. We will discuss some of these various approaches.

We mentioned above that the intrinsic fluorescence of MIANS-labeled P-gp was quenched by the presence of ATP, vinblastine, verapamil, colchicine and a variety of other P-gp substrates or chemosensitizers [30]. This quenching of fluorescence by ATP and drugs is independent and additive, indicating that each compound induces a defined change in the orientation of the protein relative to the lipid bilayer. In an extensive study of structure-activity relationships for substrates of P-gp in CHO cells [87], Litman and others found that the affinity of drugs for P-gp correlated well with their van der Waal's surface area. This suggests that binding between drug and P-glp takes place across a wide interaction surface on the protein. Studies of the competitive, noncompetitive and cooperative interactions between pairs of drug molecules [88] confirmed this picture in that, in some cases, drug molecules in pairs could be seen to act additively, as if they bound together at the wide interaction surface on P-gp.

Findings consistent with such hypotheses were reported in other studies demonstrating that P-gp possesses multiple drug-binding sites. Shapiro and Ling used reconstituted liposomes containing a purified hamster P-gp preparation from Chinese hamster ovary cells. Their study demonstrated that rhodamine 123 and Hoechst 33342 each stimulated the P-gp-mediated transport of the other compound [58], suggesting that each molecule bound to a separate and distinct binding site within P-gp. The binding of several P-gp substrates could be classified into four categories: (i) those that bound to the Hoechst-binding site (such as colchicine), (ii) those that bound to the rhodamine-binding site (such as daunorubicin), (iii) those that bound to both sites (such as vinblastine) and (iv) those that appeared to bind to neither site (such as progesterone) [89]. The apparent lack of binding to either site by progesterone and prazosin suggested that a third drug-binding site existed within P-gp [90]. Findings consistent with the two-site hypothesis were obtained by Dey and others when measuring the radiolabeling of the P-gp substrate, ^{125}I-iodoarylazidoprazosin ([^{125}I]IAAP), in the presence of the P-gp chemosensitizer *cis*-(Z)-flupentixol [91]. This study showed that the affinity of [^{125}I]IAAP was preferentially increased for the C-terminal half, but not the N-terminal half, of P-gp in the presence of *cis*(Z)-flupentixol, suggesting that at least two nonidentical drug interaction sites existed within P-gp. There is now good evidence for a minimum of four drug binding sites on P-gp; these comprise both transport and regulatory sites which are able to switch between high- and low-affinity conformations, thus allowing complex allosteric interactions [92].

Mutational analysis of P-gp has been extensively employed to determine the effects of mutations on drug binding and/or transport. Many mutations have been reported that alter affinity and/or transport of drugs by P-gp. These have been reviewed elsewhere [23, 93]. Several of these mutations have been localized to the two transmembrane domains of P-gp, further strengthening the hypothesis that P-gp interacts with its substrates from within the lipid bilayer [23]. However, the location of mutations affecting drug binding is not limited to a specific region or transmembrane segment. These muta-

tions have been found along the entire length of P-gp. In general, mutations in TM6 and TM12 seem to have the greatest impact on drug affinity and transport. Indeed, mutations within either or both of these segments eliminates the ability of P-gp to be labeled by IAAP [94] or azidopine [95]. Further, Loo and Clarke suggested that TM6 and TM12 may physically connect to their respective NBD and provide a direct link between drug transport and ATP hydrolysis [26]. Thus, the presence of P-gp transport substrates or ATP, induced differential cross-linking between cysteines introduced into TM6 and cysteines within TM12, suggesting that the conformation of P-gp is related to ligand and/or substrate binding [26].

UIC2 reactivity analysis (discussed earlier) can also be used to quantitatively characterize the interactions between P-gp, its substrates and compounds that modulate such interactions. An example of such analysis is shown in figure 1 A, which demonstrates that the low-to-high UIC2 reactivity transition in the presence of increasing concentrations of vinblastine occurs along a conventional ligand-binding curve. The apparent affinity of vinblastine, or K_m, determined for wild-type P-gp was equal to 0.79 µM (fig. 1 A). This matches the value of 0.77 µM reported for the effect of vinblastine on quenching the fluorescence of the probe MIANS bound near the nucleotide binding domains of P-gp [30]. The K_m value for the UIC2 reactivity transition is also very similar to the K_m of 0.87 µM shown in figure 1 B for the effect of vinblastine on competing with the ability of P-gp to efflux daunomycin [96–98]. In both panels, the Hill number for vinblastine, as determined using either UIC2 reactivity analysis or inhibition of daunomycin transport, suggests that two vinblastine molecules bind to P-gp [96, 99].

In contrast to vinblastine, colchicine (along with puromycin and etoposide) was previously shown to be unable to increase UIC2 reactivity in K562/i-S9 cells [37]. Similarly, colchicine was reported to induce no change in the proteolytic profile of P-gp [34]. By examining a variety of P-gp-expressing cell lines, it has been shown that colchicine increases the UIC2 reactivity in some but not all cell lines [100]. In those lines where colchicine alone did not affect UIC2 reactivity, this drug was able to reverse the vinblastine-induced increase in UIC2 reactivity. This result is consistent with the ability of colchicine to inhibit vinblastine-induced changes in the proteolytic profile of P-gp [34] and with the finding that high concentrations of colchicine inhibit vinblastine transport by P-gp [46].

In those cell lines where colchicine induces an increase in UIC2 reactivity, the increase, like vinblastine, follows a conventional ligand-binding curve. Unlike vinblastine, the Hill number for colchicine was approximately 1, a value that suggests but does not prove that only one molecule of colchicine is required to be bound during the rate-limiting step that alters UIC2 reactivity [100]. It is also similar to the Hill number of 1.1 reported for the ability

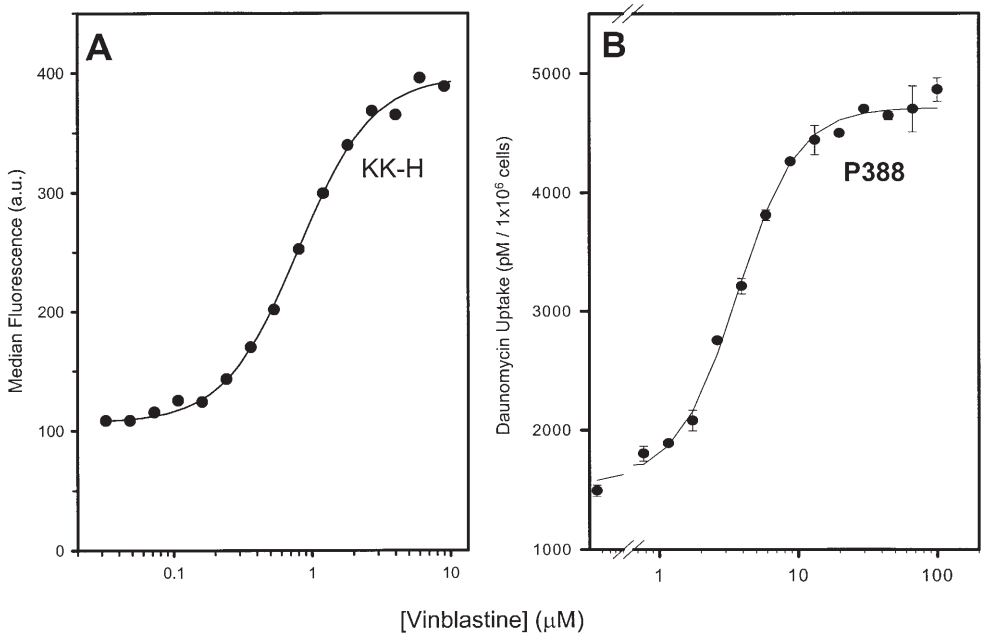

Figure 1. Increasing concentrations of vinblastine increase UIC2 reactivity (panel *A*) and daunomycin uptake (panel *B*). In panel *A*, murine fibroblasts expressing human *MDR*1 (KK-H) were incubated at 37 °C in the presence of the indicated concentration of vinblastine for 15 min prior to incubation with UIC2 for 30 min at 37 °C. In panel *B*, increasing concentrations of vinblastine competed with the efflux of daunomycin in human lymphoma cells transfected with human *MDR*1, leading to an increase in the amount of intracellular daunomycin as measured radioactively.

of vinblastine to inhibit the efflux of radiolabeled colchicine from vesicles [101]. This finding is consistent with other work reporting that colchicine has a single binding site on P-gp, as determined by the differential effects of colchicine on the efflux of rhodamine 123 or Hoechst 33342 [58]. Another elegant and classic study by Raviv and others [102] employed the use of INA, a photolabile lipophilic membrane probe, which was shown to specifically label P-gp after photoactivation by daunorubicin. This study introduced the concept of P-gp working as a 'hydrophobic vacuum cleaner' of the membrane.

One can compare the ability of P-gp expressed in different cell types to provide resistance to either vinblastine or colchicine, which are relatively good and poor P-gp substrates, respectively. By correlating this resistance with the ability of either drug to induce conformational transitions of P-gp, detectable by altered UIC2 reactivity, an interesting distinction between the way P-gp handles these substrates is observed [100]. Previous studies indicated that relative resistance to different P-gp-transported drugs in the same cellular background depends on the specific P-gp isoforms [103] and on the presence of mutations that alter the substrate specificity of P-gp [104]. It was also shown previously that resistance to vinblastine, one of the best transport substrates of P-gp, correlated with the cell-surface density of P-gp in different multidrug-resistant derivatives of the same cell line. This suggests that the P-gp expression level was the principal determinant of vinblastine resistance in such cells [105]. As shown in figure 2 A, this determination was confirmed when the analysis was expanded to include a variety of tissue types.

In contrast, figure 2 B demonstrates that the relative levels of colchicine resistance varied widely and do not correlate with the P-gp density in different cell types. One possible interpretation of this variability was that some cell-specific mechanisms of colchicine resistance, unrelated to P-gp, determine the final level of colchicine resistance in P-gp-expressing cells. Alternatively, such cell-specific factors could still act through P-gp by influencing its interactions with colchicine. Analysis of the effects of colchicine on the UIC2 reactivity shift in different cell lines supports the second interpretation. As shown in figure 2 C, the levels of colchicine resistance

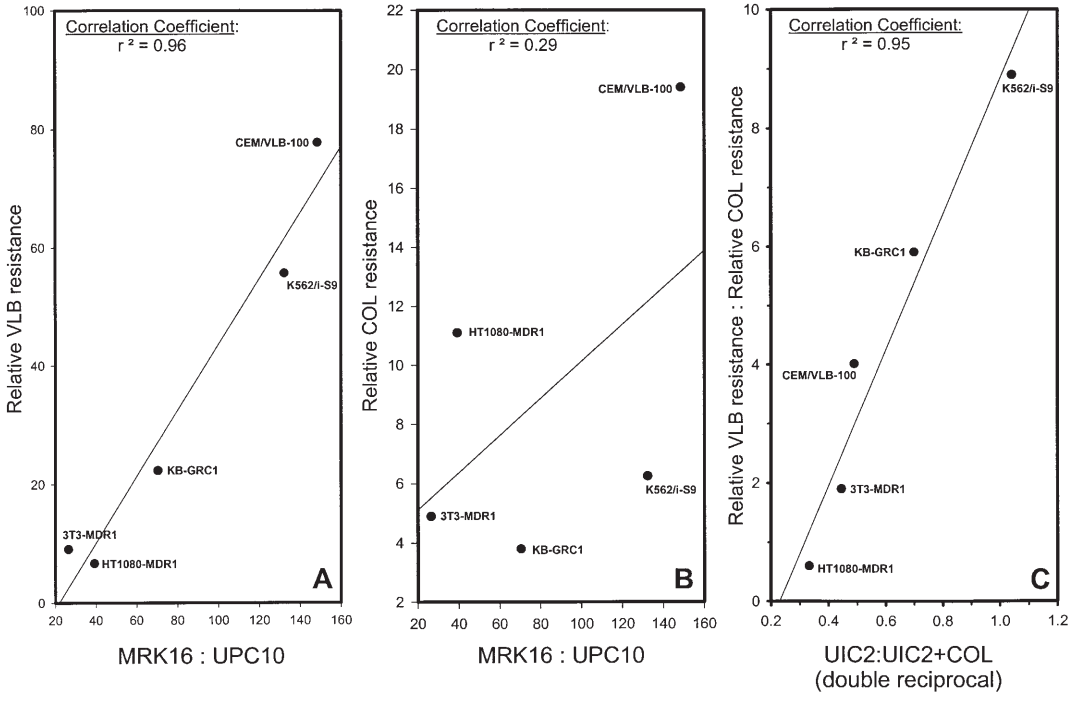

Figure 2. Colchicine resistance, unlike that for vinblastine, does not correlate to the concentration of P-gp molecules in the plasma membrane, but instead correlates to the effect of colchicine on the UIC2 reactivity of the indicated cell line. All five cell lines express human *MDR*1, and the solid line in each panel is the linear regression. The relative resistance of each cell line to either vinblastine (VLB) or colchicine (COL) was determined by cytotoxicity assays comparing the concentration of drug necessary to kill one-half of the P-gp-expressing cells against the concentration of the same drug required to kill one-half of the drug-sensitive parental cells. In panel *A*, the ratio of median MRK16 fluorescence to median UPC10 fluorescence indicates the relative concentration of P-gp on the cell surface for each cell line shown and is plotted against the relative VLB resistance. The resulting correlation coefficient (r^2) is 0.96. In panel *B*, a similar comparison yields a $r^2 = 0.29$ and demonstrates that the cell surface density of P-gp does not correlate to the relative COL resistance. In panel *C*, a double-reciprocal plot depicts the correlation between the ratio of median UIC2 reactivity to median UIC2 reactivity in the presence of a saturating concentration of COL (UIC2+COL) on the x-axis against the ratio of relative VLB resistance to relative COL resistance on the y-axis. The regression yields a $r^2 = 0.95$ and demonstrates that relative COL resistance correlates to the effect of COL on UIC2 reactivity.

relative to vinblastine resistance in different cell types show an excellent correlation in a double reciprocal plot with the ability of colchicine to induce UIC2-detectable conformational transitions. Therefore, whereas the effects of vinblastine on relative resistance and UIC2 reactivity are solely based on P-gp density, the effects of colchicine on P-gp conformation depend on the cellular environment and on ATP hydrolysis by P-gp. These effects correlate with the ability of P-gp to confer colchicine resistance in different cell types.

The cellular component involved in P-gp-colchicine interactions may be the makeup of the lipid bilayer in different cell lines. Due to the lipophilic nature of the majority of compounds transported by P-gp, many substrates of P-gp will interact directly with the enzyme upon their accumulation within the lipid bilayer [98]. In fact, fluorescence energy transfer has been used to demonstrate the efflux of Hoechst 33342 and another P-gp transport substrate, LDS-751, from the cytoplasmic leaflet of the lipid bilayer [106, 107]. In addition, several studies have reported that changes in the lipid composition of the plasma membrane alter drug and/or nucleotide binding, and therefore, the ATPase activity of P-gp. An early study of substrate binding to reconstituted P-gp in liposomes by Saeki and others found that the binding of ^{3}H-azidopine was improved when the lipid composition of the liposomes was increased for cholesterol, stigmasterol or ergosterol, in descending order [108]. Georges and others reported that ^{3}H-azidopine photolabeling of P-gp was abolished in the presence of nonionic detergents, but not in the presence of urea or a zwitterionic detergent, due presumably to disruption of the lipid bilayer [109]. The absence of lipid during reconstitution was shown to abrogate P-gp ATPase activity, which was restored to different degrees upon the addition of different lipids [110, 111]. More recently, Romsicki and Sharom [112] have found that alterations in the lipid head group and the acyl chain composition or the lipid bilayer alter the apparent affinities of vinblastine, verapamil and daunorubicin to P-gp, and also affect ATP binding and hydrolysis. These findings led these investigators to suggest that the plasma membrane may be participating directly in the interaction between the nucleotide-binding domains and drug-binding sites of P-gp [112].

Besides lipid composition, it is also possible that P-gp interactions may be affected by some cytoplasmic factors. Zhang and Ling [113] found that cytoplasmic components modulate the membrane topology of P-gp molecules produced in cell-free translation systems, suggesting that P-gp expressed in various cell types may have different topological structures. Such topological changes are likely to account for the differences in proteolytic profiles of P-gp observed in the presence of different ligands [33, 34] and may provide a plausible explanation for altered UIC2 reactivity.

Defining substrates and inhibitors of P-gp

At this point, it is necessary to ask: When is a substance a substrate, and when is it an inhibitor of a given drug efflux pump? This is not a completely trivial question, and it can be addressed by several different approaches.

One could define a typical substrate as a drug, which is being pumped by a particular ABC transporter, i.e. a transport substrate. On the other hand, one could also define a catalytic substrate, i.e. a compound that stimulates the ATPase activity of the transporter. Similarly, one could define a transport inhibitor as a drug that inhibits the efflux of other drugs, either by a competitive or non-competitive mechanism. Again, one could choose to look at the level of the catalytic site, and define P-gp-associated ATPase inhibitors as compounds that decrease the endogenous ATPase activity from its relatively high, basal level.

We presume that the catalytic activity, i.e. the energy released by splitting ATP to ADP and P_i, which takes place at the ATPase site, is directly coupled to the transport function of the enzyme. Thus, it would appear logical to conclude that inhibitors of the ATPase would also be inhibitors of transport function, and vice versa: that ATPase-stimulating drugs would also be transport substrates. That the latter is not always the case will be clear from the following examples.

Let us first look at verapamil, the first compound recognized as a chemosensitizer of P-gp mediated multidrug resistance [14]. It is beyond doubt that verapamil inhibits the transport function of P-gp, as it increases the accumulation of several drugs, such as the anthracyclines and vinca alkaloids (these being transport substrates of P-gp), in cells which overexpress P-gp [114, 115] (see fig. 3A). At $<10\,\mu M$ concentrations verapamil also decreases the accumulation of drugs into inside-out vesicles made from P-gp-expressing cells [116, 117]. In contrast, verapamil is one of the best ATPase activators known since it stimulates P-gp-associated ATPase activity by 100–500% with a K_m of 0.5–2 μM [18, 118–120] (fig. 3B). How can these two apparently conflicting findings be reconciled? One theory would suggest that verapamil acts as an uncoupler of transport activity, meaning that the effect of verapamil is to create a futile cycle of ATP hydrolysis so the energy spend does not get coupled to substrate translocation. Another explanation is that verapamil acts as a competitive substrate so that the transport of verapamil competes with the transport of other drugs. This hypothesis is not incompatible with the concept of uncoupling, because it appears that the energy is spent on translocation of verapamil and not on the other substrate. There are few data on verapamil transport by P-gp. Some studies favor verapamil as a P-gp substrate [121, 122]; others do not [123, 124]. Our data show that the fluorescent verapamil analogue BODIPY-verapamil is indeed a P-gp substrate as its

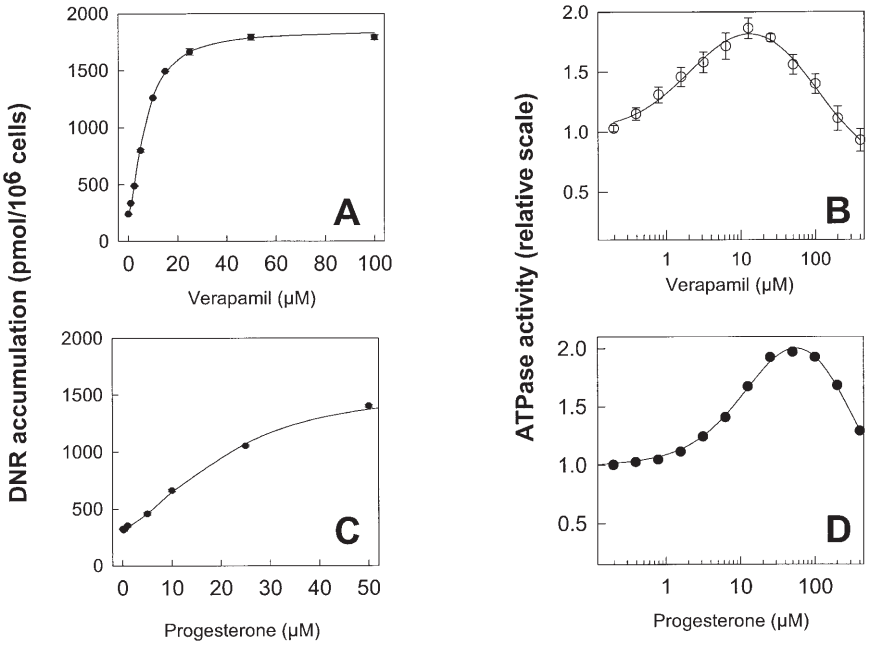

Figure 3. Verapamil (*A*) and progesterone (*C*) both increase the accumulation of daunorubicin in P-gp-expressing Ehrlich ascites tumor cells, and both drugs act as activators of the P-gp ATPase (*B* and *D*). Panels *A* and *C* are from [19]. Panels *B* and *D* are unpublished data on P-glp in microsomes prepared from Sf9 cells which have been transfected with baculovirus containing the MDR1 gene (GENTEST, Massachusetts). The ATPase assay is described in [87].

accumulation in P-gp-overexpressing cells is clearly diminished. However, BODIPY-verapamil may not represent the 'true' substrate because of the attached BODIPY moiety, and studies that have used radioactively labeled verapamil yield equivocal results [123]. The reason verapamil appears to be an inhibitor rather than a transport substrate of P-gp might be sought in its membrane permeability: verapamil is a very lipophilic compound, and thus quite membrane permeable, meaning that its diffusion rate across the plasma membrane is fast. One might assume that verapamil is indeed effluxed by P-gp, but that the drug at the extracellular side immediately diffuses back into the cell. This could explain why, in some studies, there is no apparent net efflux of verapamil, since net efflux, and not the unidirectional fluxes (passive influx, active and passive efflux) is what is being measured [123] (see fig. 4). Because verapamil is a 'fast diffuser' it will enter the cell as fast as it is being pumped out, and the ATPase activity of P-gp will indeed be futile, thus uncoupling it from transport. Gera Eytan has elaborated the concept of 'fast' versus 'slow' diffusing agents in two comprehensive texts [125, 126]. This would be compatible with the view that BODIPY-verapamil is a 'slow' diffuser (because of the additional, bulky BODIPY moiety), and thus P-gp can create a gradient before back-diffusion occurs.

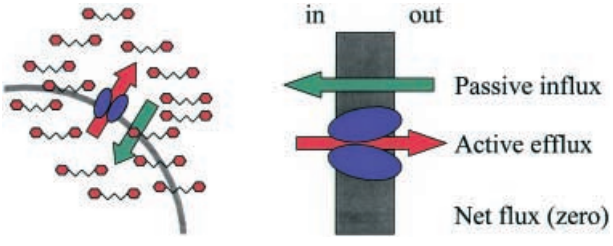

Figure 4. A cartoon showing how verapamil can uncouple the transport of other P-gp substrates by fast back-diffusion into the plasma membrane. Due to the lipophilic nature of verapamil it partitions into the plasma membrane at such a fast rate that the passive influx balances the active efflux by P-gp, so the net flux, which is the sum of these two unidirectional fluxes, equals zero.

Let us turn to another ATPase substrate, namely progesterone. This drug has been reported not to be a P-gp substrate, although it is clearly an activator of the P-gp ATPase, figure 3C, and [87], with about the same potency as verapamil. Why does this ATPase substrate not classify as a clear transport substrate? Perhaps because again progesterone is a 'fast' diffuser, and we are back to the same argument as for verapamil. Progesterone may indeed be a transport substrate of P-gp, but we are not able to observe a net efflux because of the fast back-diffusion. This view is supported by a study using different steroids, the more hydrophilic (slow diffusers) being classified as transport substrates for P-gp, whereas the more hydrophobic progesterone is classified as a drug resistance modulator [68], which is also apparent from figure 3D.

We can therefore classify the drug interactions with P-gp into the following four categories: class 1 agonists (slow

partitioning); class 2 partial agonists (fast partitioning); class 3 antagonists; class 4 nonsubstrates.

An agonist would be both an ATPase activator and a transport substrate. Some typical examples are the 'classical' substrates of P-gp: the anthracyclines, the vinca alkaloids and bisantrene ([87] and unpublished data). A partial agonist would be a molecule which stimulates the P-gp ATPase activity but which does not show any significant transport substrate features. To this group belong verapamil and progesterone, both of which activate P-gp at the catalytic level, but inhibit at the transport level (figs. 3A, C), presumably because of fast back-diffusion of the molecules. One may choose to consider this class of drugs as the fast partitioning fraction of class 1 drugs. Thus, class 1 compounds can be split in slow-partitioning (both ATPase and transport substrates) and fast-partitioning (ATPase activation, but no apparent transport) agonists.

An antagonist would inhibit the action of P-gp, and inhibit both at the ATPase and the transport level. An example of a well-known antagonist is vanadate, which inhibits the ATPase activity of P-gp by binding to the catalytic site, thus acting as a noncompetitive inhibitor of transport [88]. Another group of drugs, which inhibit the P-gp associated ATPase activity are the cyclosporins. The best-characterized is PSC-833, which at nanomolar concentrations inhibits the ATPase and transport function of P-gp. But is cyclosporin A or PSC-833 transported by P-gp? Again, the literature is not clear on this: some reports favor the view that cyclosporins are transport substrates for P-gp [127–129]; others do not [98]. Our data support the notion that PSC-833 is indeed a substrate of P-gp, but a slow one. That is, when PSC-833 competes with the presumed endogenous substrate (mentioned earlier), it will win out due to its higher affinity (a larger interaction surface, see [87]). But, since its transport rate is slower, it will slow down the turnover rate, which will then be reflected in the decrease in ATPase activity. Perhaps it is more correct to regard PSC-833 as a partial antagonist, since it does not completely block P-gp function, but just slows it down due to the bulkiness of the PSC-833 molecule acting as an 'obstructive' substrate, slowing down the P-gp machinery.

Nonsubstrates would simply be drugs that do not appear to interact with P-gp, neither at the ATPase level, nor at the transport site. Methotrexate belongs to this group.

From the above examples, it is clear that it is not safe to use only one transport substrate to screen for drug-P-gp interactions because the bi-implication that if A interacts with B, and B interacts with C, thus C must interact with A is not always valid (see fig. 5). Drugs A and B might very well overlap each others' binding site, as might B and C, but it is not at all certain that C will compete with A for binding. Especially in the case where B is a large molecule (such as PSC-833), one will find competition effects for many unrelated drugs, but these do not have to compete with each other. Allosteric interactions, both positive and negative, should also be taken into consideration [130]. The essence of all this is that it is not easy to design a reliable high throughput screening (HTS) assay for P-gp modulators if this assay is based on only one parameter. Some examples: The ATPase assay, which is a nice, fast assay, suitable for HTS, will pick up inhibitors of the P-gp ATPase, which are most likely to be inhibitors of transport as well. But when it comes to P-gp ATPase activators, it is not possible to tell whether a given activator will behave more like a substrate or more like an inhibitor of drug transport. Matters get even worse when one considers 'silent substrates", a '5th category' of P-gp drug interaction. These drugs apparently neither activate nor inhibit the basal activity of P-gp, possibly because their activation profile resembles that of an endogenous substrate. In these cases, it is necessary to repeat the experiment by (i) pre-stimulating the ATPase activity of P-gp by adding a known activator (such as verapamil) to see if the 'silent drug' has any inhibiting effect on the verapamil-stimulated ATPase activity, or by (ii) preinhibiting the ATPase with a known inhibitor (such as PSC-833) to see if any activation of the preinhibited state is possible. Fortunately, so far only a few drugs behave as silent substrates and, from our experience with hundreds of compounds, it is clear that the really good P-gp reversers are either strong activators or strong inhibitors of the P-gp associated ATPase activity.

When it comes to transport assays, the problem arises how to choose the best model substance for screening drug-P-gp interactions. A very popular P-gp substrate is calcein-acetoxymethyl (AM), mainly because it is so easy to work with: it is nonfluorescent in its AM-ester form, but once cleaved by intracellular esterases, the free calcein fluoresces, and the intracellular level can be monitored online in a fluorometer or 96-well reader [131]. No washing step is necessary, because only the intracellular calcein gives rise to the fluorescence signal. Calcein-AM is a substrate for both P-gp and MRP,

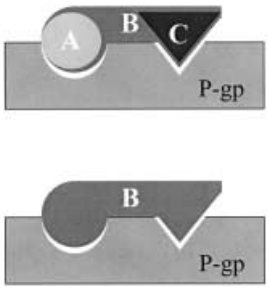

Figure 5. A model for substrate binding sites on P-gp. A relatively large molecule B, such as cyclosporin A or PSC-833, spans the binding sites of both the smaller molecules A and C (which could be verapamil or daunorubicin). Thus, although drug B competes with both drugs A and C for binding, one cannot infer from this that drugs A and C have overlapping binding sites.

whereas free calcein is also transported by MRP [54], so it is possible to screen for both drug resistance mechanisms in one assay, using cobalt as a quencher of extracellular calcein fluorescence [132]. Calcein that was able to enter the cell (as calcein AM) will stay inside the cell if MRP is absent but will be transported out of the cell if MRP is present. External calcein (quenched by cobalt) thus measures the presence of MRP in the membrane, whereas any calcein that remains within the cell reached there by blockade of P-gp. However, the calcein-AM assay will not necessarily pick up all drugs that interact with P-gp. Only drugs that are competitive (i.e. share their binding site with calcein-AM or celcein) or noncompetitive (have different/overlapping binding sites) inhibitors

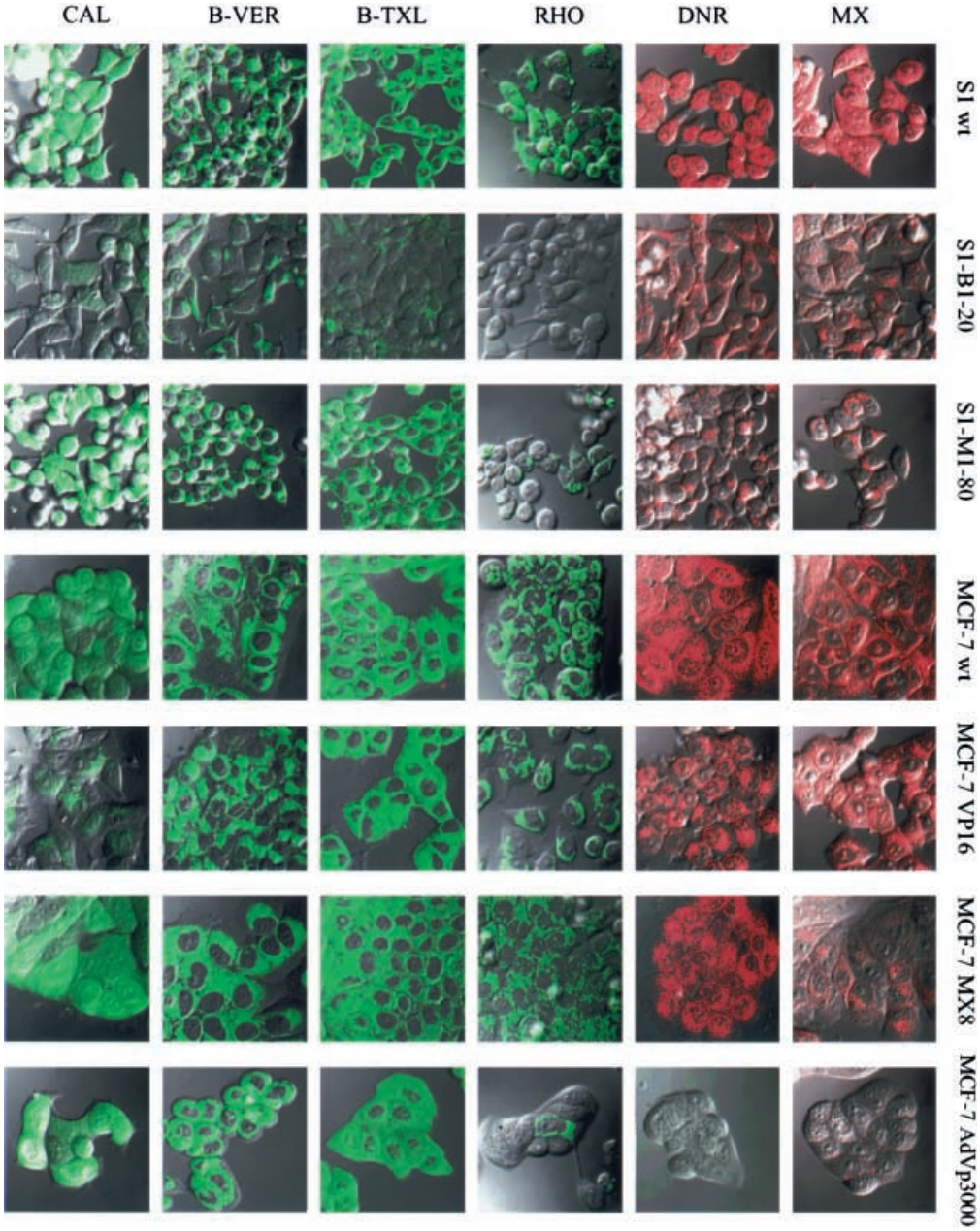

Figure 6. Identifying substrates of P-gp, MRP1, MRP2 and MXR by confocal microscopy. The micrographs show steady-state drug accumulation of fluorescent drugs after 60 min incubation at 37 °C. The levels and localization of 5 µM mitoxantrone (MX), 5 µM daunorubicin (DNR), 1 µM rhodamine 123 (RHO), 100 nM BODIPY FL-paclitaxel (B-TXL), 3 µM BODIPY-verapamil (B-VER) and 1 µM Calcein-AM (CAL) are shown. The fluorescence emission spectrum of the dyes is indicated: green (515–540 nm) and red (above 590 nm), respectively. The cell lines used are colon cells: S1 wt (parental, drug sensitive), S1-B1-20 (P-gp expressing, selected in bisantrene), S1-M1-80 (MXR expressing, selected in mitoxantrone); breast cancer cells: MCF-7 wt (parental, drug sensitive), MCF-7 VP16 (MRP1 expressing, selected in VP16), MCF-7 MX8 (MRP2 expressing, selected in low mitoxantrone concentrations), MCF-7 AdVp3000 (MXR expressing, selected in a combination of adriamycin and verapamil).

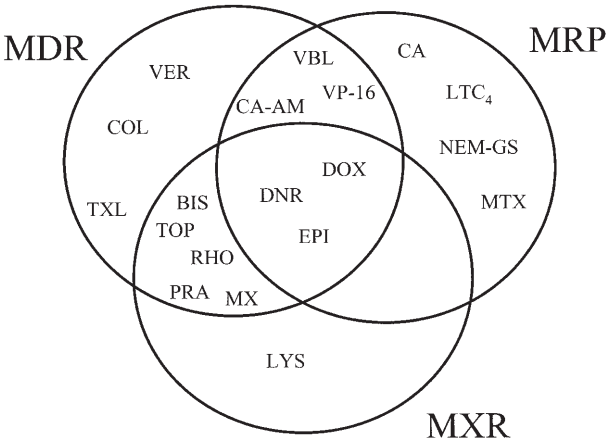

Figure 7. Venn diagram showing the substrate overlap between MDR, MRP and MXR. Abbreviations: BIS, bisantrene; CA, calcein; CA-AM, calcein-AM ester; COL, colhcicine; DNR, daunorubicin; DOX, doxorubicin; EPI, epirubicin; LTC_4, leukotriene C4; LYS, LysoTracker; MTX, methotrexate; MX, mitoxantrone; NEM-GS, *N*-ethyl maleimide glutathione; PRA, prazosin; RHO, rhodamine 123; TXL, taxol; TOP, topotecan; VBL, vinblastine; VER, verapamil; VP-16, etoposide.

of calcein transport. The problem with multiple binding sites necessitates the use of alternative model transport substrates, such as rhodamine-123, daunorubicin, or 99m-Tc-sestamibi, which have different binding sites on P-gp, to really classify a given drug as a potential transport inhibitor. A multitracer approach (either fluorescent, radiolabeled or both) might be the only way to identify a 'latent' substrate or inhibitor of P-gp. The most direct and definite way to test for interaction with a given drug resistance mechanism would be to have the compound in a radiolabeled form, but since this is not feasible for HTS, the indirect method has to be used.

In our efforts to characterize the MDR phenotype, we have used multiple fluorescent probes to identify and classify substrates and inhibitors of P-gp, MRP and MXR (fig. 6). The results are summarized in the Venn diagram, figure 7.

What is a good inhibitor of P-gp

Is a good P-gp reverser (i) a 'fast'-diffusing transport substrate, (ii) a noncompetitive inhibitor, which is not being transported itself or (iii) an ATPase inhibitor?

Among the best reversers of P-gp are PSC-833 and other cyclosporin analogs. All these compounds are characterized by a high affinity (low K_m, in the nanomolar range) for P-gp, and they are all strong ATPase inhibitors. Thus, one could deduce that a HTS for P-gp reversers should focus on group iii. However, verapamil and many other P-gp transport inhibitors (inhibitors or partial agonists, most inhibitors of P-gp probably being fast-diffusing transport substrates, and thus competitive inhibitors) are strong activators of the P-gp ATPase, thus favoring the choice of compounds in group i. Another advantage of an ATPase activator is that it might deplete the resistant cell for energy due to the futile pumping of the fast-diffusing substrate. Finally, to group ii belongs vanadate, a very efficient, albeit toxic, inhibitor of P-gp, MRP, and MXR function. Thus, vanadium compounds may provide a promising MDR reversal strategy [133].

Interactions of P-gp with nucleotides

Understanding how transport substrates and chemosensitizers interact with P-gp is only half the story. To fully comprehend the mechanism of P-gp substrate transport, an understanding of nucleotide interactions with P-gp and the coupling of nucleotide hydrolysis to substrate transport is required. The ATPase activity of P-gp was shown to be Mg^{2+}-dependent and to follow simple Michaelis-Menten kinetics with a single K_m of approximately 1 mM [119]. In contrast, a study by Sarkadi and others determined a Hill number of 1.4, suggesting that the minimum number of ATP molecules required by P-gp, following drug stimulation of the ATPase activity, is two. This is consistent with a mechanism in which ATP hydrolysis by P-gp is cooperative and where the maximal ATPase activity requires catalysis using two ATP molecules [18].

Nucleotide catalysis is relatively nonspecific and shows competitive inhibition in the presence of nonhydrolyzable adenine nucleotides such as ADP and 5′-adenylylimidodiphosphate (AMP-PNP) [134].

In a variety of ATPase enzymes, myosin being one example, vanadate had been shown to occlude nucleotide within the catalytic site by forming a stable transition-state analog [135]. Vanadate has also been used to study the ATPase activity of P-gp, which – as mentioned earlier – has a high level of basal ATPase activity (i.e. in the absence of transport substrates), compared with other energy-dependent efflux proteins. This basal ATPase activity is stimulated in the presence of P-gp transport substrates [18, 21, 119], and inhibited by vanadate [21, 136]. Urbatsch and others demonstrated that following ATP catalysis, ADP was occluded within the catalytic site of P-gp and that such occlusion inhibited further enzyme function by forming an essentially irreversible ternary complex [136]. This finding was extended to show that both NBDs of P-gp were catalytically active by using mild trypsinization to separate the halves of P-gp following the radiolabeling of vanadate-occluded 8-azido ATP (8-N_3 ATP). Autoradiography revealed that each half retained a similar content of occluded, radiolabeled dinucleotide, suggesting that both halves were equally capable of hydrolyzing ATP [137]. The same study demonstrated that the occlusion of 1 mol of ADP per mol P-gp was sufficient to block further ATP hydrolysis. This finding is consistent with results demonstrating that

mutations in either or both NBDs eliminate or reduce enzyme function [24, 138, 139]. Interestingly, despite the inability to hydrolyze nucleotide, mutants carrying a mutation in only one NBD were able to bind 8-azido ATP at a level similar to wild type [24]. This interdependence between NBDs suggests that these domains must cooperate in order to efflux drug. In addition, it was also demonstrated that the rate of vanadate-mediated occlusion is increased in the presence of transport substrates [140].

The addition of vanadate to UIC2 reactivity assays has provided further support for the hypothesis that UIC2-detectable changes in P-gp conformation are due to nucleotide binding and dissociation to the NBD of P-gp [37]. In P-gp-overexpressing cells, preincubation with increasing concentrations of vanadate inhibited the increase in UIC2 reactivity observed in the presence of vinblastine. It is not clear from UIC2 reactivity analysis whether vinblastine is able to bind to the vanadate-occluded form of P-gp. Another study, observing the direct interaction between P-gp and the substrate IAAP, has reported that treatment with vanadate and ATP blocks the binding of the substrate to P-gp [141]. However, if vinblastine were able to bind to P-gp, these findings could be interpreted as suggesting that a failure to release nucleotide, when occluded by vanadate, prevents the conformational change, that, in the absence of vanadate, results in high UIC2 reactivity.

Work with vanadate is only an indirect assessment of how ATP is being utilized by P-gp. More direct and quantitative analysis of the role of nucleotides in the mechanism of P-gp function has been performed on reconstituted P-gp expressed in vesicles, membrane fragments and permeabilized cells. Cellular permeabilization with a mild treatment with *Staphylococcus aureus* α-toxin allows the free passage of nucleotides into and out of the cell while maintaining a cellular morphology that is compatible with FACS and, therefore, UIC2 reactivity analysis. Staining with propidium iodide (PI) immediately prior to FACS analysis allowed for the segregation and independent determination of the UIC2 reactivity for cells that had been permeabilized from those that had not been permeabilized. Permeabilized cells, which are ATP depleted, demonstrate a high level of UIC2 reactivity that is comparable with the UIC2 reactivity of vinblastine-treated PI-positive cells [99]. This finding is consistent with the high level of UIC2 reactivity observed in intact cells that had been treated with ATP-depleting agents such as oligomycin and sodium azide [37].

The addition of ATP, ADP, 8-azido ATP, ATPγS and AMP-PNP to permeabilized cells induced a decrease in UIC2 reactivity that was reversed by either washing the cells thoroughly or adding vinblastine. These results are consistent with the ability of the same nucleotides to either stimulate or inhibit the ATPase activity of P-gp in the presence of vanadate, which was interpreted as the nucleotide's ability to bind to P-gp [119]. In the same study, AMP, ITP, CTP, UTP and GTP were unable to inhibit the ATPase activity of P-gp, indicating that these nucleotides did not bind to P-gp. The addition of these nucleotides similarly had no effect on the UIC2 reactivity of permeabilized cells. These findings are also consistent with the ability of ATP, ADP and AMP-PNP to alter the proteolytic profile of P-gp [33]. The P-gp proteolytic profile was either unchanged or not significantly altered in the presence of AMP, GTP, UTP or CTP. All of these results strongly indicate that UIC2-detectable P-gp conformational transitions are due to nucleotide binding and debinding, rather than nucleotide hydrolysis, and suggest that the conformational changes being detected by UIC2 are the same as those being isolated by trypsinization.

In addition to observing qualitative UIC2 reactivity changes, quantitative analysis on the ability of nucleotides to decrease UIC2 reactivity have revealed differences between the stoichiometry of P-gp binding for various nucleotides. For ATP and ADP, the Hill number describing the transition from high to low UIC2 reactivity was significantly greater than 1. This suggests that pairs of ATP and ADP molecules are required to induce the UIC2 reactivity transition and is consistent with the presence of two functional NBDs in each P-gp molecule. The binding of two nucleotides to P-gp is in agreement with reports stating that two molecules of ATP are hydrolyzed for each substrate molecule transported [142–144], and that both nucleotide-binding domains must be intact if ATP hydrolysis [24, 144, 145], vanadate trapping [140, 146] or drug efflux [147, 148] is to occur.

When the ability of nonhydrolyzable analogs of ATP to decrease the UIC2 reactivity of P-gp was analyzed, the high to low UIC2 reactivity transition yielded a Hill number of 1. The seemingly contradictory finding between the number of nonhydrolyzable nucleotide molecules required to decrease UIC2 reactivity (one) and the number of hydrolyzables so required (two) suggests the following hypothesis: in P-gp, two molecules of ATP, or its analogs, are sterically inhibited from binding to P-gp simultaneously, but no such restriction exists for the simultaneous binding of two ADP molecules. This hypothesis is in agreement with the above-cited studies reporting that both nucleotide binding domains must be functional in order for P-gp to hydrolyze ATP and with the hypothesis that hydrolysis of ATP at one nucleotide-binding domain is stimulated by ATP binding at the other domain [149]. In the presence of saturating concentrations of ADP or nonhydrolyzable ATP analogs, the ATPase activity of P-gp is unable to function. Yet, in the presence of ATP (or other hydrolyzable ATP analogs such as 8-azido ATP), P-gp is still able to actively hydrolyze ATP. Therefore, the two-site binding observed for ATP can be interpreted as the simultaneous binding of ATP

and ADP. This hypothesis is further corroborated by the findings that single nucleotide binding domain mutants of P-gp require only a single molecule of ADP to decrease UIC2 reactivity [99], and that wild-type P-gp and both single NBD mutants were photolabeled with ^{32}P-8-azido ATP to the same maximal extent [24]. This hypothesis suggests that under physiological conditions, nucleotide hydrolysis at one NBD would allow binding of ATP to the other NBD, and catalysis would thus alternate between NBDs.

A study by Hrycyna and others reported similar findings using P-gps carrying mutations in either the N-terminal or C-terminal Walker B motif. This report showed that radiolabeled 8-azido ATP bound asymmetrically to either the N-terminal or C-terminal nucleotide binding domain depending upon the concentration of nucleotide present and/or the ability of the protein to hydrolyze nucleotide [139]. These authors suggest that their findings are also compatible with a model for P-gp function in which two molecules of ATP would not bind simultaneously, but binding of ATP to one nucleotide binding domain would affect ATP hydrolysis at the second site.

A quantitative demonstration of the ability of vinblastine to reverse the nucleotide-dependent decrease in UIC2 reactivity is shown in figure 8. In the presence of ATPγS, which was shown to have the highest affinity for P-gp of all nucleotides tested [99], the presence of vinblastine decreases the apparent affinity of the nucleotide by 60-fold. Vinblastine has been shown to stimulate the ATPase activity of P-gp [18, 21]. However, these results indicate that vinblastine also promotes nucleotide dissociation from P-gp. This interpretation is also consistent with the ability of vinblastine to increase the reactivity of NBD mutants of P-gp, which are unable to hydrolyze ATP [37, 99].

An interesting and unexpected effect of colchicine on the interaction between P-gp and ATP was observed when it was noticed that the saturating concentration of colchicine (10 mM) altered the Hill number for the ability of ATP to decrease UIC2 reactivity from 2 to 1 [100]. This effect of colchicine may be interpreted in light of a recent hypothesis by Sauna and Ambudkar [150]. They suggest that hydrolysis of one ATP molecule is required to transport the P-gp-bound substrate, with an associated change in the P-gp conformation that lowers its substrate affinity, and that the second ATP molecule is then hydrolyzed to change the P-gp conformation in a way that would allow the transporter to bind a new substrate molecule. If colchicine binding shifted the equilibrium of these conformational transitions in a direction that would otherwise require the hydrolysis of one ATP molecule, then only one ATP would have been required to complete the transition, resulting in the decrease in the Hill number for ATP. Perhaps these two molecules of ATP are required to transport one molecule of compound, as stoichiometry studies imply.

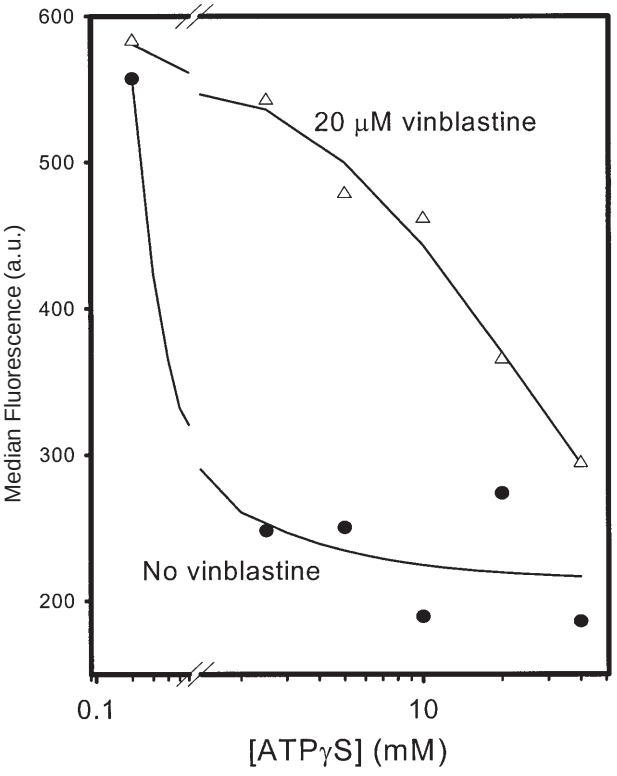

Figure 8. Vinblastine decreases the apparent nucleotide affinity for P-gp. K562/i-S9/i-S9 cells were permeabilized with *S. aureus* α-toxin, and the cells were then incubated in the presence of the indicated concentration of the nonhydrolyzable ATP analog ATPγS only (●) or ATPγS and 20 μM vinblastine (△) for 20 min at 37 °C prior to UIC2 staining for an additional 30 min at 37 °C. The solid line through each data set is the nonlinear regression. In the presence of saturating vinblastine, the apparent affinity of ATPγS was decreased over 60-fold, indicating that substrate binding to P-gp promotes nucleotide dissociation from the protein.

The mechanism of P-gp function: a series of linked equilibria?

Many of the above findings are consistent with the proposed mechanism of P-gp function depicted in figure 9. This scheme attempts to reconcile these findings in terms of a conventional kinetic model for ATP-coupled transport. Other proposed models for the action of P-gp [146, 149, 151] do not integrate the work on conformational changes. In our model, E1 refers to the state of P-gp with the substrate-binding face of the enzyme located inside the cell, and E2 refers to the state of P-gp where the substrate-binding face of the enzyme is located extracellularly. These states of P-gp correlate well to the high (E2) and low (E1) UIC2 reactivity states. Reversible conformation changes are postulated as occurring during the E1 to E2 and the E1S1 to E2S2 transitions. To account for the relatively high level of basal ATPase activity for P-gp, the 'idle cycle', depicted in figure 9, allows for ATP hydrolysis in the absence of transport substrate (S). Figure 9 also assumes that all forms in E2 are generally more stable than the corresponding form in E1 as evidenced by

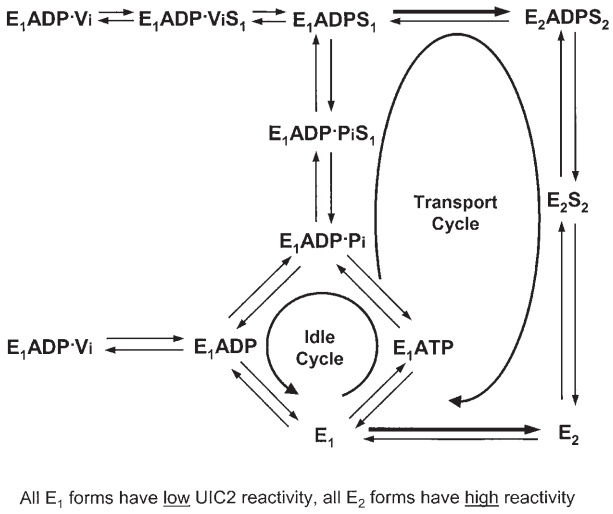

Figure 9. Proposed mechanism of P-gp function. E1 is defined as the state of the enzyme where the substrate-binding face of the protein is in an intracellular location. E2 is defined as the state of the enzyme where the substrate-binding face of the protein is in an extracellular location. In this model, E1 is equated with a low UIC2 reactivity conformation of P-gp, whereas E2 is equated with a high UIC2 reactivity conformation of P-gp. UIC2-detectable conformational transitions of P-gp occur during the E1ADPS1 to E2ADPS2 and E2 to E1 reactions. The presence of vanadate (Vi) traps P-gp in a highly stable ternary complex [136] that maintains low UIC2 reactivity [T. Druley, unpublished data]. In this scheme, the idle cycle refers to the basal level of ATP hydrolysis demonstrated by P-gp, whereas the binding of substrate (S) commits P-gp to the transport cycle that ultimately releases S on the extracellular surface of the plasma membrane.

a high level of UIC2 reactivity following nucleotide depletion. In the presence of nucleotides (according to the extent to which they bind P-gp), the E1 state is formed and the UIC2 reactivity is subsequently low. Vanadate occupies the site to which P_i would otherwise bind, promoting the formation of a stable ternary complex (leading to an increase in apparent nucleotide affinity) and stabilizing the E1 conformation in the presence of nucleotide.

Substrates such as vinblastine, and some P-gp modulators, would stabilize the E2 state, causing a decrease in apparent nucleotide affinity. In figure 9, the binding of S (e.g. vinblastine) to E1ADP · P_i will stimulate hydrolysis if the debinding of P_i from E1 is faster when S is bound than when it is unbound. It is assumed here that this loss of P_i locks S within the P-gp complex that will undergo a conformation transition to E2 and subsequently release S (the 'transport cycle'). In short, the binding of S accelerates the reaction step that is rate limiting in the absence of drug and therefore increases the rate of ATP hydrolysis. The change in conformation from E1 to E2 induced by the binding of S is assumed to promote the release of ADP followed by the release of S at the E2 face of the enzyme. Since S binds to an ATP-bound E1 form and debinds from an ADP-bound E2 form, we assume that substrate transport can only occur after ATP is hydrolyzed. The affinity of drug for P-gp at the cytoplasmic and extracellular faces of the membrane can be very different and linked to the free energy change of ATP hydrolysis. This transport cycle is proposed for functional wild-type P-gp. Mutants of P-gp that are unable to hydrolyze nucleotide will not proceed through the transport cycle but are still able to transition between E1 and E2. It is entirely possible that the parameters of substrate and/or nucleotide binding in the mutants will be very different from those of wild-type P-gp. For the MK and KM forms of P-gp, nucleotide binding promotes E1, and the binding of S will stabilize the E2 state without nucleotide hydrolysis, suggesting that these mutants transition between the steps depicted at the bottom of the scheme in figure 9.

Clearly, figure 9 is a simplification. The mechanism of P-gp function is complex and highly influenced by a variety of direct and indirect factors. Unfortunately, this complexity makes the proposal of a complete catalytic mechanism exceeding difficult. A complete mechanism must take into consideration and integrate not only conformational changes, but also multiple drug binding sites, the role of two functional nucleotide binding domains, the variable effects of competitive and noncompetitive inhibitors of P-gp, and a host of studies on P-gp mutants. We chose not to depict alternative pathways in which, for instance, substrates bind directly to P-gp before ATP is bound and split, although such a mechanism is also plausible. The findings presented here can only be applied to a small portion of the overall mechanism, and more investigation into the quantitative manner in which P-gp handles its various substrates is required in order to propose a complete mechanism.

With respect to other studies on conformational changes of P-gp, fluorescent labeling with MIANS at the cytoplasmic face of P-gp demonstrated that the effects of P-gp transport substrates and nucleotides were additive in their ability to quench the probe's fluorescence [30]. This finding is in contrast to the opposite effects of vinblastine and nucleotide observed during UIC2 reactivity analysis. Despite these differences, both studies demonstrated the same affinity for vinblastine binding to P-gp. Another study [32] observing the quenching of the intrinsic fluorescence of tryptophan residues located at the cytoplasmic face of P-gp reports findings that are complementary to our studies. In this study, P-gp was reconstituted into vesicles containing the water-soluble quenching agent acrylamide. The presence of either ATP or ATPγS resulted in quenching of tryptophan fluorescence and indicated that the cytoplasmic moieties of P-gp were found in the hydrophilic environment within the vesicle and, therefore, exposed to acrylamide. Conversely, in the presence of anthracycline substrates of P-gp, tryptophan fluorescence was not quenched, which indicated that the cytoplasmic face of P-gp had moved away from the hydrophilic environment of the vesicle interior. These results are consistent with the idea

that nucleotides drive the conformation of P-gp into a different state than transport substrates. Taken together, these two studies imply the following interpretation: With respect to the lipid bilayer, all or portions of the P-gp molecule may be physically shifted across the plane of the membrane. The presence of nucleotides moves the UIC2 epitope away from the extracellular surface while at the same time exposing more tryptophan residues at the cytoplasmic face. Nucleotide binding, stimulated in the presence of transport substrates, induces the opposite effect. Such a mechanism of P-gp movement would agree with the patterns of proteolytic digestion observed in membrane vesicles and induced in the presence of various nucleotides and transport substrates [33, 34].

Other aspects of P-gp's function

The complexity of understanding the precise nature of binding and transport is exemplified by the observation that mutation of the phosphorylation sites in P-gp results in a protein, which is still fully functional. For example, a mutation study by Goodfellow and others provided convincing evidence that phosphorylation of the linker region of P-gp by protein kinase C does not affect the rate of drug transport [152]. However, modification of substrate affinity was not really addressed since all phosphorylation sites were modified simultaneously. Other possible roles for the phosphorylation sites include effects on protein half-life or trafficking.

The mechanism by which P-gp overexpression occurs during drug selection has not yet been fully understood. In experimental models, Mickley and others have shown that the overexpressed *MDR-1* gene is frequently rearranged. Sequencing of 5′-RACE products has suggested that the rearranged *MDR-1* gene is under the control of an unrelated promoter, which results in overexpression. However, the presence of several sites of polymorphism in P-gp has allowed an examination of allelic expression patterns. These allelic expression patterns demonstrated that in lymphoma, patients who were genetically heterozygous for *MDR-1* at residues 2677 and 2995 had, in 30% of cases, expression of one allele exclusively or predominately at that site [153]. These results suggest that the rearrangement observed in the experimental models was also occurring in clinical samples. 5′-RACE of a series of clinical samples confirmed that this was the case.

Clinical correlates of P-gp function

The degree to which P-gp contributes to clinical drug resistance is still poorly understood. Largely because of the failure of clinical trials of P-gp antagonism to be successful in changing the natural history of cancer, some investigators have concluded that P-gp may contribute little to clinical drug resistance. This negative conclusion derives in part from the experimental models employed early in the study of resistance. Exposure of cells exponentially cultured in the laboratory resulted in selection of highly resistant sublines expressing enormous quantities of *MDR-1*/P-gp. These drug selections led to cell line models with several logs of resistance, and several logs of P-gp overexpression. It has been estimated that over 30% of all membrane protein is P-gp in CHO CR1R12 cells [154].

Table 2 shows the P-gp levels measured in several resistant cell lines and in clinical samples. Thus, the demonstration that a reversal agent could sensitize cells a thousandfold led to incorrect expectations on the part of scientists. It could be argued that these high expectations led to mistakes in trial design. Patients whose tumors were highly resistant to chemotherapy were treated with combined antagonist and P-gp substrate. These clinical trials were frequently organized with a 'home run' design, in which it was hoped that the results would be so significantly different from historical controls that the benefit of P-gp antagonism would be obvious. The outcome, however, failed to convincingly demonstrate a benefit for P-gp antagonism.

Despite these results, it is indisputable that studies have shown that P-gp expression is widespread in clinical cancer. In some tumor types, expression at low levels can

Table 2. Levels of P-gp in nine cell lines and in biopsy samples from tumors (the data are reported in arbitrary units, but are consistent between the various studies reported).

Tumor	Level (range)
KB 3-1	0.04
KB 8-5	108
KB 8-5-11	410
KB V1	5654
8226/S	0.0
8226/dox6	401
8226/dox40	1823
SW620	10
SW620/Ad20	1600
Breast cancer	3.4 (0.1–7)
Renal cell cancer metastases	12.1 (0.1–154)
Non-Hodgkin's lymphoma at diagnosis	1.0 (0.1–11)
Pre EPOCH	2.2 (0.1–11)
Post EPOCH	8.0 (0.2–162)

KB 3-1 is a parental lung carcinoma cell line. KB 8-5 and KB 8-5-11 are drug-resistant sublines selected in colchicine. KB V1 was selected, and is maintained, in 1 μM vinblastine. 8226/S is a drug-sensitive human leukemia cell line. 8226/dox6 and 8226/dox40 are the drug-resistant sublines grown in 6 and 40 nM doxorubicin, respectively. SW620 is a human colon adenocarcinoma cell line, and the SW620/Ad20 is its drug-resistant counterpart selected in doxorubicin (adriamycin), and maintained in 20 nM of this drug. EPOCH is a chemotherapeutic regime consisting of etoposide, vincristine, doxorubicin, cyclophosphamide and prednisone.

be demonstrated to increase following the development of resistance. In several studies P-gp expression has been shown to confer a poor prognosis at the time of diagnosis. These studies have led to a continuing level of interest in the clinical development of P-gp antagonists. The low level of P-gp expression in clinical cancer may be best served by treatment with a P-gp antagonist early in the disease course, with a trial design aimed at prevention of P-gp-mediated resistance.

The progress of reversal agents towards definitive clinical studies has been enhanced by the development of surrogate assays which confirm that P-gp antagonism can occur in patients. Imaging agents show retention in the liver with the addition of P-gp antagonists [155–158]. Circulating CD56 cells can be shown to have inhibition of P-gp-mediated efflux due to the administration of P-gp antagonists to patients [159, 160]. While not entirely predictive of P-gp antagonism, these assays are steps towards imaging tumors routinely in patients to confirm uptake of chemotherapy drugs and success of antagonism. Several reports suggest that imaging tumors with 99mT-sestamibi can confirm P-gp antagonism; highly specific imaging tools are, however, still needed.

The MDR-associated protein

The MRP family

In 1992, Cole and others described the second major ABC transporter involved in MDR: the MDR-associated protein (MRP) [161], a 190-kDa membrane protein containing 1531 amino acids. With only 15% homology to P-gp, MRP is more closely related to the cystic fibrosis transmembrane regulator gene product CFTR. Until now, six homologs of MRP have been reported; these are termed MRP2–7 [162]. Note added in proof: Recently, two new members of the MRP family, MRP8 and MRP9, have been identified. These resemble MRP5 but their physiological relevance has not yet been determinded (Michael Dean, personal communication). MRP1 is the original MRP, whereas MRP2 was originally identified as the canalicular multispecific organic anion transporter cMOAT. A new nomenclature scheme was implemented for the human ABC and mouse Abc genes in October 1999, according to which MRP1–6 is designated *ABCC1–6*, whereas MRP7 lists as *ABCC10*. An index of the new and old symbols can be found at http://www.gene.ucl.ac.uk/users/hester/abc.html. For a comparison of the different nomenclatures, expression patterns and substrates of ABC transporters associated with multidrug resistance, please see table 3, which compiles the up-to-date and referenced data on human ABC transporters at Dr Michael Müller's Web site. http://www.med.rug.nl/mdl/humanabc.htm. Another interesting and informative listing of MDR transport proteins, their substrates and inhibitors can be found at Dr Watkins's lab site at http://bigfoot. med.unc. edu/watkinsLab/info.htm.

MRP1 shares only between 34 and 58% sequence identity with MRP2–6, but the overall membrane topology is thought to be similar in all members of the MRP family. In addition to the 12 transmembrane segments characterizing P-gp, MRP has an additional 5TM (TMD_0) attached to the N-terminal. This additional domain has an apparent role in the organic anion affinity of the MRP family. Although at least six homologs of MRP have been reported, none have yet been linked as clearly with drug resistance as MRP1. Interestingly, the homolog MRP1, 2 and 3, in which resistance has been observed with transfectants also contain the 5 transmembrane segment TMD_0 [163–167]. However, the membrane topology of MRP is still under debate, some studies suggesting that the third membrane-spanning domain (equivalent to the second half of P-gp) has only four TM helices, whereas other studies favor the more 'conventional' six TM configuration of MSD3 (for a review, see [168, 169]). MRP1 contains 12 potential glycosylation sites, but these appear not to be of importance for its function [170]. On the other hand, phosphorylation of MRP seems to play a role for its transport function [171].

MRP substrates

The major issues for MRP parallel those issues raised for P-gp. The actual mechanism of transport, as with all ABC transporters, is not fully understood. In addition, the substrate specificity has been more difficult to define. Whereas P-gp has its greatest affinity for large, hydrophobic cations, MRP appears most effective in transporting organic anions. Jedlitsky and others demonstrated that MRP was able to transport glutathione, glucuronide and sulfate conjugated compounds [172, 173]. Since historically it had been reported that pretreatment of cells with BSO (buthionine sulfoximine, which depletes the intracellular GSH by inhibiting the γ-glutamylcysteine synthetase) was able to sensitize cells to anthracyclines, but difficult to demonstrate formation of glutathione conjugates, Cole and others hypothesized a model for MRP which included cotransport of GSH without actual glutathione conjugation. Such a finding would indicate that the cell did not have to conjugate compounds with glutathione in order to have the compound transported out of the cell. The data for this model are convincing; however, it is not known to what extent MRP substrates are metabolized, or cotransported [169, 174, 175].

It is well accepted that substrates for MRP include doxorubicin, vincristine and etoposide (VP-16). MRP overexpression has emerged in cell lines exposed to these compounds, and developing non-P-gp-mediated drug resistance. However, mice in which MRP has been genetically deleted display only an increased sensitivity to etoposide [176]. This finding is perhaps easily explained

Table 3. The three ABC transporter subfamilies known to be associated with multidrug resistance: MDR, MRP and MXR.

Member	*HUGO* symbol	Alternative names	Chromosome location	AA	Main tissue and cellular location	Major substrates	Associated disease
MDR1	*ABCB1*	*PGY1*, P-gp	7q21	1280	adrenal cortex, renal tubules, blood-brain barrier, apical membrane	numerous hydrophobic, amphiphilic drugs	drug resistance
MDR3	*ABCB4*	*PGY3*, MDR2/MDR3	7q21	1279	liver, apical membrane	phosphatidylcholine	progressive familial intrahepatic cholestasis
MRP1	*ABCC1*	MRP, GS-X	16p13.1	1531	lung, testis, ubiquitous, basolateral membrane	organic anions, glutathione conjugates (LTC_4)	drug resistance?
MRP2	*ABCC2*	cMOAT	10q24	1545	liver, intestine, kidney, apical membrane	organic anions, cisplatin, glucuronides (bilirubin)	Dubin-Johnson syndrome
MRP3	*ABCC3*	cMOAT2, MLP2, MOAT-D	17q21.3	1527	liver, colon, pancreas, basolateral membrane	glucuronides, bile salts	?
MRP4	*ABCC4*	MOAT-B	13q31	1325	prostate, lung, pancreas, testis, ovary, gallbladder, basolateral membrane	organic anions, nucleotide drugs (PMEA)	?
MRP5	*ABCC5*	SMRP, MOAT-C	3q27	1437	uniquitous, basolateral membrane	organic anions, nucleotide analogs, glutathione conjugates	?
MRP6	*ABCC6*	ARA, MLP1, MOAT-E	16p13.1	1503	liver, kidney, basolateral (and apical) membrane	anionic peptides (BQ-123)	Pseudoxanthoma elasticum
MRP7	*ABCC10*	EST182763	6p21	1513	?	?	?
MXR	*ABCG2*	BCRP, ABCP	4q22	655	placenta, liver, intestine, apical membrane	mitoxantrone, many other hydrophobic, amphiphilic drugs	drug resistance

by the overlap in substrate specificity between MRP and P-gp, and VP-16 being a better substrate for MRP than for P-gp. Interestingly, methotrexate was recently added to the list of transport substrates for MRP1 [165].

Clinical relevance?

The studies in P-gp served as a model for evaluating the role of MRP in clinical drug resistance. And, as with P-gp, the extent to which MRP plays a role in resistance has been difficult to define. It has been found that MRP messenger RNA (mRNA) is expressed in most cell and tumor types [177]. Levels of expression in tumors are frequently low, and correlative studies between expression and survival have frequently been unrewarding. This may be due to redundancy with the other MRPs, which have not been analyzed in the same tumor types. Whereas a functional MRP activity is detectable in acute myeloid leukemia, convincing evidence that it has a major impact on treatment outcome has not been forthcoming [178–181]. In non-small-cell lung cancer, high levels of MRP protein expression are observed in approximately one-third of cases, and in lung cancer cell lines, expression of MRP appears to correlate with resistance to MRP substrates including doxorubicin, vincristine and VP-16 [182–186]. In primary breast cancer, significant MRP protein expression has been reported in 25–30% of samples by immunohistochemistry, and in a series of 259 patients, appeared to confer an increased risk of treatment failure [187–189]. Recent development of an RT-PCR-ELISA, (reverse transcription-polymerase chain reaction multiplex assay with enzyme-linked immunosorbent assay) detection system failed to confirm that correlation in 85 patient samples [190]. Whereas expression of MRP has been detected in ovarian cancer, studies have both confirmed and rejected a correlation between outcome and expression [191–193]. Thus, the role of MRP in clinical drug resistance remains poorly defined. Asking the question in a clinical setting will require identification of an MRP-specific inhibitor. However, redundancy with the other MRPs may not allow direct determination of this question.

Interestingly, and in support of a role for MRP at least in intrinsic resistance, are the studies of Allen and others in mouse cell lines in which the ortholog for MDR1 (mdr1a and mdr1b) as well as MRP had been genetically deleted. In these studies, absence of the ortholog rendered cells severalfold times more sensitive than the expressing counterparts [194]. Knockout of MRP in these cells then resulted in an increase in sensitivity by as much as 20-fold to several drugs. Since the control cells express only low levels of MDR and MRP, it can be concluded that the low level found in unselected cells confers a significant amount of drug resistance.

MRP inhibitors

Reversal agents for MRP have not been as readily identified as for P-gp. Most of the second-generation P-gp antagonists have little or no effect on MRP-mediated drug efflux. In contrast the second-generation reversal agent VX710 does show antagonistic ability for MRP [195]. Like VX-710, the compound PAK-104P, a pyridine carboxylate, is able to inhibit both P-gp and MRP in in vitro models [196]. Thus, clinical trials could be designed to treat tumors in which both MRP and P-gp may be expressed, and drugs such as doxorubicin and VP-16 may be chosen as substrates for both transporters. MK571, a leukotriene LTD4 receptor antagonist which modulates MRP-mediated multidrug resistance, has been used as a research tool for the inhibition of MRP in vitro [197].

Functional characterization of the MRP family

Greater complexity has been added to the definition of the role of MRP in drug resistance by the identification of the six additional MRP family members. See the phylogenetic tree, figure 10. Little is known about the newest member, MRP7 [162]. The most well understood to date is MRP2 or cMOAT, which has been shown to be the bilirubin glucuronide transporter at the cannalicular membrane of the hepatocyte. Mutations in the cMOAT gene are responsible for the Dubin-Johnson syndrome of hyperbilirubinemia. Despite clear identification of this important role in normal physiology, convincing evidence for a role in drug resistance has not yet been forthcoming. There is a suggestion that cMOAT can confer resistance to cisplatin and to SN38, the active metabolite of CPT-11 [198, 199]. Recently, transfection experiments with human and rat MRP2 demonstrated that expression of MRP2 confers resistance to etoposide, vincristine, cisplatin, doxorubicin and epirubicin, and that this resistance could be partially reversed by BSO, suggesting a role of the intracellular glutathione level [200]. Antisense nucleotides to cMOAT sensitized cells 5-fold to cisplatin and over 10-fold to SN-38 [201]. Since cMOAT is normally involved in the pathway for heme metabolism, it is possible that it may be similarly involved in a mechanism of drug metabolism. Obvious overexpression of MRP2–5 was not found in a screen of cisplatin-resistant cell lines [202].

In a recent study by Bakos and others [203], both the ATPase and transport properties of MRP1 and MRP2 were compared. Leukotriene C4 and *N*-ethylmaleimide glutathione were transported by both proteins and stimulated their ATPase activity, MRP1 having the highest affinity for these substrates. On the other hand, MRP2 was a more efficient transporter of methotrexate. Interestingly, probenecid, sulfinpyrazone, indomethacin, furosemide and penicillin G activated the ATPase activity of MRP2, whereas they acted more as inhibitors of the MRP1-associated

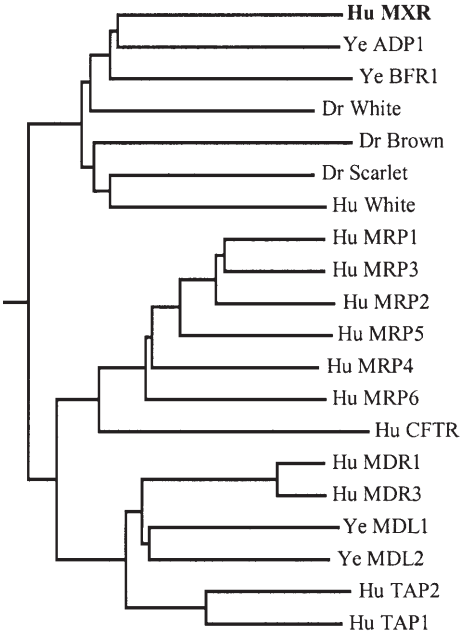

Figure 10. Phylogenetic tree showing the primary structure similarity between members of the ABC superfamily. The tree was constructed from a multiple alignment of ~130 amino acids spanning the Walker A to Walker B regions of the proteins. Abbreviations (with GenBank accession numbers): Dr Brown, *Drosophila melanogaster* Brown protein (P12428; Dr Scarlet, *Drosophila melanogaster* Scarlet Protein (P45843); Dr White, *Drosophila melanogaster* White protein (P10090); Hu CFTR, Human Cystic Fibrosis Transmembrane Conductance Regulator protein (P13569; Hu MDR1, MDR protein-1 gene product, P-glycoprotein (P08183); Hu MDR3, Human MDR3 gene product, phosphatidylcholine flippase (P21439); Hu MRP1, Human MRP1 gene product, MDR-associated protein (P33527); Hu MRP2, Human MRP2 gene product, cMOAT (Q92887); Hu MRP3, Human MRP3 gene product (O15438); Hu MRP4, Human MRP4 gene product (NM_005845); Hu MRP5, Human MRP5 gene product (U83661); Hu MRP6, Human MRP6 gene product (AF076622); Hu MXR1, Human mitoxantrone resistance associated protein (AF093771); Hu White, Human White protein homolog (P45844); Hu TAP1, Human antigen peptide transporter 1 (Q03518); Hu TAP2, Human antigen peptide transporter 2 (Q03519); Ye ADP1, *Saccharomyces cerevisiae* probable ATP-dependent permease precursor (P25371); Ye BFR1, *Schizosaccharomyces pombe* Brefeldin A resistance protein (P41820); Ye MDL1, *Saccharomyces cerevisiae* ATP-dependent permease (P33310); Ye MDL2, *Saccharomyces cerevisiae* ATP-dependant permease (P33311); Ye YOL075, *Saccharomyces cerevisiae* probable ATP-dependent transporter (Q08234).

ATPase. From this study it was concluded that MRP1 is a more efficiant glutathione conjugate transporter than MRP2, whereas the latter transports organic anions.

Transfection studies demonstrated that overexpression of MRP3 was able to confer drug resistance, whereas overexpression of MRP 5 was not [166, 167, 204, 205]. Like MRP1 and MRP2, MRP3 has been shown to transport adriamycin, vincristine, and methotrexate. MRP2 is expressed in the liver, on the cannalicular (apical) surface of the hepatocyte [206], whereas both MRP1 and MRP3 are found on the lateral membrane [205, 207–209]. The transport properties of the MRP proteins have been investigated in polarized cells. Thus, MRP5 expressed in MDCKII cells was routed to the basolateral membrane, and these cells transported both DNP-glutathione and glutathine towards the basal compartment [210]. MRP6, which apparently localizes to both the lateral and the canalicular membrane of hepatocytes, was found to transport an anionic cyclopentapeptide, BQ-123 [211].

Taken together, the findings suggest that the MRP subfamily of ABC transporters has a role in drug disposition from the liver, and probably from other normal tissues as well, with a lack of specificity that may allow subversion for drug resistance after the onset of the malignant process.

The mitoxantrone resistance protein

One protein, four names

Recently, a novel drug transporter gene was independently discovered and cloned by three different laboratories: Doyle and others applied the RNA fingerprinting technique to identify a breast cancer resistance protein (BCRP) in atypically multidrug resistant MCF-7 AdrVp cells [212], which had been selected in a combination of adriamycin and the P-gp inhibitor verapamil in order to identify mechanisms of drug resistance other than P-gp [213]. At the same time, Allikments and others mapped the gene to chromosome 4q22 and cloned this highly expressed gene from placenta, *ABCP* (ABC transporter in placenta) [214]. Simultaneously, at the National Cancer Institute, the same resistance gene was cloned by differential hybridization from the highly mitoxantrone-resistant human colon carcinoma cell line S1-M1-80 [215]; the gene was designated *MXR* for mitoxantrone resistance-associated gene. We prefer the latter name, since *MXR* is expressed in many other tissues than just breast and placenta, and because the major functional characteristic of the MXR protein is that it confers high-level resistance towards the anthracenedione drug mitoxantrone. Comparison of the three genes, *MXR, ABCP* and *BCRP,* reveals differences in the length of the 3′ untranslated region, and a polymorphism at amino acid position 482: G (MXR1)/R (ABCP)/T (BCRP).

Recently, the Human Gene Nomenclature Committee (HUGO) recommended that the *MXR* gene be designated *ABCG2,* it being the second member of the ABC superfamily subgroup G. The first member of this subfamily is *ABCG1* or *ABC8,* the human white homolog. All four synonyms for MXR can be found in the literature. For consistency, however, we will use the term ‘MXR’ throughout this review.

Structural characteristics of MXR

The *MXR* gene encodes a 655-amino acid, 72.1-kDa membrane protein with a N-terminal ATP binding

domain and a C-terminal transmembrane region consisting of six transmembrane domains. Thus, it is a half-transporter member of the ABC superfamily because it is only about half the size of full-length ABC transporters, which have two ATP binding sites and (at least) 12 transmembrane domains; for a structural comparison with P-gp and MRP, see figure 11. Half-transporters probably require dimerization in order to become functional full-transporter molecules.

The proteins with which MXR shares the highest sequence homology (see figure 10 for a phylogenetic tree), the white, scarlet and brown gene products of *Drosophila melanogaster*, form heterodimers. These transport eye pigment precursors into the eye pigment cells: a white-brown heterodimer transports guanine, whereas the substrate for the white-scarlet complex is tryptophan. Homodimers of these three proteins, on the other hand, apparently do not form functional transporters [216]. The ATP binding site of the white family members resides in the cytoplasm, whereas the substrate binding site is extracellular, as the transport complex pumps eye-pigment precursors across the plasma membrane and into the cell. Thus, the white family proteins differ in at least two ways from MXR: (i) from transfection experiments with MXR, we have reason to believe that MXR can form a functional homodimer for drug transport, and (ii) MXR apparently translocates its substrate from the cytoplasm (or plasma membrane) out of the cell, not into it.

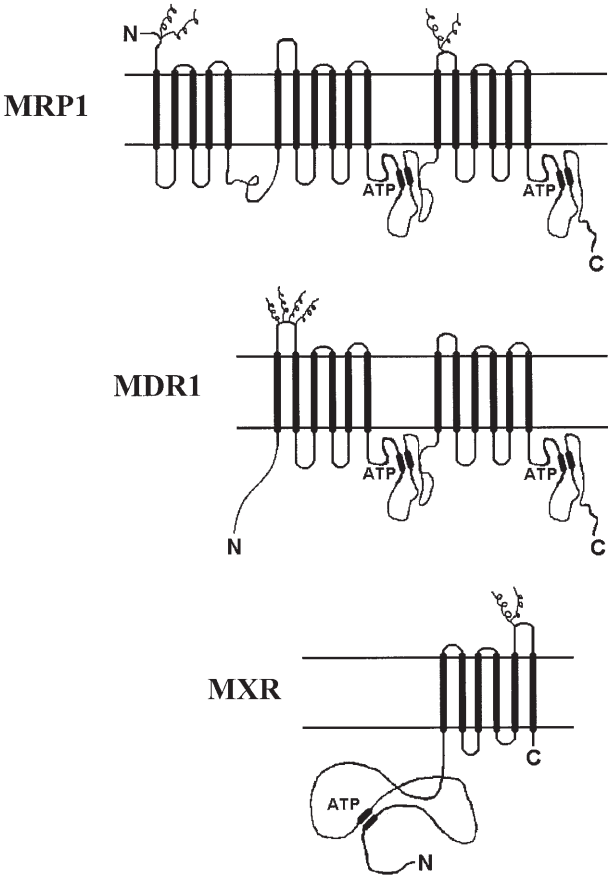

Figure 11. Models for the predicted structures of MRP1, MDR1 and MXR. The transmembrane helixes, the ATP-binding fold (filled bars) and glycosylation sites (ξ) are indicated. Note that the orientation of MXR is 'reverse' to that of P-gp (MDR1) and MRP1. The latter proteins are oriented in the order MSD-NBD-MSD-NBD, whereas the orientation of MXR is NBD-MSD (MSD, membrane spanning domain; NBD, nucleotide binding domain). It should be noted that these figures are highly schematic, and that the transmembrane segments probably form some kind of a pore in the membrane [29].

Another human half-transporter subfamily consists of the TAP1/TAP2 (the transporters associated with antigen presentation) heterodimer, which resides in the membrane of the endoplasmatic reticulum and actively transports peptides into this cellular compartment [217]. One might speculate that MXR, by analogy with TAP1/TAP2, might reside, physiologically, on the endoplasmatic reticulum to facilitate drug metabolism. Indeed, glucuronidation of anticancer drugs, such as mitoxantrone, the anthracyclines and SN-38 has been reported to occur in tumor cells [121, 218, 219]. In the case of the high expression levels found in many drug-resistant cells, the excess protein might redistribute to the plasma membrane, which indeed is what we observe by immunohistochemical staining for MXR protein expression.

The mouse homolog of the human *MXR* gene, *Bcrp1/Mxr/Abcp,* was recently cloned and shown to encode a 657-amino acid protein with 81% identity to human MXR [220]. Interestingly, a murine putative partner protein, Abcp2 or MXR2, has now been identified [221, 222]. In these studies, computer searches of the expressed sequence tag (EST) databases with the BLAST program led to the identification of several mouse and rat sequences that have high homology to *MXR* but that appear to encode a unique gene. RACE was used to amplify and clone the entire coding region of the gene using mouse spleen RNA, and the sequence revealed a single open reading frame encoding a 644-amino acid protein, designated Abcp2. Clear homology to MXR was seen throughout the coding region. Abcp2 is most closely related to MXR with 54% amino acid identity overall, 64% in the nucleotide binding domain and 50% in the transmembrane region. No human homologue for Abcp2 has been identified. However, in chromosome cytological studies, a murine BAC probe containing *Abcp2* localized with a single signal on human chromosome 8p12 [222]. It will be recalled that *MXR* itself resides on chromosome 4 at 4q22.

The *Abcp2* gene is, to date, clearly the gene most closely related to MXR. However, variability in mouse expression patterns between *Abcp1* and *Abcp2* suggest that the two are not obligate heterodimers. As for the human MXR protein, current transfection data [with complementary DNA (cDNA) encoding MXR only] suggest that

it is functional as a homodimer, and no putative dimerization partner has been identified so far. Also, cytogenetic analysis of *MXR*-overexpressing cell line showed that only the *MXR*-containing region of chromosome 4 was amplified, whereas coamplification of any other region was absent, supporting the notion that MXR functions as a homodimer [223]. Finally, expression of MXR in a heterologous expression system such as Sf9 insect cells, which are very unlikely to contain an endogenous partner protein, demonstrates MXR transport and ATPase activity [T. Litman, (unpublished data)].

MXR expression in cells and tissue

A number of different cell lines have now been shown to express MXR by Northern blotting [224] and immunostaining [225] techniques. Most of these cell lines have been selected in mitoxantrone, including several MCF-7 breast cancer cells, the 8226 myeloma cell line, S1-M1 and HT29RNOV colon cells, EPG85-257RNOV gastric cells, EPF86-079RNOV fibrosarcoma cells, and Igrov1 MX3 ovarian cancer cells. In addition, cells selected in topotecan, such as the Igrov T8 ovarian cells, and MCF-7 cells grown in flavopiridol [226] overexpress MXR, which is in agreement with the fact that both of these drugs are MXR transport substrates.

Using immunohistochemical staining, we have localized MXR to the plasma membrane of several tumor cell lines [227], and this observation has been confirmed by others [121, 225, 228]. We also see MXR expression in intracellular vesicles, which could be lysosomes. The tissue with highest expression of MXR is placenta (the syncytio-trophoblasts), followed by liver (in the canalicular membrane, as in the case for MRP2), the small intestine and colon (in the apical membrane, suggesting a secretory role), lung, kidney, adrenal, sweat glands, and the endothelia of veins and capillaries. Only low or no expression of MXR is seen in the brain, heart, stomach, prostate, spleen and cervix [S. Bates, (unpublished PCR data)].

MXR substrates

The resistance phenotype conferred by MXR appears to overlap considerably with that for P-gp (please refer to the Venn diagram, fig. 7), although with differing efficiencies. The substrates for which MXR has the highest affinity are mitoxantrone, topotecan, and, perhaps less so, flavopiridol, which conforms to the observation that these compounds select for cells overexpressing MXR. Another excellent transport substrate is prazosin and its fluorescent analog BODIPY-prazosin, which can be used in a functional FACS assay to screen for drugs that interact with MXR function [229]. Prazosin is also a P-gp substrate, and its analog IAAP has been used in an elegant photoaffinity labeling study of P-gp [91].

Several other fluorescent compounds are transported by MXR [227]. Among these are bisantrene, the anthracyclines, rhodamine-123 and LysoTracker Green, which is a stain for acidic compartments. Both rhodamine-123 and LysoTracker Green can be used in a FACS-based assay to measure accumulation of these drugs as a function of MXR activity [229].

MXR inhibitors

So far, only one potent MXR antagonist, fumitremorgin C (FTC), has been identified [230, 231]. FTC is derived from *Aspergillus fumigatus* cultures and, while being relatively nontoxic, is able to completely inhibit resistance at submicromolar concentrations in cell lines with highest levels of MXR-mediated resistance. More than 20 FTC analogs have been synthesized, but none of these have as strong an activity and high selectivity ($K_m < 1$ μM) for MXR as has FTC [232]. However, in a recent structure-activity relationship study of 42 fumitremorgin-type indolyl diketopiperazines, several potent analog were recognized [233]. Additional inhibitors have been identified, including the acridonecarboxamide P-gp antagonist GF120918 [81] and the dipyridamole analog BIB-E [234]. FTC and GF120918 induce the greatest increase in rhodamine accumulation, whereas the P-gp inhibitor PSC 833 has no effect.

MXR ATPase activity

We have studied the ATPase activity of MXR and found that it is variably modulated by different drugs [T. Litman, unpublished observations]. Most notably, the MXR ATPase is vanadate sensitive, and has a high background activity comparable to that of the P-gp ATPase, perhaps again due to the presence of an endogenous substrate. The ATPase activation profile, however, differs from that of P-gp: for example, verapamil at high concentrations is a weak inhibitor of the MXR ATPase (whereas it is one of the best activators of the P-gp ATPase). On the other hand, mitoxantrone, daunorubicin, prazosin and flavopiridol are all good activators of the MXR-associated ATPase activity, confirming their role as MXR transport substrates. Finally, the MXR reversing agent FTC is also a strong inhibitor of the MXR-associated ATPase activity, which confirms that this drug is an excellent transport inhibitor (but refer here to the discussion of substrates versus reversers of ATPase and transport in the P-gp section). Interestingly, FTC is a good activator of the P-gp ATPase [T. Litman, unpublished observations].

Clinical implications

MXR is expressed in the gastrointestinal tract; thus, it may affect the bioavailability of drugs as demonstrated for P-gp. One can also speculate that MXR might be important in the placenta, since it is highly expressed there. This notion is supported by recent experiments on pregnant mice, suggesting that MXR indeed has a function in the maternal-fetal barrier of the placenta; MXR was shown to mediate apically directed transport of topotecan, thus reducing its bioavailability and protecting the fetus against the drug [235]. Because MXR expression in cancer cells confers a multidrug-resistant phenotype, the relevance of this new protein in clinical drug resistance needs to be defined.

Future perspectives

The prevailing paradigm for the last decade has been that P-gp-mediated drug resistance could account for the refractory nature of many cancers. The broad nature of clinical resistance in cancer suggests, however, that intrinsic resistance is likely to be multifactorial. As we have seen, some of these possibilities include the existence of an array of ABC transporters, such as the MRP1 and MXR proteins, able to transport a wider range of substrates than encompassed by P-gp alone. While such drug transporters will directly affect intracellular concentrations of drug, other mechanisms of drug resistance have been identified that confer resistance at the level of cell survival pathways, drug metabolism and the drug target. Many of these have been examined in solid cancers, but the findings are preliminary and still require validation. There is also a line of investigation which suggests that drug delivery may be impaired in certain tumors. Factors that influence drug delivery include blood flow, permeability of tumor vasculature and drug diffusion into the interstitium, which is affected both by properties of the drug and by interstitial pressure within the tumor. In addition, tumors in patients frequently are nutrient deprived and hypoxic, factors which promote drug resistance as they reduce the progress of cells through the cell cycle. These 'tumor factors' have not been systematically examined. The existence of profound resistance in even the tiniest metastatic nodules suggests, however, that cellular mechanisms will prove to be of great importance. But impaired drug delivery to the cell interior, brought about by the MDR transport systems, will always need to be overcome, even when other cellular mechanisms are identified and become targets for chemotherapy. Thus, it is most likely that new agents directed against novel targets will be subject to the same mechanisms of resistance that have plagued treatment of this disease for decades.

A detailed understanding of drug resistance in the solid cancers, and how to overcome it, is therefore a major challenge for the new millennium. Many questions remain to be answered: How can one design the best combination of chemotherapy? Should we select agents that are not transport substrates for these redundant mechanisms? Which reversal agent(s) is the best choice for a given tumor? And how can we deal with and predict the possible side effects due to therapy? Some of these questions may be addressed with the recent developments in genotyping and proteomics. Today, it is possible to analyze a tumor at the time of its diagnosis. Thus, it is possible to measure the expression levels of relevant drug resistance proteins (these also include topoisomerases and apoptosis proteins), and on the basis of this knowledge to tailor an 'individual' treatment protocol, which should be aimed at reversing resistance upfront.

As we have seen, the analysis of P-gp structure and function, and the definition and testing of its antagonists for use in the clinic, has taken more than a decade. That progress was painstaking and still has not fully answered the question of the importance of P-gp in clinical drug resistance. However, these studies with P-gp have provided the experience necessary to shorten the process of defining the structure and function of the newer transporters, and will aid in the identification and adoption of appropriate antagonists. The process of moving from bench to bedside will thus be abbreviated, and should result in properly designed trials in the optimal clinical setting.

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1 Stein W. D. (1967) The Movement of Molecules across Cell Membranes, Academic Press, New York
2 Stein W. D. (1986) Transport and Diffusion across Cell Membranes, Academic Press, San Diego
3 Higgins C. F., Gallagher M. P., Mimmack M. M. and Pearce S. R. (1988) A family of closely related ATP-binding subunits from prokaryotic and eukaryotic cells. Bioessays **8:** 111–116
4 Walker J. E., Saraste M., Runswick M. J. and Gay N. J. (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:** 945–951
5 Danø K. (1972) Cross resistance between vinca alkaloids and anthracyclines in Ehrlich ascites tumor in vivo. Cancer Chemother. Rep. **56:** 701–708
6 Danø K. (1973) Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. Biochim. Biophys. Acta **323:** 466–483
7 Skovsgaard T., Danø K. and Nissen N. I. (1984) Chemosensitizers counteracting acquired resistance to anthracyclines and vinca alkaloides in vivo. A new treatment principle. Cancer Treat. Rev. **11:** 63–72
8 Biedler J. L. and Riehm H. (1970) Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic and cytogenetic studies. Cancer Res. **30:** 1174–1184

9 Beck W. T., Mueller T. J., and Tanzer L. R. (1979) Altered surface membrane glycoproteins in vinca alkaloid-resistant human leukemic lymphoblasts. Cancer Res. **39:** 2070–2076

10 Ling V. (1975) Drug resistance and membrane alteration in mutants of mammalian cells. Can. J. Genet. Cytol. **17:** 503–515

11 Juliano R. L. and Ling V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim. Biophys. Acta **455:** 152–162

12 Chen C., Chin J. E., Ueda K., Clark D. P., Pastan I., Gottesman M. M. et al. (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell **47:** 381–389

13 Gros P., Croop J. and Housman D. E. (1986) Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell **47:** 371–380

14 Tsuruo T., Iida H., Tsukagoshi S. and Sakurai Y. (1981) Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res. **41:** 1967–1972

15 Tsuruo, T., Iida, H., Tsukagoshi, S., and Sakurai, Y. (1982) Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res. **42:** 4730–4733

16 Tsuruo T., Iida H., Nojiri M., Tsukagoshi S., and Sakurai Y. (1983) Circumvention of vincristine and adriamycin resistance in vitro and in vivo by calcium channel blockers. Cancer Res. **43:** 2905–2910

17 Tsuruo T., Iida H., Kitatani Y., Yokota K., Tsukagoshi S. and Yakurai Y. (1984) Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. Cancer Res. **44:** 4303–4307

18 Sarkadi B., Price E. M., Boucher R. C., Germann U. A. and Scarborough G. A. (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J. Biol. Chem. **267:** 4854–4858

19 Litman T., Nielsen D., Skovsgaard T., Zeuthen T., and Stein W. D. (1997) ATPase activity of P-glycoprotein related to emergence of drug resistance in Ehrlich ascites tumor cell lines. Biochim. Biophys. Acta **1361:** 147–158

20 Senior A. E., Al-Shawi M. K. and Urbatsch, I. L. (1995) ATP hydrolysis by multidrug-resistance protein from Chinese hamster ovary cells. J. Bioenerg. Biomembr. **27:** 31–36

21 Ambudkar S. V., Lelong I. H., Zhang J., Cardarelli C. O., Gottesman M. M. and Pastan I. (1992) Partial purification and reconstitution of the human multidrug-resistance pump: Characterization of the drug-stimulatable ATP hydrolysis. Proc. Natl. Acad. Sci. USA **89:** 8472–8476

22 Sharom F. J., Yu X. and Doige C. A. (1993) Functional reconstitution of drug transport and ATPase activity in proteoliposomes containing partially purified P-glycoprotein. J. Biol. Chem. **268:** 24197–24202

23 Ambudkar S. V., Dey S., Hrycyna C. A., Ramachandra M., Pastan I. and Gottesman M. M. (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu. Rev. Pharmacol. Toxicol. **39:** 361–398

24 Müller M., Bakos É., Welker I., Váradi A., Germann U. A., Gottesman M. M. et al. (1996) Altered drug-stimulated ATPase activity in mutants of the human multidrug resistance protein. J. Biol. Chem. **271:** 1877–1883

25 Ford J. M. and Hait W. N. (1990) Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev. **42:** 155–199

26 Loo T. W. and Clarke D. M. (1997) Drug-stimulated ATPase activity of human P-glycoprotein requires movement between transmembrane segments 6 and 12. J. Biol. Chem. **272:** 20986–20989

27 Loo T. W. and Clarke D. M. (1999) Identification of residues in the drug-binding domain of human P-glycoprotein. J. Biol. Chem. **274:** 35388–35392

28 Loo T. W. and Clarke D. M. (2000) Identification of residues within the drug-binding domain of the human multidrug resistance P-glycoprotein by cysteine-scanning mutagenesis and reaction with dibromobimane. J. Biol. Chem. **275:** 39272–39278

29 Loo T. W. and Clarke D. M. (2000) The packing of the transmembrane segments of human multidrug resistance P-glycoprotein is revealed by disulfide cross-linking analysis. J. Biol. Chem. **275:** 5253–5256

30 Liu R. and Sharom F. J. (1996) Site-directed fluorescence labeling of P-glycoprotein on cysteine residues in the nucleotide binding domains. Biochemistry **35:** 11865–11873

31 Sonveaux N., Shapiro A. B., Goormaghtigh E., Ling V. and Ruysschaert J. M. (1996) Secondary and tertiary structure changes of reconstituted P- glycoprotein. A Fourier transform attenuated total reflection infrared spectroscopy analysis. J. Biol. Chem. **271:** 24617–24624

32 Sonveaux N., Vigano C., Shapiro A. B., Ling V. and Ruysschaert J. M. (1999) Ligand-mediated tertiary structure changes of reconstituted P- glycoprotein. A tryptophan fluorescence quenching analysis. J. Biol. Chem. **274:** 17649–17654

33 Wang G., Pincheira R., Zhang M. and Zhang J.-T. (1997) Conformational changes of P-glycoprotein by nucleotide binding. Biochem. J. **328:** 897–904

34 Wang G., Pincheira R. and Zhang J. T. (1998) Dissection of drug-binding-induced conformational changes in P-glycoprotein. Eur. J. Biochem. **255:** 383–390

35 Mechetner E. B. and Roninson I. B. (1992) Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. Proc. Natl. Acad. Sci. USA **89:** 5824–5828

36 Hamada H. and Tsuruo T. (1986) Functional role for the 170- to 180-kDa glycoprotein specific to drug- resistant tumor cells as revealed by monoclonal antibodies. Proc. Natl. Acad. Sci. USA **83:** 7785–7789

37 Mechetner E. B., Schott B., Morse B. S., Stein W. D., Druley T., Davis K. A. et al. (1997) P-glycoprotein function involves conformational transitions detectable by differential immunoreactivity. Proc. Natl. Acad. Sci. USA **94:** 12908–12913

38 Schinkel A. H., Arceci R. J., Smit J. J., Wagenaar E., Baas F., Dolle M. et al. (1993) Binding properties of monoclonal antibodies recognizing external epitopes of the human MDR1 P-glycoprotein. Int. J. Cancer **55:** 478–484

39 Zhou Y., Gottesman M. M. and Pastan I. (1999) The extracellular loop between TM5 and TM6 of P-glycoprotein is required for reactivity with monoclonal antibody UIC2. Arch. Biochem. Biophys. **367:** 74–80

40 Georges E., Tsuruo T., and Ling V. (1993) Topology of P-glycoprotein as determined by epitope mapping of MRK-16 monoclonal antibody. J. Biol. Chem. **268:** 1792–1798

41 Kaye S. and Merry S. (1985) Tumour cell resistance to anthracyclines: a review. Cancer Chemother. Pharmacol. **14:** 96–103

42 Bogush T., Smirnova G., Shubina I., Syrkin A. and Robert J. (1995) Direct evaluation of intracellular accumulation of free and polymer-bound anthracyclines. Cancer Chemother. Pharmacol. **35:** 501–505

43 Mülder H. S., Dekker H., Pinedo H. M., and Lankelma J. (1995) The P-glycoprotein-mediated relative decrease in cytosolic free drug concentration is similar for several anthracyclines with varying lipophilicity. Biochem. Pharmacol. **50:** 967–974

44 Zhang X. P., Ritke M. K., Yalowich J. C., Slovak M. L., Ho J. P., Collins K. I. et al. (1994) P-glycoprotein mediates profound resistance to bisantrene. Oncol. Res. **6:** 291–301

45 Taylor C. W., Dalton W .S., Parrish P. R., Gleason M. C., Bellamy W. T., Thompson F. H. et al. (1991) Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF7 human breast cancer cell line. Br. J. Cancer **63:** 923–929

46 Horio M., Gottesman M. M. and Pastan I. (1988) ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proc. Natl. Acad. Sci. USA **85:** 3580–3584

47 Fojo A. T., Akiyama S., Gottesman M. M. and Pastan I. (1985) Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. Cancer Res. **45:** 3002–3007

48 Hoki Y., Fujimori A. and Pommier Y. (1997) Differential cytotoxicity of clinically important camptothecin derivatives in P-glycoprotein-overexpressing cell lines. Cancer Chemother. Pharmacol. **40:** 433–438

49 Maare C., Nielsen D. and Skovsgaard T. (1995) Comparison of the accumulation of the epipodophyllotoxins VP-16 and VM-26 in a sensitive and a multidrug-resistant Ehrlich ascites tumour cell line detected by HPLC. Cell Pharmacol. **2:** 35–42

50 Doige C. A. and Sharom F. J. (1992) Transport properties of P-glycoprotein in plasma membrane vesicles from multidrug-resistant Chinese hamster ovary cells. Biochim. Biophys. Acta **1109:** 161–171

51 Gottesman M. M. and Pastan I. (1993) Modulation of the multidrug resistance phenotype. Cell Pharm. **1:** S111–S112

52 Horio M., Chin K.-V., Currier S. J., Goldenberg S., Williams C., Pastan I. et al. (1989) Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney cell epithelia. J. Biol. Chem. **264:** 14880–14884

53 Lee C. G. L., Gottesman M. M., Cardarelli C. O., Ramachandra M., Jeang K.-T., Ambudkar S. V. et al. (1998) HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. Biochemistry **37:** 3594–3601

54 Holló Z., Homolya L., Davis C. W. and Sarkadi B. (1994) Calcein accumulation as a fluorometric functional assay of the multidrug transporter. Biochim. Biophys. Acta **1191:** 384–388

55 Brezden C. B., Hedley D. W. and Rauth A. M. (1994) Constitutive expression of P-glycoprotein as a determinant of loading with fluorescent calcium probes. Cytometry **17:** 343–348

56 Homolya L., Holló Z., Germann U. A., Pastan I., Gottesman M. M. and Sarkadi B. (1994) Fluorescent cellular indicators are extruded by the multidrug resistance protein. J. Biol. Chem. **268:** 21493–21496

57 Kessel D., Beck W. T., Kukuruga D.,and Schulz V. (1991) Characterization of multidrug resistance by fluorescent dyes. Cancer Res. **51:** 4665–4670

58 Shapiro A. B. and Ling V. (1997) Extraction of Hoechst 33342 from the cytoplasmic leaflet of the plasma membrane by P-glycoprotein. Eur. J. Biochem. **250:** 122–129

59 Ganapathi R., Grabowski D., Turinic R. and Valenzuela R. (1984) Correlation between potency of calmodulin inhibitors and effects on cellular levels and cytotoxic activity of doxorubicin (adriamycin) in resistant P388 mouse leukemia cells. Eur. J. Cancer Clin. Oncol. **20:** 799–806

60 Ford J. M., Prozialeck W. C. and Hait W. N. (1989) Structural features determinig activity of phenotiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance. Mol. Pharmacol. **35:** 105–115

61 Ford J. M., Bruggemann E. P., Pastan I., Gottesman M. M. and Hait W. N. (1990) Cellular and biochemical characterization of thioxanthenes for reversal of multidrug resistance in human and murine cell lines. Cancer Res. **50:** 1748–1756

62 Strobl J. S. and Peterson V. A. (1992) Tamoxifen-resistant human breast cancer cell growth: inhibition by thioridazine, pimozide and the calmodulin antagonist, W-13. J. Pharmacol. Exp. Therap. **263:** 186–193

63 Chauffert B., Martin M., Hammann A., Michel M. F. and Martin F. (1986) Amiodarone-induced enhancement of doxorubicin and 4'-deoxyrubicin cytotoxicity to rat colon cancer cells in vitro and in vivo. Cancer Res. **46:** 825–830

64 Chiba P., Burghofer S., Richter E., Tell B., Moser A. and Ecker G. (1995) Synthesis, pharmacologic activity, and structure-activity relationships of a series of propafenone-related modulators of multidrug resistance. J. Medicin. Chem. **38:** 2789–2793

65 Pearce H. L., Safa A. R., Bach N. J., Winter M. A., Cirtain M. C. and Beck W. T. (1989) Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance. Proc. Natl. Acad. Sci. USA **86:** 5128–5132

66 Zamora J. M., Pearce H. L. and Beck W. T. (1988) Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. Mol. Pharmacol. **33:** 454–462

67 Yang C.-P.H., DePinho S. H., Greenberger L. M., Arceci R. J. and Horwitz S. B. (1989) Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. J. Biol. Chem. **264:** 782–788

68 Barnes K. M., Dickstein B., Cutler G. B. Jr, Fojo A. T., and Bates S. E. (1996) Steroid transport, accumulation, and antagonism of P-glcoprotein in multidrug-resistant cells. Biochemistry **35:** 4820–4827

69 Ramu A., Glaubiger D. and Fuks Z. (1984) Reversal of acquired resistance to doxorubicin in P388 murine leukemia cells by tamoxifen and other triparanol analogues. Cancer Res. **44:** 4392–4395

70 Meador J., Sweet P., Stupecky M., Wetzel M., Murray S., Gupta S. et al. (1987) Enhancement by cyclosporin A of daunorubicin efficacy in Ehrlich ascites carcinoma and murine hepatoma 129. Cancer Res. **47:** 6216–6219

71 Boesch D., Muller K., Pourtier-Manzanedo A., and Loor F. (1991) Restoration of daunomycin retention in multidrug-resistant P388 cells by submicromolar concentrations of SDZ PSC 833, a nonimmunosuppressive cyclosporin derivative. Exp. Cell Res. **196:** 26–32

72 Borrel M. N., Pereira E., Fiallo M. and Garnier-Suillerot A. (1994) Mobile ionophores are a novel class of P-glycoprotein inhibitors. Eur. J. Biochem. **223:** 125–133

73 Assaraf Y. G. and Borgnia M. (1994) Probing the interaction of the multidrug-resistance phenotype with the polypeptide ionophore gramicidin D via functional channel formation. Eur J. Biochem. **222:** 813–824

74 Patel R. B. and Welling N. J. (1982) High-pressure liquid chromatographic determination of promethazine plasma levels in the dog after oral, intramuscular and intravenous dosage. J. Pharmaceut. Sci. **71:** 529–532

75 Hait W. N., Gesmonde J. F., Murren J. R., Yang J. M., Chen H. X. and Reiss M. (1993) Terfenadine (Seldane): a new drug for restoring sensitivity to multidrug resistant cancer cells. Biochem. Pharmacol. **45:** 401–406

76 ter Kuile F. O., Nosten F., Thieren M., Luxemburger C., Edstein M. D., Chongsuphajaisiddhi T. et al. (1992) High-dose mefloquine in the treatment of multidrug-resistant falciparum malaria. J. Infect. Dis. **166:** 1393–1400

77 Weaver J. L., Szabo G. Jr, Pine P. S., Gottesman M. M. and Goldenberg S. (1993) The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. Int. J. Cancer **54:** 456–461

78 Breyer-Pfaff U., Gaertner H. J. and Giedke H. (1982) Plasma levels, psychophysiological variables and clinical response to amitriptyline. Psychiatry. Res. **6:** 223–234

79 Howell S. B., Hom D. K., Sanga R., Vick J. S. and Chan T. C. K. (1989) Dipyridamole enhancement of etoposide sensitivity. Cancer Res. **49:** 4147–4153

80 Thornes R. D. and Sheehan M. V. (1966) Fucidin therapy in carcinoma of the breast. (Preliminary report). J. Irish Med. Assoc. **58:** 52–54

81 de Bruin M., Miyake K., Litman T., Robey R. and Bates S. E. (1999) Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. Cancer Lett. **146:** 117–126

82 Skovsgaard T. (1980) Circumvention of resistance to daunorubicin by N-acetyldaunorubicin in Ehrlich ascites tumor. Cancer Res. **40:** 1077–1083

83 Bates S. E., Lee J. S., Dickstein B., Spolyar M. and Fojo A. T. (1993) Differential modulation of P-glycoprotein transport by protein kinase inhibition. Biochemistry **32:** 9156–9164

84 Alvarez M., Paull K., Monks A., Hose C., Lee J.-S., Weinstein J. et al. (1995) Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the National Cancer Institute anticancer drug screen. J. Clin. Invest. **95:** 2205–2214

85 Lee J.-S., Paull K., Alvarez M., Hose C., Monks A., Grever M. et al. (1994) Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. Mol. Pharmacol. **46:** 627–638

86 Scala S., Akhmed N., Rao U. S., Paull K., Lan L. B., Dickstein B. et al. (1997) P-glycoprotein substrates and antagonists cluster into two distinct groups. Mol. Pharmacol. **51:** 1024–1033

87 Litman T., Zeuthen T., Skovsgaard T. and Stein W. D. (1997) Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity. Biochim. Biophys. Acta **1361:** 159–168

88 Litman T., Zeuthen T., Skovsgaard T. and Stein W. D. (1997) Competitive, non-competitive and cooperative interactions between substrates of P-glycoprotein as measured by its ATPase activity. Biochim. Biophys. Acta **1361:** 169–176

89 Shapiro A. B. and Ling V. (1997) Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. Eur. J. Biochem. **250:** 130–137

90 Shapiro A. B., Fox K., Lam P. and Ling V. (1999) Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. Eur. J. Biochem. **259:** 841–850

91 Dey S., Ramachandra M., Pastan I., Gottesman M. M. and Ambudkar S. V. (1997) Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. PNAS **94:** 10594-10599

92 Martin C., Berridge G., Higgins C. F., Mistry P., Charlton P., and Callaghan R. (2000) Communication between multiple drug binding sites on P-glycoprotein. Mol Pharmacol **58**: 624–632

93 Germann U. A., Chambers T. C., Ambudkar S. V., Licht T., Cardarelli C. O., Pastan I. et al. (1996) Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. J. Biol. Chem. **271:** 1708–1716

94 Greenberger L. M. (1993) Major photoaffinity drug labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. J. Biol. Chem. **268:** 11417–11425

95 Bruggemann E. P., Currier S. J., Gottesman M. M. and Pastan I. (1992) Characterization of the azidopine and vinblastine binding site of P-glycoprotein. J. Biol. Chem. **267:** 21020–21026

96 Ayesh S., Shao Y.-M. and Stein W. D. (1996) Co-operative, competitive and non-competitive interactions between modulators of P-glycoprotein. Biochim. Biophys. Acta **1316:** 8–18

97 Shao Y. M., Ayesh S. and Stein W. D. (1997) Mutually cooperative interactions between modulators of P-glycoprotein. Biochim. Biophys. Acta **1360:** 30–38

98 Stein W. D. (1997) Kinetics of the multidrug transporter (P-glycoprotein) and its reversal. Physiol. Rev. **77:** 545–590

99 Druley T. E., Stein W. D. and Roninson I. B. (2001) Analysis of MDR1 P-glycoprotein conformational changes in permeabilized cells using differential immunoreactivity. Biochemistry, in press

100 Druley T. E., Stein W. D., Ruth A. C. and Roninson I. B. (2001) P-glycoprotein mediated colchicine resistance in different cell lines correlates with the effects of colchicine on P-glycoprotein conformation, Biochemistry, in press

101 DiDiodato G. and Sharom F. J. (1997) Interaction of combinations of drugs, chemosensitizers and peptides with the P-glycoprotein multidrug transporter. Biochem. Pharmacol. **53:** 1789–1797

102 Raviv Y., Pollard H. B., Bruggemann E. P., Pastan I. and Gottesman M. M. (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. J. Biol. Chem. **265:** 3975–3980

103 Tang-Wai D. F., Kajiji S., DiCapua F., de Graaf D., Roninson I. B. and Gros P. (1995) Human (MDR1) and mouse (mdr1, mdr3) P-glycoproteins can be distinguished by their respective drug resistance profiles and sensitivity to modulators. Biochemistry **34:** 32–39

104 Choi K., Chen C., Kriegler M. and Roninson I. B. (1988) An altered pattern of cross-resistance in multidrug-resistant human cells results from spontaneous mutations in the mdr1 (P- glycoprotein) gene. Cell **53:** 519–529

105 Choi K., Frommel T., Stern R., Perez C., Kriegler M., Tsuruo T. et al. (1991) Multidrug resistance after retroviral transfer of the human MDR1 gene correlates with P-glycoprotein density in the plasma membrane and is not affected by cytotoxic selection. Proc. Natl. Acad. Sci. USA **88:** 7386–7390

106 Shapiro A. B., Corder A. B. and Ling V. (1997) P-glycoprotein-mediated Hoechst 33342 transport out of the lipid bilayer. Eur. J. Biochem. **250:** 115–121

107 Shapiro A. B. and Ling V. (1998) Transport of LDS-751 from the cytoplasmic leaflet of the plasma membrane by the rhodamine-123-selective site of P-glycoprotein. Eur. J. Biochem. **254:** 181–188

108 Saeki T., Shimabuku A. M., Ueda K. and Komano, T. (1992) Specific drug binding by purified lipid-reconstituted P-glycoprotein: dependence on the lipid composition. Biochim. Biophys. Acta **1107:** 105–110

109 Zordan-Nudo T., Ling V., Liu Z. and Georges E. (1993) Effects of non-ionic detergents on P-glycoprotein drug binding and reversal of multidrug resistance. Cancer Res. **53:** 5994–6000

110 Urbatsch I. L. and Senior A. E. (1995) Effects of lipids on ATPase activity of purified Chinese hamster P-glycoprotein. Arch. Biochem. Biophys. **316:** 135–140

111 Callaghan R., Berridge G., Ferry D. R. and Higgins C. F. (1997) The functional purification of P-glycoprotein is dependent on maintenance of a lipid-protein interface. Biochim. Biophys. Acta **1328:** 109–124

112 Romsicki Y. and Sharom F. J. (1999) The membrane lipid environment modulates drug interactions with the P- glycoprotein multidrug transporter. Biochemistry **38:** 6887–6896

113 Zhang J. T. and Ling V. (1995) Involvement of cytoplasmic factors regulating the membrane orientation of P-glycoprotein sequences. Biochemistry **34:** 9159–9165

114 Willingham M. C., Cornwell M. M., Cardarelli C. O., Gottesman M. M. and Pastan, I. (1986) Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: effects of verapamil and other drugs. Cancer Res. **46:** 5941–5946

115 Cass C. E., Janowska-Wieczorek A., Lynch M. A., Sheinin H., Hindenburg A. A. and Beck W. T. (1989) Effect of duration of

exposure to verapamil on vincristine activity against multidrug-resistant human leukemic cell lines. Cancer Res. **49:** 5798–5804
116 Schlemmer S. R. and Sirotnak F. M. (1994) Functional studies of P-glycoprotein in inside-out plasma membrane vesicles derived from murine erythroleukemia cells overexpressing MDR3. J. Biol. Chem. **269:** 31059–31066
117 Horio M., Lovelace E., Pastan I. and Gottesman M. M. (1991) Agents which reverse multidrug-resistance are inhibitors of [^{3}H]vinblastine transport by isolated vesicles. Biochim. Biophys. Acta **1061:** 106–110
118 Shapiro, A.B. and Ling, V. (1994) ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. J. Biol. Chem. **269:** 3745–3754
119 Urbatsch I. L., Al-Shawi M. K. and Senior A. E. (1994) Characterization of the ATPase activity of purified Chinese hamster P-glycoprotein. Biochemistry **33:** 7069–7076
120 Sharom F. J., Yu X., Chu J. W. K. and Doige C. A. (1995) Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant Chinese hamster ovary cells. Biochem. J. **308:** 381–390
121 Brangi M., Litman T., Ciotti M., Nishiyama K., Kohlhagen G., Takimoto C. et al. (1999) Camptothecin resistance: role of the ATP-binding cassette (ABC), mitoxantrone-resistance half-transporter (MXR), and potential for glucuronidation in MXR-expressing cells. Cancer Res. **59:** 5938–46
122 Lelong I. H., Guzikowski A. P., Haugland R. P., Pastan I., Gottesman M. M.,and Willingham M. C. (1991) Fluorescent verapamil derivative for monitoring activity of the multidrug transporter. Mol. Pharmacol. **40:** 490–494
123 Ayesh S., Litman T. and Stein W. D. (1997) Drug accumulation in the presence of the multidrug resistance pump: dissociation between verapamil accumulation and the action of P-glycoprotein. Receptors and Channels **5:** 175–183
124 Sehested M., Skovsgaard T., Jennings M. L., Demant E. J. F., Friche E. and Bindslev N. (1990) Transport of the multidrug resistance modulators verapamil and azidopine in wild type and daunorubicin resistant Ehrlich ascites tumour cells. Br. J. Cancer **62:** 37–41
125 Eytan G. D. and Kuchel P. W. (1999) Mechanism of action of P-glycoprotein in relation to passive membrane permeation. Int. Rev. Cytol. **190:** 175–250
126 Eytan G. D., Regev R., Oren G. and Assaraf Y. G. (1996) The role of passive transbilayer drug movement in multidrug resistance and its modulation. J. Biol. Chem. **271:** 12897–12902
127 Saeki T., Ueda K., Tanigawara Y., Hori R., and Komano T. (1993) Human P-glycoprotein transports cyclosporin A and FK506. J. Biol. Chem. **268:** 6077–6080
128 Shirai A., Naito M., Tatsuta T., Dong J., Hanaoka K., Mikami K. et al. (1994) Transport of cyclosporin A across the brain capillary endothelial cell monolayer by P-glycoprotein. Biochim. Biophys. Acta **1222:** 400–404
129 Schramm U., Fricker G., Wenger R. and Miller D. S. (1995) P-glycoprotein-mediated secretion of a fluorescent cyclosporin analogue by teleost renal proximal tubules. Am. J. Physiol. **268:** F46–F52
130 Orlowski S. and Garrigos M. (1999) Multiple recognition of various amphiphilic molecules by the multidrug resistance P-glycoprotein: molecular mechanisms and pharmacological consequences coming from funcional interactions between various drugs. Anticancer Res. **19:** 3109–3124
131 Eneroth,A., Astrom E., Hoogstraate J., Schrenk D., Conrad S., Kauffmann H. et al. (2001) Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. Eur. J. Pharm. Sci. **12:** 205–214
132 Essodaïgui M., Broxterman H. J., and Garnier-Suillerot A. (1998) Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and P-glycoprotein. Biochemistry **37:** 2243–2250
133 Colin M., Madoulet C., Baccard N., Arsac F. and Jardillier J.C. (1994) Study of sodium orthovanadate as a reverser of multidrug resistance on lymphoblastic leukemic CEM/VLB100 cells. Anticancer Res. **14:** 2383–2387
134 Al-Shawi M. K., Urbatsch I. L. and Senior A. E. (1994) Covalent inhibitors of P-glycoprotein ATPase activity. J. Biol. Chem. **269:** 8986–8992
135 Goodno C. C. (1979) Inhibition of myosin ATPase by vanadate ion. Proc. Natl. Acad. Sci. USA **76:** 2620–2624
136 Urbatsch I. L., Sankaran B., Weber J. and Senior A. E. (1995) P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. J. Biol. Chem. **270:** 19383–19390
137 Urbatsch I. L., Sankaran B., Bhagat S. and Senior A. E. (1995) Both P-glycoprotein nucleotide-binding sites are catalytically active. J. Biol. Chem. **270:** 26956–26961
138 Loo T. W. and Clarke D. M. (1995) Rapid purification of human P-glycoprotein mutants expressed transiently in HEK 293 cells by nickel-chelate chromatography and characterization of their drug-stimulated ATPase activities. J. Biol. Chem. **270:** 21449–21452
139 Hrycyna C. A., Ramachandra M., Germann U. A., Cheng P. W., Pastan I. and Gottesman M. M. (1999) Both ATP sites of human P-glycoprotein are essential but not symmetric. Biochemistry **38:** 13887–13899
140 Szabó K., Welker E., Bakos É., Müller M., Roninson I. B., Váradi A. (1998) Drug-stimulated nucleotide trapping in the human multidrug transporter MDR1. J. Biol. Chem. **273:** 10132–10138
141 Ramachandra M., Ambudkar S. V., Chen D., Hrycyna C. A., Dey S., Gottesman M. M. et al. (1998) Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. Biochemistry **37:** 5010–5019
142 Eytan G. D., Regev R. and Assaraf Y. G. (1996) Functional reconstitution of P-glycoprotein reveals an apparent near stoichiometric drug transport to ATP hydrolysis. J. Biol. Chem. **271:** 3172–3178
143 Ambudkar S. V., Cardarelli C. O., Pashinsky I. and Stein W. D. (1997) Relation between the turnover number for vinblastine transport and for vinblastine-stimulated ATP hydrolysis by human P-glycoprotein. J. Biol. Chem. **272:** 21160–21166
144 Shapiro A. B. and Ling V. (1998) Stoichiometry of coupling of rhodamine 123 transport to ATP hydrolysis by P-glycoprotein. Eur. J. Biochem. **254:** 189–193
145 Loo T. W. and Clarke D. M. (1993) Functional consequences of proline mutations in the predicted transmembrane domain of P-glycoprotein. J. Biol. Chem. **268:** 3143–3149
146 Urbatsch I. L., Beaudet L., Carrier I., and Gros P. (1998) Mutations in either nucleotide-binding site of P-glycoprotein (Mdr3) prevent vanadate trapping of nucleotide at both sites. Biochemistry **37:** 4592–4602
147 Azzaria M., Schurr E. and Gros P. (1989) Discrete mutations introduced in the predicted nucleotide-binding sites of the mdr1 gene abolish its ability to confer multidrug resistance. Mol. Cell Biol. **9:** 5289–5297
148 Loo T. W. and Clarke D. M. (1995) Membrane topology of a cysteine-less mutant of human P-glycoprotein. J. Biol. Chem. **270:** 843–848
149 Senior A. E., Al-Shawi M. K. and Urbatsch I. L. (1995) The catalytic cycle of P-glycoprotein. FEBS Lett. **377:** 285–289
150 Sauna Z. E. and Ambudkar S. V. (2000) Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. Proc. Natl. Acad. Sci. USA **97:** 2515–2520
151 van Veen H. W., Margolles A., Muller M., Higgins C. F., and Konings W. N. (2000) The homodimeric ATP-binding cassette transporter LmrA mediates multidrug transport by an alter-

nating two-site (two-cylinder engine) mechanism. EMBO J. **19:** 2503–2514
152 Goodfellow H. R., Sardini A., Ruetz S., Callaghan R., Gros P., McNaughton P. A. et al. (1996) Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. J. Biol. Chem. **271:** 13668–13674
153 Mickley L. A., Lee J. S., Weng Z., Zhan A., Alvarez M., Wilson W. et al. (1998) Genetic polymorphism in P-glycoprotein: a tool for examining allelic expression in normal cells, unselected and drug-selected cell lines, and human tumors. Blood **91:** 1749–1756
154 Al-Shawi, M.K. and Senior, A.E. (1993) Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. J. Biol. Chem. **268:** 4197–4206
155 Piwnica-Worms D., Chiu M. L., Budding M., Kronaauge J. F., Kramer R. A. and Croop J. M. (1993) Functional imaging of multidrug-resistant P-glycoprotein with an organotechnetium complex. Cancer Res. **53:** 977–984
156 Luker G. D., Facasso P. M., Dobkin J. and Piwnica-Worms D. (1997) Modulation of the multidrug resistance p-glycoprotein: detection with technetium-99m-sestamibi in vivo. J. Nucl. Med. **38:** 369–372
157 Bakker M., van der Graaf W. T., Piers D. A., Franssen E. J., Groen H. J., Smit E. F. et al. (1999) 99mTc-Sestamibi scanning with SDZ PSC 833 as a functional detection method for resistance modulation in patients with solid tumours. Anticancer Res. **19:** 2349–2353
158 Chen C. C., Meadows B., Regis J., Kalafsky G., Fojo T., Carrasquillo J. A. et al. (1997) Detection of in vivo p-glycoprotein inhibition by PSC 833 using Tc-99m sestamibi. Clin. Cancer. Res. **4:** 545–552
159 Robey R., Bakke S., Stein W., Meadows B., Litman T., Patil S. et al. (1999) Efflux of rhodamine from CD56+ cells as a surrogate marker for reversal of P-glycoprotein-mediated drug efflux by PSC 833. Blood **93:** 306–314
160 Witherspoon S. M., Emerson D. L., Kerr B. M., Lloyd T. L., Dalton W. S. and Wissel P. S. (1996) Flow cytometric assay of modulation of P-glycoprotein function in whole blood by the multidrug resistance inhibitor GG918. Clin. Cancer Res. **2:** 7–12
161 Cole S. P. C., Bhardwaj G., Gerlach J. H., Mackie J. E., Grant C. E., Almquist K. C. et al. (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science **258:** 1650–1654
162 Borst P., Evers R., Kool M. and Wijnholds J. (2000) A family of drug transporters: the multidrug resistance-associated proteins. J. Natl. Cancer Inst. **92:** 1295–1302
163 Cole S. P. C., Sparks K. E., Fraser K., Loe D. W., Grant C. E., and Wilson G. M. (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res. **54:** 5902–5910
164 Cui Y., Konig J., Buchholz J. K., Spring H., Leier I., and Keppler D. (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol. Pharmacol. **55:** 929–937
165 Hooijberg J. H., Broxterman H. J., Kool M., Assaraf Y. G., Peters G. J., Noordhuis P. et al. (1999) Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res. **59:** 2532–2535
166 Zeng H., Bain L. J., Belinsky M. G. and Kruh G. D. (1999) Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. Cancer Res. **59:** 5964–5967
167 Kool M., van der Linden M., de Haas M., Scheffer G. L., de Vree J. M., Smith A. et al. (1999) MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc. Natl. Acad. Sci. USA **96:** 6914–6919
168 Deeley R. G. and Cole S. P. C. (1997) Function, evolution and structure of multidrug resistance protein (MRP). Seminars in Cancer Biology **8:** 193–204
169 Hipfner D. R., Deeley R. G. and Cole S. P. (1999) Structural, mechanistic and clinical aspects of MRP1. Biochim. Biophys. Acta **1461:** 359–376
170 Almquist K. C., Loe D. W., Hipfner D. R., Mackie J. E., Cole S. P. C. and Deeley R. G. (1995) Characterization of the M_r 190,000 multidrug resistance protein (MRP) in drug-selected and transfected human tumor cells. Cancer Res. **55:** 102–110
171 Feller N., Kuiper C. M., Lankelma J., Ruhdal J. K., Scheper R. J., Pinedo H. M. et al. (1995) Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry. Br. J. Cancer **72:** 543–549
172 Jedlitschky G., Leier I., Buchholz U., Center M. and Keppler D. (1994) ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. Cancer Res. **54:** 4833–4836
173 Jedlitschky G., Leier I., Buchholz U., Barnouin K., Kurz G., and Keppler D. (1996) Transport of glutathione, glucuronate and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res. **56:** 988–994
174 Loe D. W., Deeley R. G. and Cole S. P. (1998) Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res. **58:** 5130–5136
175 Rappa G., Lorico A., Flavell R. A. and Sartorelli A. C. (1997) Evidence that the multidrug resistance protein (MRP) functions as a co- transporter of glutathione and natural product toxins. Cancer Res. **57:** 5232–5237
176 Wijnholds J., Evers R., van Leusden M. R., Mol C. A., Zaman G. J., Mayer U. et al. (1997) Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. Nat. Med. **3:** 1275–1279
177 Nooter K., Westerman A. M., Flens M. J., Zaman G. J., Scheper R. J., van Wingerden K. E. et al. (1995) Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. Clin. Cancer Res. **1:** 1301–1310
178 van der Kolk D. M., de Vries E. G., van Putten W. J., Verdonck L. F., Ossenkoppele G. J., Verhoef G. E. et al. (2000) P-glycoprotein and multidrug resistance protein activities in relation to treatment outcome in acute myeloid leukemia. Clin. Cancer Res. **6:** 3205–3214
179 Borg A. G., Burgess R., Green L. M., Scheper R. J. and Liu Yin J. A. (2000) P-glycoprotein and multidrug resistance-associated protein, but not lung resistance protein, lower the intracellular daunorubicin accumulation in acute myeloid leukaemic cells. Br. J. Haematol. **108:** 48–54
180 Leith C. P., Kopecky K. J., Chen,I. M., Eijdems L., Slovak M. L., McConnell T. S. et al. (1999) Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood **94:** 1086–1099
181 Filipits M., Stranzl T., Pohl G., Suchomel R. W., Zochbauer S., Brunner R. et al. (1999) MRP expression in acute myeloid leukemia. An update. Adv. Exp. Med. Biol. **457:** 141–150
182 Young L. C., Campling B. G., Voskoglou-Nomikos T., Cole S. P., Deeley R. G. and Gerlach J. H. (1999) Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. Clin. Cancer Res. **5:** 673–680
183 Sugawara I., Yamada H., Nakamura H., Sumizawa T., Akiyama S., Masunaga A. et al. (1995) Preferential expression of the multidrug-resistance-associated protein (MRP) in adenocarcinoma of the lung. Int. J. Cancer **64:** 322–325
184 Oshika Y., Nakamura M., Tokunaga T., Fukushima Y., Abe Y., Ozeki Y. et al. (1998) Multidrug resistance-associated protein

and mutant p53 protein expression in non-small cell lung cancer. Mod. Pathol. **11:** 1059–1063
185 Ota E., Abe Y., Oshika Y., Ozeki Y., Iwasaki M., Inoue H. et al. (1995) Expression of the multidrug resistance-associated protein (MRP) gene in non-small-cell lung cancer. Br. J. Cancer **72:** 550–554
186 Nooter K., Bosman F. T., Burger H., van Wingerden K. E., Flens M. J., Scheper R. J. et al. (1996) Expression of the multidrug resistance-associated protein (MRP) gene in primary non-small-cell lung cancer. Ann. Oncol. **7:** 75–81
187 Nooter K., Brutel de la Riviere G., Look M. P., van Wingerden K. E., Henzen-Logmans S. C., Scheper R. J. et al. (1997) The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer. Br. J. Cancer **76:** 486–493
188 Filipits M., Suchomel R. W., Dekan G., Haider K., Valdimarsson G., Depisch D. et al. (1996) MRP and MDR1 gene expression in primary breast carcinomas. Clin. Cancer Res. **2:** 1231–1237
189 Ito K., Fujimori M., Nakata S., Hama Y., Shingu K., Kobayashi S. et al. (1998) Clinical significance of the increased multidrug resistance-associated protein (MRP) gene expression in patients with primary breast cancer. Oncol. Res. **10:** 99–109
190 Ferrero J. M., Etienne M. C., Formento J. L., Francoual M., Rostagno P., Peyrottes I., Ettore F. et al. (2000) Application of an original RT-PCR-ELISA multiplex assay for MDR1 and MRP, along with p53 determination in node-positive breast cancer patients. Br. J. Cancer **82:** 171–177
191 Arts H. J., Katsaros D., de Vries E. G., Massobrio M., Genta F., Danese S. et al. (1999) Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2 and lung resistance protein as prognostic factors in ovarian carcinoma. Clin. Cancer Res. **5:** 2798–2805
192 Izquierdo M. A., van der Zee A. G., Vermorken J. B., van der Valk P., Belien J. A., Giaccone G. et al. (1995) Drug resistance-associated marker Lrp for prediction of response to chemotherapy and prognoses in advanced ovarian carcinoma. J. Natl. Cancer Inst. **87:** 1230–1237
193 Yokoyama Y., Sato S., Fukushi Y., Sakamoto T., Futagami M., and Saito Y. (1999) Significance of multi-drug-resistant proteins in predicting chemotherapy response and prognosis in epithelial ovarian cancer. J. Obstet. Gynaecol. Res. **25:** 387–394
194 Allen J. D., Brinkhuis R. F., van Deemter L., Wijnholds J. and Schinkel A. H. (2000) Extensive contribution of the multidrug transporters p-glycoprotein and Mrp1 to basal drug resistance. Cancer Res. **60:** 5761–5766
195 Germann U. A., Ford P. J., Shlyakhter D., Mason V. S. and Harding M. W. (1997) Chemosensitization and drug accumulation effects of VX-710, cyclosporin A, MS-209 and GF120918 in multidrug resistant HL60/ADR cells expressing the multidrug resistance-associated protein MRP. Anticancer Drugs **8:** 141–155
196 Chen Z. S., Furukawa T., Sumizawa T., Ono K., Ueda K., Seto K. et al. (1999) ATP-dependent efflux of CPT-11 and SN-38 by the multidrug resistance protein (MRP) and its inhibition by PAK-104P. Mol. Pharmacol. **55:** 921–928
197 Gekeler V., Ise W., Sanders K. H., Ulrich W. R. and Beck J. (1995) The leukotriene LTC4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem. Biophys. Res. Comm. **208:** 345–352
198 Taniguchi K., Wada M., Kohno K., Nakamura T., Kawabe T., Kawakami M. et al. (1996) A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res. **56:** 4124–4129
199 Chu X. Y., Kato Y., Niinuma K., Sudo K. I. Hakusu, H. and Sugiyama Y. (1997) Multispecific organic anion transporter is responsible for the biliary excretion of the camptothecin derivative irinotecan and its metabolites in rats. J. Pharmacol. Exp. Ther. **281:** 304–314
200 Cui Y., König J., Buchholz U., Spring H., Leier I. and Keppler D. (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol. Pharmacol. **55:** 929–937
201 Koike K., Kawabe T., Tanaka T., Toh S., Uchiumi T., Wada M. et al. (1997) A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res. **57:** 5475–5479
202 Kool M., de Haas M., Scheffer G. L., Scheper R. J., van Eijk M. J., Juijn J. A. et al. (1997) Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene in human cancer cell lines. Cancer Res. **57:** 3537–3547
203 Bakos E., Evers R., Sinko E., Varadi A., Borst P. and Sarkadi B. (2000) Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. Mol. Pharmacol. **57:** 760–768
204 McAleer M. A., Breen M. A., White N. L. and Matthews N. (1999) pABC11 (also known as MOAT-C and MRP5), a member of the ABC family of proteins, has anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. J. Biol. Chem. **274:** 23541–23548
205 Konig J., Nies A. T., Cui Y., Leier I. and Keppler D. (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity and MRP2-mediated drug resistance. Biochim. Biophys. Acta **1461:** 377–394
206 Keppler D. and König J. (2000) Hepatic secretion of conjugated drugs and endogenous substances. Semin. Liv. Dis. **20:** 265–272
207 Roelofsen H., Vos T. A., Schippers I. J., Kuipers F., Koning H., Moshage H. et al. (1997) Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells. Gastroenterology **112:** 511–521
208 Ortiz D. F., Li S., Iyer R., Zhang X., Novikoff P., and Arias I. M. (1999) MRP3, a new ATP-binding cassette protein localized to the canalicular domain of the hepatocyte. Am. J. Physiol. **276:** G1493–500
209 Konig J., Rost D., Cui Y. and Keppler D. (1999) Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. Hepatology **29:** 1156–1163
210 Wijnholds J., Mol C. A. A. M., van Deemter L., de Haas M., Scheffer G. L., Baas F. et al. (2000) Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. Proc. Natl. Acad. Sci. USA **97:** 7476–7481
211 Madon J., Hagenbuch B., Landmann L., Meier P. J. and Stieger, B. (2001) Transport function and hepatocellular localization of mrp6 in rat liver. Mol. Pharmacol. **57:** 634–641
212 Doyle L. A., Yang W., Abruzzo L. E., Krogmann T., Gao Y., Rishi A. K. et al. (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. PNAS **95:** 15665–15670
213 Chen Y.-N., Mickley L. A., Schwartz A. M., Acton E. M., Hwang J. and Fojo A. T. (1990) Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. J. Biol. Chem. **265:** 10073–10080
214 Allikmets R., Schrim L. M., Hutchinson A., Romano-Spica V., and Dean M. (1998) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res. **58:** 5337–5339

215 Miyake K., Mickley L. A., Litman T., Greenberger L., Zhan Z., Robey R. et al. (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells. Demonstration of homology to ABC transport genes. Cancer Res. **59:** 8–13
216 Ewart G. D. and Howells A. J. (1998) ABC transporters involved in transport of eye pigment precursors in *Drosophila melanogaster*. Methods Enzymol. **292:** 213–224
217 Townsend A. and Trowsdale J. (1993) The transporters associated with antigen presentation. Seminars in Cell Biology **4:** 53–61
218 Burchell B., Baird S. and Coughtrie M. W. (1990) The role of xenobiotic glucuronidating enzymes in drug resistance of tumour tissues and cells. Princess Takamatsu Symp. **21:** 263–275
219 Takahashi T., Fujiwara Y., Yamakido M., Katoh O., Watanabe H. and Mackenzie P. I. (1997) The role of glucuronidation in 7-ethyl-10-hydroxycamptothecin resistance in vitro. Jpn J. Cancer Res. **88:** 1211–1217
220 Allen J. D., Brinkhuis R. F., Wijnholds J. and Schinkel, A. H. (2000) The mouse bcrp/mxr/abcp gene: amplification and overexperssion in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. Cancer Res. **59:** 4237–4241
221 Schriml L. M. and Dean M. (2000) Identification of 18 mouse ABC genes and characterization of the ABC superfamily in *Mus musculus*. Genomics **64:** 24–31
222 Mickley L., Jain P., Miyake K., Schriml L., Rao V. K., Fojo T. et al. (2001) An ATP-binding cassette gene (ABCG5) closely related to the multidrug transporter (MXR/ABCP) ABCG2 has an unusual ATP-binding domain. Mammilian Genome **2:** 86–88
223 Knutsen T., Rao V. K., Ried T., Mickley L., Schneider E., Miyake, K. et al. (2000) Amplification of 4q21-q22 and the MXR gene in independently derived mitoxantrone-resistant cell lines. Genes, Chromosomes and Cancer **27:** 110–116
224 Ross D. D., Yang W., Abruzzo L. V., Dalton W. S., Schneider E., Lage H. et al. (1999) Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. J. Natl. Cancer Inst. **91:** 429–433
225 Scheffer G. L., Maliepaard M., Pijnenborg A. C. L. M., van Gastelen M. A., de Jong M. C., Schroeijers, A. B. et al. (2000) Breast cancer resistance protein is localized at the plasma membrane in mitoxantrone- and topotecan-resistant cell lines. Cancer Res. **60:** 2589–2593
226 Robey R. W., Medina-Perez W. Y., Nishiyama K., Lahusen T., Miyake K., Litman T. et al. (2001) Overexpression of the ATP-binding cassette half-transporter, ABCG2 (MXR/BCRP/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin. Cancer Res., in press
227 Litman T., Brangi M., Hudson E., Fetsch P., Abati A., Ross D. D. et al. (2000) The multidrug resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J. Cell Sci. **113:** 2011–2021
228 Rocchi E., Khodjakov A., Volk E. L., Yang C.-H., Litman T., Bates S. E. et al. (2000) The product of the ABC half-transporter gene *ABCG2* (BCRP/MXR/ABCP) is expressed in the plasma membrane. Biochem. Biophys. Res. Commun. **271:** 42–46
229 Robey R. W., Miyake K., van de Laar A., Honjo Y., Regis J. T., Litman T. et al. (2001) A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). Biochim. Biophys. Acta, in press
230 Rabindran S. K., Ross D. D., Doyle L. A., Yang W. and Greenberger L. M. (2000) Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res. **60:** 47–50
231 Rabindran S. K., He H., Singh M., Brown E., Collins K. I., Annable T. et al. (1998) Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. Cancer Res. **58:** 5850–5858
232 He H., Rabindran S. G., Greenberger L. M. and Carter G. T. (1999) Fumitremorgin C analogs that reverse mitoxantrone resistance in human colon carcinoma cells. Med. Chem. Res. **9:** 424–437
233 van Loevezijn A., Allen J. D., Schinkel A. H. and Koomen G. J. (2001) Inhibition of BCRP-mediated drug efflux by fumitremorgin-type indolyl diketopiperazines. Bioorg. Med. Chem. Lett. **11:** 29–32
234 Schlegel S., Klimecki W. and List A. F. (1999) Breast cancer resistance protein (BCRP) associated drug export and p53 inactivation impact flavopiridol (FLA) antitumor activity. Proc. Amer. Assoc. Cancer Res. 40: Abstr. 4415
235 Jonker J. W., Smit J. W., Brinkhuis R. F., Maliepaard M., Beijnen J. H., Schellens J. H. and Schinkel A. H. (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J. Natl. Cancer Inst. **92:** 1651–1656