Structurally Distinct MDR Modulators Show Specific Patterns of Reversal against P-Glycoproteins Bearing Unique Mutations at Serine$^{939/941}$ †

Shama Kajiji,*,‡ J. Andrew Dreslin,‡ Kärt Grizzuti,‡ and Philippe Gros§

Department of Cancer, Pfizer Central Research, Groton, Connecticut 06340, and Department of Biochemistry, McGill University, Montreal, Canada H3G 1Y6

Received November 8, 1993; Revised Manuscript Received February 7, 1994®

ABSTRACT: The mechanism by which P-glycoprotein (P-gp) interacts with a number of structurally unrelated substrates or inhibitors remains unknown. We have recently shown that a serine residue within the predicted transmembrane (TM) domain 11 of P-gps encoded by mouse *mdr1* (Ser^{941}) and *mdr3* (Ser^{939}) plays an important role in the substrate specificity of P-gp. We wished to determine if Ser$^{939/941}$ is also important for efficient interaction of P-gp with structurally different modulating agents, a cyclic peptide (cyclosporin A, CsA), a diaminoquinazoline (CP100356), and a chiral, tricyclic structure (CP117227). For this, the capacity of these compounds to modulate the vinblastine (VBL) resistance phenotype of transfected cells expressing similar levels of P-gps bearing either the wild-type Ser or a mutant Phe at position 941 (*mdr1*) or 939 (*mdr3*) was initially tested. The Ser → Phe substitution indeed affected the potency and P-gp isoform specificity of some of the modulators, in particular that of CP117227 (racemic mixture and enantiomers), which were active against wild-type but not mutant *mdr3*. The modulatory effect of the mutation on CP117227-mediated reversal of VBL resistance was paralleled by a comparable modulation of the steady-state levels of VBL accumulation in Ser^{939}- and Phe^{939}-expressing cells, but was not linked to differential cellular accumulation of the modulator, which was identical in both cell types. To further assess the role of this amino acid residue in P-gp interactions with modulators, the effect of additional mutations (Ala, Cys, Thr, Asp, Tyr, Trp) at that site on potencies of CsA, CP117227 enantiomers, and CP100356 was evaluated. Each modulator was found to have a unique pattern of preferential activity for wild-type and individual mutant P-gps tested, with individual mutations having no effect or causing either an increase or a decrease in potency of the modulator. Together, these results indicate that (1) the three modulators tested interact directly with P-gp and the Ser$^{939/941}$ residue is an important determinant; (2) the similar effect of mutations at that site (either increasing, decreasing, or leaving intact) on either P-gp-mediated drug resistance or modulator potency indicates that drug molecules and reversal agents are recognized by a similar complex binding site and mechanism; (3) the recognition of modulators by P-gp at that site is independent of their capacity to be transported (CsA) or not (CP117227) by P-gp.

Multidrug resistance (MDR)[1] represents a major obstacle to the successful clinical therapy of cancer. A common mechanism of cellular resistance to structurally distinct chemotherapeutants like Adriamycin (ADM), etoposide, actinomycin D (ACT), and the *Vinca* alkaloids is the overexpression of a group of integral membrane proteins, P-glycoproteins (P-gps). P-gps belong to the superfamily of structurally related ABC (ATP binding cassette) membrane transport proteins that bind covalently to ATP and drug analogs and that are thought to function as energy dependent efflux pumps to reduce net drug accumulation in drug resistant cells [reviewed in Gottesman and Pastan (1993)]. P-gps are encoded by a family of closely related *mdr* genes composed of two members in humans (*MDR1* and *MDR2*) and three members in rodents (*mdr1*, *mdr2*, and *mdr3*). Sequence analyses have shown that P-gps contain ~1280 amino acids, organized in a tandem repeat of about 640 amino acids. Each repeat consists of an amino-terminal hydrophobic domain containing six predicted transmembrane helices, followed by a hydrophilic domain containing a nucleotide binding site (Gros et al., 1986, 1988; Devault & Gros, 1990; Hsu et al., 1990; Chen et al., 1986; Van der Bliek et al., 1988). Recently, alternative biological functions of P-gps have been proposed, including channels for chloride ions (Valverde et al., 1992) and ATP (Abraham et al., 1993), pH regulators (Roepe, 1992), and lipid flippases (Higgins & Gottesman, 1992).

The clinical need to overcome MDR has prompted the search and evaluation of compounds capable of blocking *in vivo* the activity of P-gp. The inclusion of modulators such as CsA, verapamil (VRP), and quinine in chemotherapy protocols has produced encouraging clinical responses (Dalton et al., 1989; Solary et al., 1992; Yahanda et al., 1992; List et al., 1993; Sikic, 1993), and efforts to develop more potent and more specific and well-tolerated P-gp inhibitors are ongoing. The precise molecular mechanism of action for reversing P-gp-mediated MDR and the structural determinants of chemically and pharmacologically distinct MDR modulators remain largely unknown. Although relative lipophilicity, molecular refractivity, molecular weight, and presence of a basic nitrogen atom have all been suggested to be important for efficient interaction of modulators with P-gp (Pearce et al., 1989; Ford

† This work was supported by grants to P.G. from the Medical Research Council of Canada. P.G. is supported by a scientist award from the Fonds de Recherche en Santé du Québec and a Howard Hughes Medical Institute International Scholarship.

* To whom all correspondence should be addressed: Shama Kajiji, Department of Cancer, Pfizer Central Research, Eastern Point Rd., Groton, CT 06340. Telephone: 203-441-4997. FAX: 203-441-4111.

‡ Pfizer Central Research.

§ McGill University.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: ACT, actinomycin D; ADM, Adriamycin; COL, colchicine; CsA, cyclosporin A; FCS, fetal calf serum; MDR, multidrug resistance; MEM, minimal essential medium; P-gp, P glycoprotein; PRG, progesterone; pst, position; TM, transmembrane; VBL, vinblastine; VRP, verapamil.

0006-2960/94/0433-5041$04.50/0

& Hait, 1990; Selassie et al., 1990; Hait & Aftab, 1992; Ramu & Ramu, 1992; Tang-Wai et al., 1993), an optimal structure necessary for recognition by P-gp and chemosensitization activity has yet to emerge.

The functional analysis of P-gp suggests that MDR modulators can be divided into two types. The first type of modulators, e.g., CsA, VRP, azidopine, diltiazem, and FK-506, are transported by P-gp and exert their inhibitory effect not only by blocking the initial binding of the anticancer drugs but also throughout the course of the transport process (Yusa & Tsuruo, 1989; Tamai & Safa, 1991; Naito et al., 1993; Saeki et al., 1993a). The second type of MDR modulators, like progesterone and nitrendipine, are not transported by P-gp and reverse MDR by only blocking the initial binding of the cytotoxic drug to P-gp (Ueda et al., 1992; Saeki et al., 1993b). Photoaffinity labeling studies suggest that modulators which are transported may interact at different binding sites on P-gp. For example, CsA and VRP compete with the *Vinca* alkaloid binding to P-gp (Beck & Qian, 1992; Safa, 1993) whereas azidopine, a noncompetitive inhibitor of CsA and VBL transport and binding, was shown to interact at a site distinct from that apparently recognized by CSA, VRP, and the *Vinca* alkaloids (Tamai & Safa, 1991).

The biochemical analysis of P-gp with photoactivatable drug analogs has suggested that the membrane-associated (TM) segments of the protein are the key determinants for drug recognition and binding. This was shown by energy-transfer experiments using ADM or rhodamine 123 to activate the photolabile membrane probe 5-[^{125}I]iodonaphthalenyl 1-azide (Raviv et al., 1990), and epitope mapping studies of P-gp proteolytic fragments photolabeled with analogs of azidopine, prazosin, and forskolin. Two major drug binding sites located in each half of P-gp have been delineated as two ≤5-kDa V8 protease fragments symmetrically located in, or immediately C-terminal to, predicted TMs 6 and 12 and in close proximity to the ATP binding regions (Bruggeman et al., 1989, 1992; Yoshimura et al., 1989; Greenberger et al., 1991; Greenberger, 1993).

The genetic analysis of chimeric and mutant P-gps showing altered substrate specificities has also identified the TM regions as important sites for substrate interactions. In human *MDR1*, mutation of Gly^{185} to Val^{185} within the TM2 to TM3 segment was shown to decrease VBL and increase colchicine (COL) resistance (Choi et al., 1988), whereas the replacement of either Pro^{223} (TM4) or Pro^{886} (TM10) with Ala drastically reduced the ability of the mutant proteins to confer resistance to ADM, COL, and ACT, but not VBL (Loo & Clarke, 1993a). Also, substituting Phe^{335} (TM6) and Phe^{978} (TM12) to Ala drastically altered the substrate specificity of *MDR1* and showed opposed effects of the two mutations on VBL/ACT and COL/ADM resistance (Loo & Clarke, 1993b). In hamster *pgp1*, the double substitution of Gly^{338}/Ala^{339} to Ala^{338}/Pro^{339} in TM6 increased resistance to ACT and provided survival advantage to these cells during continuous drug selection (Devine et al., 1992). We have recently demonstrated that the Ser residue at pst 941 and 939 in the TM11 domain of mouse *mdr1* and *mdr3*, respectively, is a key determinant for substrate specificity of P-gp (Gros et al., 1991). Individual mutations at that site have opposite effects on the profiles of drug resistance conferred by the mutant proteins, causing either a small (VBL) or drastic (ADM, COL) reduction in resistance to certain drugs while increasing resistance to others (ACT). The modulatory effect of mutations at pst 939/941 on drug resistance was paralleled by alterations of drug binding and transport by mutant P-gps (Kajiji et al., 1993). The analysis of multiple mutations at pst 939/941 and structure–activity studies using COL analogs together showed that efficient interaction of COL at that site involves a minimal size requirement for both the side chain of the residue and the drug molecule itself (Tang-Wai et al., 1993; Dhir et al., 1993).

Since experimental evidence suggests that MDR drugs and P-gp modulators may interact with similar sites on the protein, we have initiated studies to analyze the possible role of Ser$^{939/941}$ residue in P-gp interactions with modulators of its drug efflux function. For this, three structurally and pharmacologically distinct modulators, a cyclic peptide (cyclosporin A, CsA), a chiral, tricyclic derivative CP117227 which has been described by Sato et al. (1991) as MS-073, and the novel diaminoquinazoline CP100356 (Coe et al., 1992), were studied. The capacity of these compounds to reverse the multidrug resistance phenotype of CHO cells stably transfected with and overexpressing similar levels of wild-type and mutant P-gps, bearing unique substitutions at pst 939/941, was measured.

MATERIALS AND METHODS

Drugs and Chemicals. Vinblastine (VBL) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma. [^{3}H]VBL (specific activity 9 Ci/mmol) was from Moravek Biochemicals. The MDR modulators, cyclosporin A (CsA), CP100356, and CP117227 racemate and its *R*-(+) and *S*-(−) enantiomers were prepared at Pfizer Central Research, Groton, CT. The *R*-(+) and *S*-(−) enantiomers of CP117227 were purified to optical purities >95% enantiomeric excess. Tissue culture medium was purchased from Gibco/BRL, and serum and supplements were from JRH Biosciences.

Cell Lines and Tissue Culture. Chinese hamster LR73 ovary cells and their drug resistant clonal derivatives transfected with wild-type *mdr1* (1S) or *mdr3* (3S) cDNAs or with mutant *mdr1* (1F) or *mdr3* (3F) cDNAs bearing a single Ser to Phe substitution at position 941 (*mdr1*) or 939 (*mdr3*) in the predicted TM11 (Gros et al., 1991) were maintained in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal calf serum (FCS), penicillin (50 units/mL), streptomycin (100 μg/mL), and gentamycin (25 μg/mL). Drug resistant transfected clones were maintained in medium containing VBL at 25 ng/mL (1F), 50 ng/mL (1S, 3F), and 100 ng/mL (3S). Mass populations of LR73 drug resistant derivatives expressing individual *mdr3* mutants bearing unique substitutions at Ser^{939} (Ala, Asp, Cys, Thr, Trp, or Tyr) were generated and characterized as we have previously described (Dhir et al., 1993). These cell lines were maintained in α-minimum essential medium (α-MEM) including the above supplements and VBL (50 ng/mL). Western blotting analyses of membrane-enriched fractions have shown that these drug resistant, clonal and mass populations transfected with either wild-type or mutant *mdr* stably express similar levels of P-gp (Dhir et al., 1993; Kajiji et al., 1993).

Drug Cytotoxicity Assays. Cell survival was determined by the MTT method as previously described (Mosmann, 1983). Briefly, 3 × 10^3 cells were seeded in the absence of VBL in 96-well microtiter plates 24 h prior to drug treatment. Cells were exposed for 72 h to increasing concentrations of VBL in the presence or absence of modulators. Cells were treated with MTT (500 μg/mL) for 4 h, and the resulting formazan was dissolved in dimethyl sulfoxide. The optical density was measured at 570 nm on a Bio-Tek Instrument EL320 microplate reader. Values were normalized to 100% for the

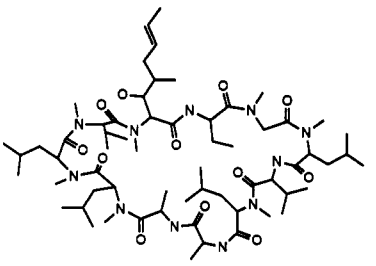
control group plated without drugs, and linear regression was used to calculate IC_{50}s of cytotoxic drugs (defined as the drug concentration which inhibits cell growth by 50%). All values represent the average of at least three independent experiments performed in duplicate.

Drug Transport Assays. Twenty-four hours prior to experimentation, cells were seeded in 6-well plates (1.2×10^6 cells/well) in α-MEM lacking VBL but supplemented with 5% FCS and antibiotics. In VBL accumulation experiments, cells were exposed for 0.5 h to 20 nM [^{3}H]VBL in the absence or presence of modulators. Cells were washed twice with ice-cold PBS and collected by trypsinization. Cell number and viability were determined and cell-associated radioactivity was measured by suspending collected cells in liquid scintillation fluid (Beckman Ready Safe), vortexing, and scintillation counting. Cellular accumulation of CP117227 (racemate and enantiomers) was determined after incubating cells for 0.5 h with the modulator. Cells were washed and harvested by trypsinization, and cell-associated modulator concentrations were determined by HPLC. Separation of compound was achieved using a 250 × 4.6 mm Zorbax RX C-8 column, a mobile phase consisting of acetonitrile and 0.05 M ammonium acetate buffer pH 6.5 (80:20), and a flow rate of 1.5 mL/min. Peaks were detected by fluorescence excitation at 297 nm and emission at 370 nm. Quantitation was done by measuring area under the elution peak (lower limit of quantitation was 0.05 μg/mL).

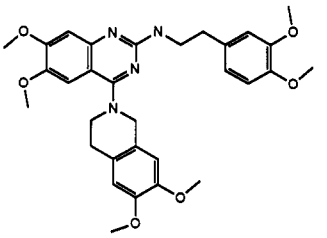
RESULTS

We have previously shown that a Ser to Phe substitution in the predicted TM11 of P-gp encoded by *mdr1* (pst 941) and *mdr3* (pst 939), respectively, strongly modulated the substrate specificity of the two P-gps, by altering drug transport and drug binding characteristics of the mutant P-gps (Gros et al., 1991; Kajiji et al., 1993). While mutations at this site had little effect on VBL resistance, they significantly altered the degree of COL, ADM, and ACT resistance. The Ser to Phe substitution also modulated the capacity of known P-gp modulators verapamil (VRP) and progesterone (PRG) to inhibit P-gp function in both drug cytotoxicity and drug transport assays. These preliminary studies suggested that this residue may be important for interaction of P-gp with both drugs and modulators. In order to test this possibility, and to gain further insight pertaining to the molecular parameters of interaction between P-gp and its modulators, we tested the capacity of structurally and pharmacologically distinct modulators to inhibit the activity of wild-type and mutant P-gps bearing discrete amino acid substitutions at pst 939/941, and thereby reverse VBL resistance. The structural formulas of the modulators tested are shown in Figure 1. CsA is a naturally occurring cyclic peptide and one of the few MDR modulators lacking a basic nitrogen atom. It is a substrate for P-gp-mediated drug transport and has been shown to be efficacious as a MDR modulator in the clinic. On the other hand, CP100356 and CP117227 are novel synthetic P-gp inhibitors which are more potent that CsA in preclinical models of multidrug resistance (S. Kajiji, unpublished observations). CP100356 is an achiral diaminoquinazoline having phenylethylamine substructure elements in the 2 and 4 positions and a pK_a of 7.6, while CP117227 is a 1,4-disubstituted piperazine derivative having multiring moieties attached at each end; chirality at its β-hydroxy center can be resolved into the individual enantiomers. CP117227 has pK_a values of 4.5 and 7.3.

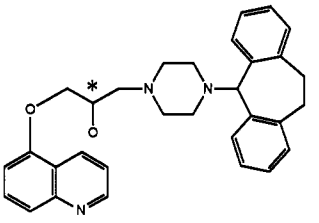
The capacity of these compounds to modulate P-gp-mediated VBL resistance in cell clones stably expressing either



Cyclosporin A



CP100356



CP117227

FIGURE 1: Molecular structures of cyclosporin A, CP100356, and CP117227.

the wild-type (*mdr1*Ser^{941}, *mdr3*Ser^{939}) or a mutant form of P-gp (*mdr1*Phe^{941}, *mdr3*Phe^{939}) was initially tested. The four cell lines (1S, 1F, 3S, 3F) expressed similar levels of VBL resistance in drug cytotoxicity assays which represented an increase of about 30× above background levels measured in drug sensitive LR73 control cells (Figure 2A, Table 1). Modulation of VBL resistance in these clones, expressed as fold reversal, was the ratio of the VBL IC_{50}s calculated in the presence or absence of modulator. The modulators were tested at concentrations which were not toxic to the cells in the absence of VBL. CsA at a concentration of 0.5 μM could modulate VBL resistance of 1S and 1F cells by 3× and 19×, and of 3S and 3F cells by 6× and 26×, respectively, suggesting that at this concentration CsA had a greater modulatory effect for Phe$^{939/941}$ than Ser$^{939/941}$ bearing P-gps. However, titration of CsA over a broad concentration range produced similar dose dependent reversal of VBL resistance of both 3S and 3F cells (Figure 2B), demonstrating that the Ser to Phe substitution does not dramatically influence the activity of CsA against *mdr3*. CP100356 at 0.1 μM produced a 17× vs 78× potentiation of VBL cytotoxicity in 1S cells and 1F expressing cells, respectively, but failed to modulate VBL resistance in 3S and 3F cells (Table 1). In addition, titration of CP100356 over a broad concentration range produced dose dependent reversal effects that were similar for 3S and 3F cells (Figure 2B), but that showed preferential activity of CP100356 against 1F cells (data not shown). These data indicate that the two P-gp isoforms may have distinct pharmacological specificities for MDR modulators. In addition, replacement of Ser by Phe in the TM11 domain could alter interaction of the modulator with *mdr1* but not *mdr3*, suggesting that Ser^{941} is an important determinant for interaction of CP100356 with

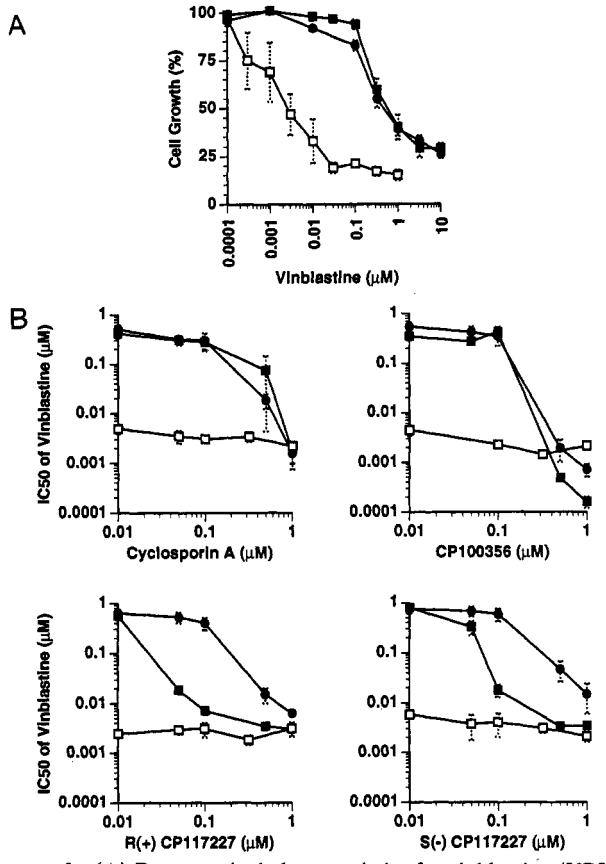


FIGURE 2: (A) Drug survival characteristics for vinblastine (VBL) of LR73 control drug sensitive cells (□) and LR73 cell clones transfected and overexpressing wild-type (*mdr3S*, ■) or mutant (*mdr3F*, ●) *mdr3* cDNAs. Survival of individual clones in increasing concentrations of VBL is expressed as a percentage of growth measured for the same clone in control wells devoid of VBL (percent cell growth). Cell survival was determined by a 4-h MTT incubation, with colorimetric evaluation at 570 nm as described in Materials and Methods. (B) Effect of increasing concentrations of cyclosporin A, CP100356, and enantiomers of CP117227 on the VBL resistance characteristics of control drug sensitive LR73 cells (□) and cell clones transfected and overexpressing either wild-type (*mdr3S*, ■) or mutant *mdr3* (*mdr3F*, ●). The IC_{50} was the VBL dose required to decrease cell growth by 50% in the presence of increasing concentrations of the modulators. Data represent the mean and standard errors of at least three independent experiments, each carried out in duplicate.

mdr1. On the other hand, evaluation of the tricyclic derivative CP117227 under similar assay conditions revealed a dramatically different activity profile and specificity for *mdr* isoforms: CP117227 at a concentration of 0.1 μM completely abrogated VBL resistance (45× reduction) of 3S cells but had a negligible effect on 1S, 1F, and 3F cells (Table 1). Since CP117227 is a racemic mixture, the capacity of its two stereoisomers to modulate these P-gps was tested (Figure 2B). Both stereoisomers of CP117227 produced similar dose dependent reversal of VBL resistance of 3S and 3F cells, and both were substantially more active against wild-type (3S) than mutant *mdr3* (3F) (Figure 2). Indeed, these dose–response experiments showed that, over a broad concentration range, equal reductions in VBL IC_{50}s required doses (potency) of both enantiomers that were 10 times higher for 3F than 3S cells, with the *R*-(+) enantiomer appearing slightly more potent than the *S*-(−) form. These data demonstrate that structurally distinct MDR modulators interact differentially with the P-gp isoforms tested, and suggested that one of the drug binding sites recognized by these modulators includes TM11, in particular, the $Ser^{941/939}$ residue. Recognition of this residue appears to be critical for the interaction of CP117227 (racemic and both enantiomers) and CP100356 with P-gp while it may not be as critical for CsA-mediated reversal of MDR.

The apparent selectivity of CP117227 and its enantiomers for 3S cells detected in drug cytotoxicity assays may result from differential intracellular accumulation of VBL in the two cell populations. In turn, this could be due to differences either in competitive interactions between the cytotoxic drug and the modulator at a shared binding/transport site on P-gp or in steady-state cellular concentration of the modulator in the two cell types. To address these issues, we first assayed the effect of increasing concentrations of (*R*)-(+)- and (*S*)-(−)-CP117227 on accumulation of [^{3}H]VBL in 3S and 3F cells (Table 2). Drug accumulation in cells is expressed as picomoles/million cells. In the absence of modulator, 3S and 3F cells accumulate approximately 10-fold less VBL than the LR73 drug sensitive parent, confirming that the Ser to Phe substitution has little effect on the capacity of *mdr3* to reduce cellular VBL accumulation. However, addition of increasing concentrations of the (*R*)-(+)- and (*S*)-(−)-CP117227 to the transport assay produced dose dependent increases in VBL accumulation that were quite different for 3S and 3F cells. Although high concentrations of the modulators (>1 μM) completely restored VBL accumulation in both 3S and 3F cells, comparison of modulator effects within the linear dose range (concentrations of ≤1 μM) showed that both enantiomers were much more efficacious in increasing VBL accumulation in 3S than 3F cells (Table 2). Again, the (*R*)-(+)-CP117227 enantiomer was about 2-fold more potent than its *S*-(−) isomer for both *mdr3* isoforms. Therefore, the apparent selectivity of CP117227 enantiomers for modulating VBL resistance in 3F and 3S cells in drug cytotoxicity assays (Figure 2) is reflected by a concomitant capacity of these compounds to preferentially increase VBL accumulation in 3S vs 3F cells. Thus, preferential activity of CP117227 for 3S cells involves direct modulation of P-gp-mediated drug transport. Whether the differential activity of CP117227 enantiomers against wild-type and mutant *mdr3* was caused by differences in steady-state levels of the modulator in the two cell populations was examined by exposing control LR73 and 3S and 3F cells to 1 μM and 5 μM of (*R*)-(+)- and (*S*)-(−)-CP117227, and determining steady-state levels of accumulation by HPLC analyses. As shown in Table 3, treatment with 5 μM of either stereoisomer produced a 3–5-fold-higher level of cellular accumulation of the modulator than treatment with 1 μM. Further, cellular concentrations of (*R*)-(+)- and (*S*)-(−)-CP117227 in *mdr3* transfectants were similar to those measured in LR73 cells, strongly suggesting that these compounds are not substrates for P-gp dependent transport. More importantly, cellular concentrations of the CP117227 enantiomers were similar in both 3S and 3F cells. Hence, the CP117227-mediated preferential modulation of VBL resistance and transport detected in 3S cells is not caused by variation in cellular accumulation of the modulator but probably reflects differences in the physical interaction of CP117227 enantiomers with the Ser^{939}- and Phe^{939}-containing P-gps encoded by *mdr3*.

Whether the Ser residue at pst 939 in TM11 of *mdr3* is a critical determinant for the recognition of these structurally diverse MDR modulators was further evaluated using mass populations of transfected LR73 cells stably expressing six additional *mdr3* mutants modified at Ser^{939} (Dhir et al., 1993). The IC_{50}s for VBL determined for the various *mdr3* transfectants indicate that replacing the Ser^{939} with Cys (*mdr3C*), Ala (*mdr3A*), Thr (*mdr3T*), and Asp (*mdr3D*) did not alter the degree of VBL resistance (40–50-fold), whereas the

Table 1: Effects of Structurally Distinct MDR Modulators on Vinblastine Resistance of LR73 Control Cells and Cell Clones Transfected with Either Wild-Type (*mdr1S*, *mdr3S*) or Mutant (*mdr1F*, *mdr3F*) *mdr* cDNAs

P-gp isoform	position 939/941	control	cyclosporin A, 0.5 μM	CP100356, 0.1 μM	(+/−)-CP117227, 0.1 μM
mdr1	Ser (S)	495 ± 150[a]	150 ± 130 (3.3×)[b,c]	30 ± 20 (17×)[c]	270 ± 100 (1.8×)[c]
	Phe (F)	310 ± 140	16 ± 8.5 (19×)[c]	4.2 ± 2.5 (78×)[c]	420 ± 230 (0.7×)
mdr3	Ser (S)	450 ± 90	74 ± 70 (6.1×)[c]	420 ± 110 (1.1×)	10 ± 7 (45×)[c]
	Phe (F)	470 ± 90	18 ± 6.0 (26×)[c]	340 ± 120 (1.4×)	520 ± 230 (0.9×)
LR73		15.0 ± 5.0	3.4 ± 0.7 (4.4×)	2.3 ± 0.3 (6.5×)	3.1 ± 1.0 (4.8×)

[a] IC_{50} values (nanomoles/liter) for vinblastine were calculated for each cell line, and they represent the drug dose necessary to reduce cell growth by 50%, using the MTT assay described in Materials and Methods. The mean and standard errors were obtained from at least three independent experiments carried out in duplicate. [b] The reversal index is the decrease in IC_{50} values (fold decrease shown in parentheses) of control and transfected cell clones determined in the presence of modulators at the indicated concentrations. [c] Significance of $p < 0.005$ compared to its control IC_{50}.

Table 2: [^{3}H]Vinblastine Accumulation in LR73 Control Cells and Transfected Cell Clones Expressing Either Wild-Type (*mdr3S*) or Mutant *mdr3* (*mdr3F*)

		concentration of CP117227 (μM)					
modulator	cell type	0	0.1	0.5	1.0	5.0	10
(+)-CP117227	3S	0.3 ± 0.0[a]	0.7 ± 0.3	2.3 ± 0.7	2.8 ± 0.7	3.0 ± 0.6	3.5 ± 0.8
	3F	0.2 ± 0.0	0.3 ± 0.1	0.8 ± 0.2	1.4 ± 0.5	2.6 ± 0.7	3.5 ± 0.6
	LR73	2.1 ± 0.3	2.9 ± 0.7	3.0 ± 0.6	3.2 ± 0.7	3.2 ± 0.6	3.6 ± 1.0
(−)-CP117227	3S	0.3 ± 0.0	0.3 ± 0.1	0.9 ± 0.4	1.8 ± 0.7	2.8 ± 0.7	3.5 ± 0.8
	3F	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	1.5 ± 0.4	2.5 ± 0.1
	LR73	2.1 ± 0.3	2.7 ± 3.1	3.1 ± 0.6	3.3 ± 0.7	3.3 ± 0.6	3.7 ± 0.8

[a] LR73 control cells and transfected cell clones expressing either wild-type (*mdr3S*) or mutant (*mdr3F*) *mdr3* were exposed for 30 min to vinblastine, with or without CP117227 enantiomers, and accumulated radioactivity was measured as described in Materials and Methods. Values represent the cell-associated accumulation of vinblastine in picomoles/10^6 cells (± standard errors) and are from three experiments carried out in duplicate.

Table 3: Accumulation of CP117227 Enantiomers in LR73 Control Cells and Transferred Cell Clones Expressing Either Wild-Type (*mdr3S*) or Mutant (*mdr3F*) *mdr3*

	(+)-CP117227		(−)-CP117227	
cell type	1 μM	5 μM	1 μM	5 μM
LR73	0.29 ± 0.09[a]	1.29 ± 0.49	0.19 ± 0.01	0.66 ± 0.04
3S	0.26 ± 0.06	0.90 ± 0.33	0.18 ± 0.00	0.78 ± 0.22
3F	0.20 ± 0.02	0.72 ± 0.14	0.13 ± 0.09	0.55 ± 0.24

[a] LR73 control cells and transfected cell clones expressing either wild-type (*mdr3S*) or mutant (*mdr3F*) *mdr3* were exposed for 30 min to two concentrations (1 μM, 5 μM) of CP117227 enantiomers. The cellular accumulation of the modulators was measured by HPLC as described in Materials and Methods. Values represent the cell-associated accumulation of modulators in nanomoles/10^6 cells (± standard errors) and are from three experiments carried out in duplicate.

introduction of amino acids with aromatic side chains, Trp (*mdr3W*) and Tyr (*mdr3Y*), resulted in a 2–5-fold decrease in resistance to this drug (Table 4). The effect of such mutations in a putative drug binding domain of P-gp on the capacity of CsA, CP100356, and CP117227 to reverse VBL resistance was determined in drug cytotoxicity assays (Table 4, Figure 3). Results shown in Figure 3 indicate that treatment with increasing concentrations of either CsA, CP100356, or CP117227 enantiomers produced dose dependent effects for all the cell populations tested. The modulatory effects of these compounds on VBL IC_{50}s obtained in the linear range of the dose–response curves (Figure 3) are summarized for all mutants in Table 4. These results showed that each modulator had its own activity profile against the individual mutants tested. CsA, at 0.32 μM, was poorly efficacious (≤6×) in reversing VBL resistance of either wild-type *mdr3S* or mutant *mdr3C* or *mdr3Y* expressing cells. However, the introduction of either Ala (*mdr3A*), Thr (*mdr3T*), or Asp (*mdr3D*) at this position drastically increased the capacity of CsA to modulate VBL resistance expressed by the mutant proteins by factors of 23×, 98×, and 78×, respectively. Finally, the introduction of large residues with aromatic side chains either abrogated (Trp, *mdr3W*) or did not influence (Tyr, *mdrY*) the action of CsA (Table 4). At a concentration of 0.32 μM, CP100356, like CsA, was minimally efficacious in modulating VBL resistance of wild-type *mdr3S*, and insertion of larger amino acids, Asp and Thr, resulted in a 72× and 102× modulation of VBL resistance of *mdr3D* and *mdr3T* cells, respectively. However, unlike CsA, CP100356 appeared to interact preferentially with the Ala (*mdr3A*) and Trp (*mdr3W*) containing P-gps, causing 90× and 72× reductions in VBL resistance, respectively. It was also 2–3-fold more efficacious than CsA at this concentration in modulating VBL resistance of *mdr3C* and *mdr3T*. In contrast, CP117227 stereoisomers were highly selective inhibitors of P-gp encoded by wild-type *mdr3* (*mdr3S*). Exposure to 0.1 μM (*R*)-(+)-CP117227 and 0.32 μM (*S*)-(−)-CP117227 modulated VBL resistance of *mdr3S* cells by 300×. This activity was reduced by 3–10-fold when Ser^{939} was replaced by either Cys, Ala, Asp, or Thr. In addition, insertion of nonpolar amino acids at this position significantly altered enantiospecific interaction of CP117227 with P-gp, such that 0.1 μM of the *R*-(+) stereoisomer reversed *mdr3C* by 108× and *mdr3A* by 31× whereas 0.32 μM of (*S*)-(−)-CP117227 showed the reverse order of preferential activity. As previously seen with the Phe-containing *mdr3* mutant (3F), replacement of Ser^{939} with aromatic side chain containing residues such as Trp (*mdr3W*) and Tyr (*mdr3Y*) significantly reduced the capacity of either enantiomer to modulate P-gp.

Although in several instances CP100356 and CsA showed similarities in their activity profiles against wild-type and mutant *mdr3*, the activity profiles of these compounds differed markedly from that of CP117227. An important difference was that both CP117227 enantiomers were highly selective for wild-type *mdr3*. In addition, the three modulators tested had distinct preferences for interacting with mutant P-gps containing structural modifications at position 939. Taken together, these data establish that structurally unrelated modulators directly interact with P-gp, and that the Ser^{939} residue is an important determinant for optimal interaction and therefore inhibition of P-gp function.

Table 4: Effects of Structurally Distinct MDR Modulators on Vinblastine Resistance of LR73 Control Cells and Cell Clones Transfected with Either Wild-Type (*mdr3S*) or Mutant (*mdr3C*, *mdr3A*, *mdr3T*, *mdr3D*, *mdr3W*, *mdr3Y*) *mdr3* cDNAs

cell lines	control	cyclosporin A, 0.32 μM	CP100356, 0.32 μM	(+)-CP117227, 0.1 μM	(−)-CP117227, 0.32 μM
3S	690 ± 80[a]	110 ± 60 (6.3×)[b,c]	140 ± 130 (4.9×)[c]	2.3 ± 0.8 (300×)[c]	2.1 ± 0.8 (330×)[c]
3C	660 ± 80	120 ± 30 (5.5×)[c]	54 ± 40 (12×)[c]	5.2 ± 0.5 (108×)[c]	16 ± 7.6 (41×)[c]
3A	560 ± 110	24 ± 4.9 (23×)[c]	6.3 ± 1.3 (90×)[c]	24 ± 20 (31×)[c]	5.4 ± 0.4 (104×)[c]
3T	570 ± 50	7.3 ± 1.4 (78×)[c]	5.5 ± 1.4 (102×)[c]	13 ± 5.3 (44×)[c]	8.9 ± 3.4 (64×)[c]
3D	570 ± 110	5.8 ± 0.9 (98×)[c]	7.9 ± 2.1 (72×)[c]	6.7 ± 1.5 (85×)[c]	7.5 ± 2.5 (76×)[c]
3W	380 ± 12	240 ± 50 (1.6×)	5.3 ± 0.9 (72×)[c]	250 ± 60 (1.5×)	160 ± 50 (2.4×)[c]
3Y	140 ± 20	18 ± 5.6 (7.8×)[c]	5.5 ± 0.4 (25×)[c]	80 ± 20 (1.8×)	44 ± 7.8 (3.2×)[c]
LR73	15 ± 5	3.4 ± 0.7 (4.4×)	1.4 ± 0.2 (10×)	3.1 ± 1.0 (4.8×)	3.0 ± 0.6 (5.0×)

[a] IC_{50} values (nanomoles/liter) for vinblastine were calculated for each cell line, and they represent the drug dose necessary to reduce cell growth by 50%, using the MTT assay described in Materials and Methods. The mean and standard errors were obtained from at least three independent experiments carried out in duplicate. [b] The reversal index is the decrease in IC_{50} values (fold decrease shown in parentheses) of control and transfected cell clones determined in the presence of modulators at the indicated concentrations. [c] Significance of $p < 0.005$ compared to its control IC_{50}.

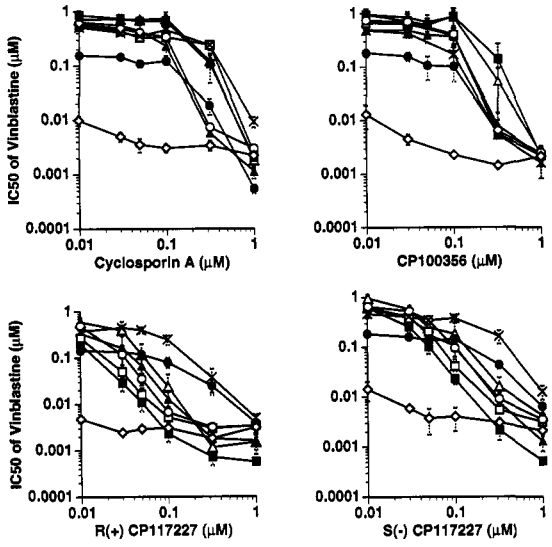


FIGURE 3: Effect of increasing concentrations of cyclosporin A, CP100356, and enantiomers of CP117227 on the VBL resistance characteristics of control drug sensitive LR73 cells (◇) and mass populations of cell clones transfected and overexpressing either wild-type (*mdr3S*, ■) or mutant *mdr3* (*mdr3A*, □; *mdr3C*, △; *mdr3D*, ▲; *mdr3T*, ○; *mdr3W*, ×; *mdr3Y*, ●) bearing single amino acid substitutions at position 939. The IC_{50} for each line was the VBL dose required to decrease cell growth by 50% in the presence of increasing concentrations of modulators. Data represent the mean and standard errors of at least three independent experiments, each carried out in duplicate.

DISCUSSION

The biochemical analysis of P-glycoprotein proteolytic fragments photolabeled with drug analogs has suggested that at least two major determinants located in each homologous half of P-gp contribute to the formation of one or more complex sites implicated in initial recognition/binding of drug molecules (Bruggeman et al., 1989, 1992; Yoshimura et al., 1989; Greenberger et al., 1991; Greenberger, 1993). Genetic analysis of discrete P-gp mutants with altered substrate specificities for MDR drugs indicates that the membrane-associated domains of the proteins are most likely implicated in the formation of such a site, in particular the TM5–6 and TM11–12 regions (Choi et al., 1988; Gros et al., 1991; Devine et al., 1992; Loo & Clarke, 1993a,b). Indeed mutations in these regions have dramatic effects on the activity of P-gp against individual classes of MDR drugs, the same mutation either leaving intact, decreasing, or enhancing P-gp-mediated resistance against a specific class of drugs. Examples of such mutations include a Phe^{335} to Ala^{335} substitution in TM6 which impairs ACT and VBL resistance in human *MDR1*, while leaving intact the capacity of this protein to confer COL and ADM resistance. Conversely, a parallel Phe^{978} to Ala^{978} substitution in the same protein had a more pronounced effect on COL and ADM resistance than on VBL and ACT resistance (Loo & Clarke, 1993b). In addition, such opposite effects of mutations on individual classes of drugs can be further modulated by the protein background onto which these unique mutations are introduced. We have recently observed that mutations at $Ser^{939/941}$ of mouse *mdr1* and *mdr3* alter the substrate specificity of these two P-gps, drastically reducing resistance to ADM and COL, while having little effect on VBL resistance in both proteins (Gros et al., 1991). Biochemical and pharmacological analyses of cell lines expressing these mutant proteins indicate that mutations at that site impair drug transport by altering the drug binding characteristics of the mutants (Kajiji et al., 1993). Further substitutions at that site showed that the replacement of the wild-type Ser by a Tyr caused either an 8-fold reduction in ACT resistance conferred by the mutant *mdr3* or, surprisingly, a 3-fold increase in ACT resistance conferred by the mutant *mdr1*, respectively (Dhir et al., 1993). These observations are compatible with the proposal that recognition and transport of structurally heterogeneous drug molecules by P-gp implicates several determinants within the TM domains of homologous halves of the proteins which come together to form a complex binding pocket.

Preliminary studies also indicated that mutations in TM11 not only affected the capacity of P-gp to recognize and transport drug molecules but also affected its interaction with known modulators, such as VRP and PRG (Kajiji et al., 1993). Substitutions at that site also interfered with the ability of mouse *mdr3* to complement the biological activity of its yeast homolog, the *STE6* peptide pheromone transporter, in a yeast cell bearing a null mutation at that locus (Raymond et al., 1992). The intriguing possibility that a single residue within TM11 be implicated in recognition/binding and transport of cytotoxic drugs and peptide substrates but also modulators of P-glycoprotein was further investigated. For this, the ability of three structurally distinct modulators to reverse the VBL resistance of CHO cells transfected and expressing similar levels of either wild-type or mutant P-gps bearing unique substitutions at position 939/941 was determined. Our results indicate that mutations at that site drastically altered the activity of these reversal agents, suggesting that this site is indeed important for efficient interaction of P-gp with its inhibitors. Each modulator demonstrated a specific activity profile against individual mutants, with the introduced mutations either producing no effect or enhancing or reducing the potency of a specific P-gp inhibitor: For instance, replacement of Ser by Tyr did not affect the activity of CsA, but improved the potency of CP100356, while it abrogated

the capacity of CP117227 to block VBL resistance (Figure 3; Table 4). On the other hand, introduction of Thr at that site had very different effects on the activity of the three modulators. The effect of these mutations on the efficacy of these compounds was also dependent on the protein background onto which they were constructed (Table 1, *mdr1* vs *mdr3*), in agreement with previous results by Yang et al. (1990), who observed preferential activity of PRG against mouse *mdr1* (*mdr1b*) over *mdr3* (*mdr1a*) encoded proteins. Likewise, we noted that CP117227 was much more efficacious in blocking the activity of P-gp encoded by human *MDR1* than P-gps encoded by either of its *mdr1* and *mdr3* counterparts (D. Tang-Wai et al., unpublished results). Taken together, these results indicate that mutations at pst 939 produce similar types of effects on the interaction of P-gp with both cytotoxic drugs and modulating agents. Recent data indicate that mutations at residues flanking the $Ser^{939/941}$ residue on either side are without consequences on the overall activity and substrate specificity of P-gp (M. Hana and P. Gros, unpublished observations). Furthermore, the effect of these mutations on modulation by CsA, CP100356, and CP117227 enantiomers was independent of whether the compound was transported (CsA) or not (CP117227) by P-gp. Finally, the two stereoisomers of CP117227 had similar activity profiles in most cases, but also differed in their activity against certain *mdr3* mutants, suggesting specificity of interaction at that site.

Our observations with *mdr3* mutants and modulators, together with the combined genetic and biochemical characterization of P-gp, are compatible with a model for secondary structure of P-gp previously proposed by Greenberger (1993) and others (Georges et al., 1993; Gottesman & Pastan, 1993). Packing of membrane helices of P-gp would allow the formation of a complex binding site that would bring into immediate proximity some of the protein segments and amino acid residues that have been previously identified as important for substrate specificity, in particular the two C-terminal portions of the membrane-associated region of both halves of P-gp. This complex binding site would be capable of accommodating very distinct molecules such as different classes of MDR drugs and structurally heterogeneous modulators such as those used in the current study. Protein domains involved in the formation of that site may be different from those implicated in transport, since compounds interacting at that site may (such as CsA) or may not be (CP117227) subsequently transported by P-gp. Although this proposal remains speculative at this stage, the segregation of an "on" and "off" rate of drug binding to P-gp in individual *mdr* mutants bearing single amino acid substitutions within predicted TM11 (Kajiji et al., 1993) and near TM3 (Safa et al., 1990), respectively, is in agreement with this proposal. Irrespective of the proposed mechanism of drug binding and transport by P-gp, our study clearly identifies Ser^{939} as an important component of the initial binding of drug and reversal agents to the protein. The identification of such protein domains and specific amino acids should help in the design of more potent and efficacious compounds capable of blocking its drug transport activity.

ACKNOWLEDGMENT

Special thanks to Dr. Anton Fliri (Pfizer Central Research) for preparing CP100356 and the valuable discussions during the writing of this manuscript. We also thank Dr. Takushi Kaneko (Pfizer Central Research) for preparing CP117227 and cyclosporin A.

REFERENCES

Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R., Kramer, R., Guidotti, G., & Cantiello, H. F. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 312–316.
Beck, W. T., & Qian, X. D. (1992) *Biochem. Pharmacol. 43*, 89–93.
Bruggemann, E. P., German, U. A., Gottesman, M. M., & Pastan, I. (1989) *J. Biol. Chem. 264*, 15483–15488.
Bruggemann, E. P., Currier, S. J., Gottesman, M. M., & Pastan, I. (1992) *J. Biol. Chem. 267*, 21020–21026.
Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., & Roninson, I. B. (1986) *Cell 47*, 381–389.
Choi, K., Chen, C. J., Kriegler, M., & Roninson, I. B. (1988) *Cell 53*, 519–529.
Coe, J. W., Fliri, A. F., Kaneko, T., & Larson, E. R. (1992) World Intellectual Property Organisation Publication WO 92/07844.
Dalton, W. S., Grogan, T. M., Meltzer, P. S., Scheper, R. J., Durie, B. G. M., Taylor, C. W., Miller, T. E, & Salmon, S. E. (1989) *J. Clin. Oncol. 7*, 415–424.
Devault, A., & Gros, P. (1990) *Mol. Cell. Biol. 10*, 1652–1663.
Devine, S. E., Ling, V., & Melera, P. W. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4564–4568.
Dhir, R., Grizzuti, K., Kajiji, S., & Gros, P. (1993) *Biochemistry 32*, 9492–9498.
Ford, J. M., & Hait, W. N. (1990) *Pharmacol. Rev. 42*, 155–199.
Georges, E., Tsuruo, T., & Ling, V. (1993) *J. Biol. Chem. 268*, 1792–1798.
Gottesman, M. M., & Pastan, I. (1993) *Annu. Rev. Biochem. 62*, 385–428.
Greenberger, L. M. (1993) *J. Biol. Chem. 268*, 11417–11425.
Greenberger, L. M., Lisanti, C. J., Silva, J. T., & Horwitz, S. B. (1991) *J. Biol. Chem. 266*, 20744–20751.
Gros, P., Croop, J., & Housman, D. E. (1986) *Cell 47*, 371–380.
Gros, P., Raymond, M., Bell, J., & Housman, D. E. (1988) *Mol. Cell. Biol. 8*, 2770–2778.
Gros, P., Dhir, R., Croop, J., & Talbot, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7289–7293.
Hait, W. N., & Aftab, D. T. (1992) *Biochem. Pharmacol. 43*, 103–107.
Higgins, C. F., & Gottesman, M. M. (1992) *Trends Genet. 17*, 18–21.
Hsu, S. I. H., Cohen, D., Kirschner, L. S., Lothstein, L., Harstein, M., & Horwitz, S. B. (1990) *Mol. Cell. Biol. 10*, 3596–3606.
Kajiji, S., Talbot, F., Grizzuti, K., Van Dyke-Phillips, V., Agresti, M., Safa, A., & Gros, P. (1993) *Biochemistry 32*, 4185–4194.
List, A. V., Spier, C., Greer, J., Wolf, S., Hutter, J., Dorr, R., Salmon, S., Futscher, B., Baier, M., Dalton, W. (1993) *J. Clin. Oncol. 11*, 1652–1663.
Loo, T. W., & Clark, D. M. (1993a) *J. Biol. Chem. 268*, 3143–3149.
Loo, T. W., & Clark, D. M. (1993b) *J. Biol. Chem. 268*, 19965–19972.
Mosmann, T. (1983) *J. Immunol. Methods 65*, 55–63.
Naito, M., Tsuge, H., Kuroko, C., Koyama, T., Tomida, A., Tatsuta, T., Heike, Y., & Tsuruo, T. (1993) *J. Natl. Cancer Inst. 85*, 311–316.
Pearce, H. L., Safa, A. R., Bach, N. J., Winter, M. A., Cirtain, M. C., & Beck, W. T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5128–5132.
Ramu, A., & Ramu, N. (1992) *Cancer Chemother. Pharmacol. 30*, 165–173.
Raviv, Y., Pollard, H. B., Bruggemann, E. P., Pastan, I., & Gottesmann, M. M. (1990) *J. Biol. Chem. 265*, 3975–3980.
Raymond, M., Gros, P., Whiteway, M., & Thomas, D. Y. (1992) *Science 256*, 232–234.
Roepe, P. D. (1992) *Biochemistry 31*, 12555–12564.
Saeki, T., Ueda, K., Tanigawara, Y., Hori, R., & Komano, T., (1993a) *J. Biol. Chem. 268*, 6077–6080.

Saeki, T., Ueda, K., Tanigawara, Y., Hori, R., & Komano, T. (1993b) *FEBS 324*, 99–102.

Safa, A. R. (1993) *Cancer Invest. 11*, 46–56.

Safa, A. R., Stern, R. K., Choi, K., Agresti, M., Tamai, I., Mehta, N. D., & Roninson, I. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7225–7229.

Sato, W., Fukazawa, N., Suzuki, T., Yusa, K., & Tsuruo, T. (1991) *Cancer Res. 51*, 2420–2424.

Selassie, C. D., Hansch, C., & Khwaja, T. A. (1990) *J. Med. Chem. 33*, 1914–1919.

Sikic, B. I. (1993) *J. Clin. Oncol. 11*, 1629–1635.

Solary, E., Caillot, D., Chauffert, B., Casasnovas, R., Dumas, M., Maynadie, D., & Guy, H. (1992) *J. Clin. Oncol. 10*, 1730–1736.

Tamai, I., & Safa, A. R. (1991) *J. Biol. Chem. 266*, 16797–16800.

Tang-Wai, D., Brossi, A., Arnold, L. D., & Gros, P. (1993) *Biochemistry 32*, 6470–6476.

Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T., & Hori, R. (1992) *J. Biol. Chem. 267*, 24248–24252.

Valverde, M. A., Diaz, M., Sepulveda, F. V., Gill, D. R., Hyde, S. C., & Higgins, C. F. (1992) *Nature 355*, 830–833.

Van der Bliek, A. M., Kooiman, P. H., Schneider, C., & Borst, P. (1988) *Gene 71*, 407–411.

Yahanda, A. M., Adler, K. M., Fisher, G. A., Brophy, N. A., Halsey, J., Hardy, R. I., Gosland, M. P., Lum, B. L., & Sikic, B. (1992) *J. Clin. Oncol. 10*, 1624–1634.

Yang, C. P. H., Cohen, D., Greenberger, L. M., Hsu, S. I. H., & Horwitz, S. B. (1990) *J. Biol. Chem. 265*, 10282–10288.

Yoshimura, A., Kuwazuru, Y., Sumizawa, T., Ishikawa, M., Ikeda, S. I., Ueda, T., & Akiyama, S. I. (1989) *J. Biol. Chem. 264*, 16282–16291.

Yusa, K., & Tsuruo, T. (1989) *Cancer Res. 49*, 5002–5006.