Human (*MDR1*) and Mouse (*mdr1,mdr3*) P-glycoproteins Can Be Distinguished by Their Respective Drug Resistance Profiles and Sensitivity to Modulators†

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ABSTRACT: Possible functional differences between P-glycoproteins (P-gps) encoded by the human *MDR1* and mouse *mdr1* and *mdr3* genes with respect to drug resistance profiles and sensitivity to known modulators have been investigated. For this, the three genes were introduced and overexpressed in the same cellular background, that of Chinese hamster LR73 ovary cells, and drug-resistant clones expressing comparable amounts of the corresponding P-gps were selected under the same conditions. Analysis of the specific drug resistance profiles encoded by each P-gp for colchicine, adriamycin, vinblastine, and actinomycin D revealed overlapping but distinct patterns of drug resistance for the three isoforms. While all three P-gps conferred levels of resistance to vinblastine that did not vary by more than 2.5-fold, each isoform could be clearly distinguished by its capacity to confer resistance to colchicine and actinomycin D. Likewise, the study of structurally related and unrelated P-gp modulators indicated strong isoform-specific differences in the capacity of individual modulators to abrogate vinblastine resistance in the corresponding *mdr* transfectants. The study of several disubstituted piperazine analogs indicated that minor chemical modifications of the linker region of this modulator had strong effects on the sensitivity profile of each isoform to the modulator. Together, these results indicate that the three P-gp isoforms analyzed have specific and distinguishable functional characteristics with respect to interactions with drugs and modulators. These findings also suggest that P-gp positive murine transplantable tumors should be used with caution in the design and *in vivo* testing of novel P-gp modulators to be used to reverse multidrug resistance to tumor cells expressing human *MDR1*.

P-glycoproteins are encoded by a small family of closely related *mdr* genes which has three members in rodents (*mdr1*, *mdr2*, *mdr3*) and two members in humans (*MDR1*, *MDR2*). The typical P-gp is composed of two homologous halves, each encoding six putative transmembrane (TM) domains, and one nucleotide binding (NB) site (Gros et al., 1986a, 1988; Devault & Gros, 1990; Hsu et al., 1990; Chen, et al., 1986; Van der Bliek et al., 1988). This basic structural unit defines a superfamily of ABC membrane transport proteins with members in higher and lower eukaryotes as well as prokaryotes, implicated in the transport of unrelated substrates such as cations, anions, peptides, and sugars (Higgins, 1992). The biochemical analysis of photoaffinity-labeled tryptic P-gp peptides identified by epitope mapping has revealed that two short segments near TM6 and TM12 are major drug binding sites (Bruggeman et al., 1989, 1992; Yoshimura et al., 1989; Greenberger et al., 1991; Greenberger, 1993). Likewise, the analysis of mutant P-gps showing altered substrate specificity has shown that membrane-associated regions are important for drug recognition and transport by P-gp (Choi et al., 1988; Gros et al., 1991; Devine et al., 1992; Loo & Clarke, 1993a,b). On the other hand, mutational analysis of the two phylogenically conserved NB sites has shown that both are essential for P-gp function and that these sites probably underlie a mechanistic aspect common to all P-gps (Azzaria et al., 1989).

Studies in MDR cell lines obtained by stepwise drug selection and analyses of *mdr* gene transfectants have shown that *mdr* genes encode functionally distinct P-gps. Indeed, mouse *mdr1* (*mdr1b*) and *mdr3* (*mdr1a*) and human *MDR1* can confer MDR while mouse *mdr2* and human *MDR2* cannot (Gros et al., 1986b; Devault & Gros, 1990; Buschman & Gros, 1991; Ueda et al., 1987; Schinkel et al., 1991). Mouse *mdr2* is expressed almost exclusively in liver bile canaliculi (Cordon-Cardo et al., 1990; van der Valk et al., 1990; Buschman et al., 1992), and the analysis of mutant mice carrying null alleles at this locus has suggested that although this P-gp does not transport MDR drugs, it may transport phosphatidylcholine (Smit et al., 1993), working as a lipid translocase (Ruetz & Gros, 1994). In addition, P-gps encoded by mouse *mdr1* and *mdr3* confer different degrees of resistance to structurally distinct MDR drugs (Kajiji et al., 1993) and seem to show different sensitivity to the reversal effect of structurally distinct inhibitors (Yang et al., 1990; Kajiji et al., 1994a). A similar comparison of functional similarities and differences between the mouse and human P-gps has been very difficult to carry out. Indeed, human MDR cell lines which overexpress *MDR1* show large quantitative and qualitative variations in their drug resistance phenotypes [reviewed by Beck and Danks (1991)]. Underly-

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ing parameters perhaps contributing to these differences include (1) the tissue origin of the parental cell line (sarcoma, carcinoma, leukemia), (2) the specific properties of the drug used in selection of the resistant variant, (3) the type of drug survival assay used to measure resistance (short or long exposure to drugs), and (4) the presence of discrete mutations in P-glycoprotein overexpressed in these cells (Beck & Danks, 1991). This phenotypic heterogeneity renders very difficult a comparative analysis of the published drug resistance profiles and response to modifiers associated with human and mouse P-gps. Identifying such putative differences would be very important since MDR cell lines and tumors of rodent origins are currently used as models/systems for the production and screening of novel drugs and reversal agents capable of bypassing or blocking the activity of the human P-gp expressed in clinical tumors [reviewed by Tew et al. (1993)]. Moreover, localizing in chimeric and mutant proteins the specific segments and amino acids implicated in substrate specificity and response to modulators would help the structure/function analysis of P-gp.

To address these questions unambiguously, we have introduced and overexpressed in Chinese hamster ovary cell line LR73 P-gps encoded by the wild-type human *MDR1* and mouse *mdr1* and *mdr3* genes. We have then compared the drug resistance profiles of cell clones expressing similar amounts of each P-gp isoform for several MDR drugs in cytotoxicity assays. We have analyzed the capacity of structurally distinct P-gp modulators to abrogate vinblastine resistance encoded by the three proteins. Our results show that the human and mouse P-gps confer overlapping but distinct drug resistance phenotypes and show dramatically different responses to modulators.

MATERIALS AND METHODS

Drugs and Chemicals. Vinblastine, colchicine, sulforhodamine B, and rhodamine 123 were purchased from Sigma Chemical Co. (St. Louis, MO). Adriamycin and actinomycin D were provided by Dr. C. Shushtik (Royal Victoria Hospital, Montreal). Stock solutions of vinblastine, colchicine, and adriamycin were prepared at 1.0 mg/mL in phosphate-buffered saline (PBS), while actinomycin D was prepared at 0.5 mg/mL and stored at −80 °C protected from light. The MDR modulators cyclosporin A (CsA) and a diaminoquinazoline CP100356 (Kajiji et al., 1994b) and the disubstituted piperazine analogs CP162398 (*R* enantiomer), CP147795, CP215545, CP215548, and CP147478 (*R* enantiomer) were synthesized at Pfizer Central Research, Groton, CT. CP162398 has been previously described in the literature as MS-073 (racemic mixture; Sato et al., 1991).

Cell Lines and Tissue Culture. Chinese hamster LR73 ovary cells and their drug-resistant derivatives transfected and overexpressing either *mdr1* (clone 1S) or *mdr3* (clone 3S) cDNAs were prepared and maintained as previously described (Gros et al., 1991). LR73 cells transfected with and overexpressing the human *MDR1* gene were prepared as follows. LR73 cells were electroporated with 2 μg of plasmid pCMV*MDR1neo*, a plasmid containing the cytomegalovirus promoter which drives the expression of a bicistronic mRNA combining the wild-type coding sequence of human *MDR1* cDNA (Choi et al., 1988) with the *neo* (G418 resistance) gene (B. S. Morse and I. B. Roninson, unpublished procedures). Since pCMV*MDR1neo* expresses the *neo* gene only at a low level, this plasmid was cotransfected with 0.1 μg of indicator plasmid pSV2*neo*. Transfectants were selected with 500 μg/mL G418. Twenty-four colonies were expended and tested for *MDR1* expression first by a functional assay based on the efflux of rhodamine 123 and then by immunostaining with monoclonal antibody UIC2, specific to the human *MDR1* gene product (Mechetner & Roninson, 1992), as previously described (Chaudhary & Roninson, 1991). The *MDR1* positive clones were then selected in medium containing vinblastine (50 ng/mL) to increase their *MDR1* expression to levels similar to those of the mouse *mdr1* and *mdr3* transfectants. All cell lines were grown in α-minimal essential medium (MEM) supplemented with 10% fetal calf serum, 3 mM glutamine, penicillin (50 units/mL), and streptomycin (50 mg/mL). Tissue culture medium, serum, and supplements were purchased from Gibco/BRL.

Detection of P-glycoproteins. Membrane-enriched fractions from parental LR73 cells or drug-resistant transfectants were prepared by ultracentrifugation, as previously described (Devault & Gros, 1990). Protein concentrations in the extracts were measured using an amido black based commercial assay (Bio-Rad). Forty micrograms of membrane proteins form control and *mdr*-transfected cells was separated by sodium dodecyl sulfate−polyacrylamide (final concentration 7.5%) gel electrophoresis (SDS−PAGE) and transferred to a nitrocellulose membrane by electroblotting. For dilution analyses, 40, 20, and 10 μg of crude membrane extract were used. The Western blot was incubated with the monoclonal mouse anti-P-gp antibody C219 (Centocor Corp., Philadelphia, PA) at a dilution of 1:300 for 1 h at 20 °C, and specific immune complexes were revealed with goat anti-mouse IgG antiserum coupled to alkaline phosphatase used at a 1:3000 dilution, as described (Devault & Gros, 1990).

Cytotoxicity Assay. A modification of a cell survival assay (Skehan et al., 1989) based on sulforhodamine B (SRB) staining of total cell protein was used. Briefly, 5×10^3 drug-sensitive LR73 control cells or *mdr* transfected clones were plated in 96-well titer plates in complete medium containing increasing concentrations of either vinblastine, colchicine, adriamycin, or actinomycin D and incubated for 72 h at 37 °C. Cells were then washed once in ice-cold PBS, fixed in 17% trichloroacetic acid in PBS for 1 h at 4 °C, and then washed extensively in tap water. Cellular protein was stained with a solution of 0.4% SRB in 1% acetic acid for 15 min at room temperature, followed by four washes with 1% acetic acid to remove excess stain. After the plates were dried, the stain was dissolved in 10 mM Tris (pH 9.0), and quantitation was carried out using an automated ELISA plate reader (Bio-Rad Model 450) set at 490 nm. The relative plating efficiency of each clone was calculated by dividing the absorbance observed at a given drug concentration by the absorbance detected in the same clone in medium devoid of drug and is expressed as a percentage. IC_{50} is defined as the drug dose required to reduce plating efficiency of each clone in a given drug by 50%. The inhibitory activity of modulators was determined by calculating the vinblastine IC_{50} of each clone in increasing concentrations of modulators (range 0.01−1 μM). The dose of modulator required to reduce the IC_{50} for vinblastine of each clone by 90% was defined as the MC_{90} (90% modulatory concentration). The results shown were the average of three independent experiments, each carried out in duplicate.

Molecular Modeling. The structure of cyclosporin A was obtained from the Cambridge Crystallographic Databank. The

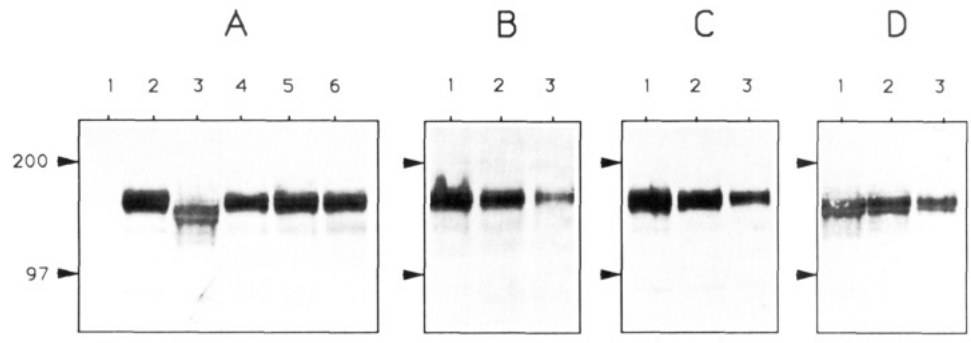


FIGURE 1: Identification of P-glycoproteins encoded by human *MDR1* and mouse *mdr1* and *mdr3* cDNAs transfected in LR73 drug-sensitive cells. (A) Forty micrograms of crude membrane extracts from either LR73 control cells (1), stable drug-resistant transfectants expressing mouse *mdr1* (2) or mouse *mdr3* (3), or three independent drug-resistant transfectants expressing human *MDR1* cDNAs (4−6) was analyzed by immunoblotting with the mouse anti-P-glycoprotein antibody C219. A 2-fold dilution of the same extracts corresponding to 40 (1), 20 (2), or 10 (3) μg of membrane proteins from either human *MDR1* (B), mouse *mdr1* (C), or mouse *mdr3* (D) transfectants was also analyzed by Western blotting with C219. The four blots were developed simultaneously and under identical conditions. The molecular mass markers used were myosin (200 kDa) and phosphorylase *b* (97 kDa), and their positions are indicated by arrowheads on each blot.

remaining structures were generated and minimized using the SYBYL program from TRIPOS Associates. The Connoly surfaces were generated using the MOLCAD module of SYBYL. Electrostatic potentials are based on Gasteiger−Huckel charges.

RESULTS

Expression of P-glycoproteins Encoded by the Mouse mdr1 and mdr3 and Human MDR1 Genes in Transfected CHO Cells. Members of the human and mouse *mdr* gene family can be classified in two groups. While full-length cDNA clones for the mouse *mdr1* and *mdr3* and human *MDR1* genes confer drug resistance to transfected cells, cDNAs for mouse *mdr2* and human *MDR2* cannot do so. To further characterize possible functional differences between the MDR type of human and mouse isoforms, we have overexpressed full-length cDNAs for mouse *mdr1* and *mdr3* and for human *MDR1* in the same cellular background, that of LR73 Chinese hamster ovary (CHO) cells. The LR73 transfectants overexpressing mouse *mdr1* and *mdr3* isoforms have been previously described (Gros et al., 1991; Kajiji et al., 1993). LR73 transfectants stably expressing human *MDR1* were prepared as described in Materials and Methods. Briefly, CHO cells were cotransfected with the indicator plasmid pSV2*neo* and a mammalian expression vector directing high levels of expression of a full-length human *MDR1* cDNA placed under the control of a cytomegalovirus enhancer (pCMV-MDR). Neomycin-resistant colonies were first selected in G418, and *MDR1* positive cell clones were then selected on the basis of a rhodamine 123 efflux assay (Chaudhary & Roninson, 1991) and survival in vinblastine (50 ng/mL). Several independent VBL-resistant colonies were isolated, expanded in culture, and frozen.

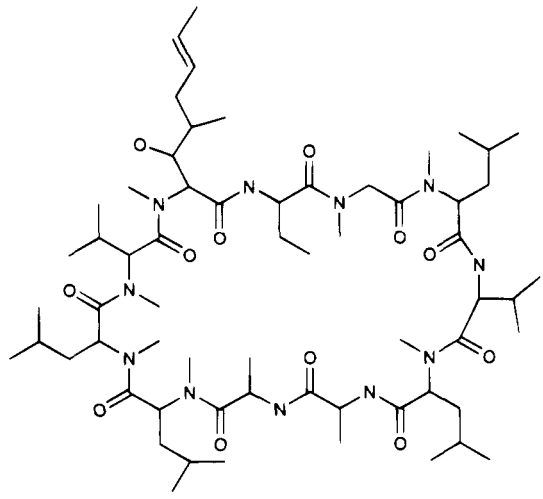
Enriched membrane fractions from three independently isolated *MDR1* transfectants were prepard and analyzed for their level of P-gp expression by Western blotting using the anti-P-glycoprotein monoclonal antibody C219 (Kartner et al., 1985). Levels of expression were compared to those detected in membrane fractions from mouse *mdr1* and *mdr3* CHO transfectants isolated under the same drug selection conditions (Figure 1A). All cell clones expressed readily detectable amounts of specific immunoreactive species of molecular mass 160−165 kDa for mouse *mdr1* and human *MDR1* transfactants and 150−155 kDa for mouse *mdr3* transfectants which were absent in control untransfected LR73 cells. The three human *MDR1* transfectants seemed to express comparable amounts of the human P-gp, and this level of expression was comparable to that detected for the mouse isoforms present in the *mdr1* and *mdr3* expressing clones (Figure 1A). To further evaluate the relative level of expression of each isoform, three serial dilutions of membrane fractions from human *MDR1* (Figure 1B), mouse *mdr1* (Figure 1C), or mouse *mdr3* (Figure 1D) expressing clones were analyzed by immunoblotting with C219. These experiments showed that the level of expression of the three isoforms was comparable in these cells, although the level of human *MDR1* expression was slightly lower than that of the two mouse isoforms and this by a factor of no more than 2-fold.

Drug Resistance Profiles of Transfected Cell Clones Stably Expressing either Mouse mdr1 or mdr3 or Human MDR1. The three transfected cell clones analyzed in Figure 1 were then tested for their levels of cellular resistance to four structurally unrelated MDR drugs, actinomycin D (ACT), adriamycin (ADM), colchicine (COL), and vinblastine (VBL). This was done in a 72-h assay which monitored cell survival in medium containing increasing concentrations of each drug and used total cellular protein staining of fixed cells with sulforhodamine B as an indicator of cell survival. These results are shown in Table 1 and are presented as the

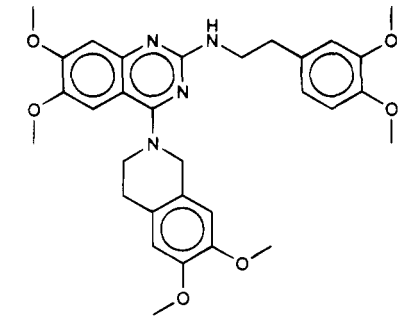
Table 1: Drug Survival Characteristics of Cell Clones Stably Transfected with cDNAs for Mouse *mdr1* and *mdr3* or Human *MDR1* Genes

	LR73	*mdr1*	*mdr3*	*MDR1*	*n*[c]
actinomycin D	1.3 ± 0.4[a]	6 ± 2 (4×)[b]	110 ± 30 (114×)	30 ± 10 (24×)	6
adriamycin	17 ± 2	310 ± 90 (18×)	600 ± 300 (35×)	130 ± 50 (7×)	8
colchicine	30 ± 6	800 ± 200 (27×)	1600 ± 200 (57×)	73 ± 8 (2×)	6
vinblastine	6 ± 1	150 ± 50 (24×)	310 ± 60 (53×)	110 ± 60 (19×)	7

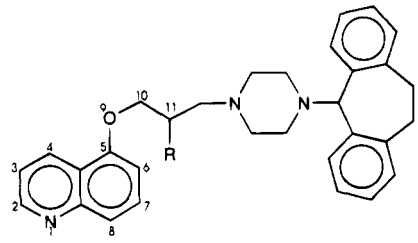
[a] The drug survival of Chinese hamster LR73 drug-sensitive cells (LR73) and of cell clones transfected with either wild-type mouse *mdr1* or *mdr3* or wild-type human *MDR1* is expressed as the IC_{50} (in ng/mL) or the dose necessary to reduce the plating efficiency of the control and transfected cells by 50%. [b] The resistance index is the degree of resistance above background levels expressed in LR73 cells and is shown in parentheses. [c] Number of independent experiments.



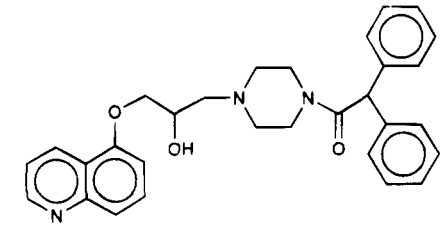
Cyclosporin A



CP100356



Compound	R
CP147795	H
CP162398	OH
CP215545	OCH_3
CP215548	CN



CP147478

FIGURE 2: Chemical structure of P-gp modulators.

IC_{50}, or the drug dose necessary to reduce plating efficiency by 50%, when compared to control wells containing medium devoid of drug. Both quantitative and qualitative differences were noted in the specific drug resistance profile expressed by each cell clone. At similar levels of protein expression, the mouse *mdr3* isoform seemed to be a more efficient drug efflux pump, as the *mdr3* transfectant showed levels of resistance that were superior to those expressed by *mdr1* or *MDR1* transfectants by a factor of at least 2-fold, and this for all drugs tested. In addition, important qualitative differences between each clone were evident for some of the four drugs tested. While the three transfectants demonstrated robust levels of resistance to VBL, human *MDR1* conferred lower levels of resistance to ADM than did mouse *mdr1* and *mdr3*. More striking differences were detected for COL and ACT resistance levels. In the case of COL, human *MDR1* was poorly active against this drug (2× resistance), while mouse *mdr1* (27×) and *mdr3* (57×) transfectants expressed strong levels of resistance. Finally, the situation was reversed for ACT, as mouse *mdr1* transfectants were only poorly resistant to this drug (4×), while human *MDR1* (24×) and mouse *mdr3* (114×) expressing clones were resistant and highly resistant to this drug, respectively. These results indicate that although the three P-gp isoforms analyzed showed overlapping patterns of drug resistance, they could be distinguished by obvious differences in the level of resistance they could each confer to individual drugs of the MDR spectrum, such as COL and ACT.

Efficacy of Modulators in Reversing VBL Resistance Expressed by Mouse mdr1 and mdr3 and Human MDR1 Transfectants. The previously analysis suggested that P-glycoproteins encoded by mouse *mdr1* and *mdr3* and human *MDR1* may have distinct substrate specificities, with respect to MDR drugs. Since many of the known modulators of drug efflux of P-gp appear to mediate their effect by competing for drug binding sites or transport by P-gp [reviewed by Safa (1993)], the functional differences detected among the three isoforms with respect to drug resistance profiles may also underlie different sensitivity of the three isoforms to known P-gp modulators. To address this issue, we tested the capacity of structurally related and structurally unrelated P-gp modulators to reverse the drug resistance phenotype encoded by each of the human and mouse P-gp isoforms expressed in LR73 CHO transfectants.

The structurally distinct modulators tested included a cyclic peptide (cyclosporin A, CsA), a diaminoquinazoline (CP100356), and two disubstituted piperazine analogs, CP162398 and CP147478 (Figure 2). These molecules are also distinct with respect to shape and charge distribution, as shown by the analysis of surface electrostatic potentials (Figure 3). Also tested were a series of close-in analogs of CP162398, in which the hydroxyl group at the chiral center (position 11) has been substituted with either a proton (H; CP147795), a methoxy group (OCH_3; CP215545), or a cyano group (CN; CP215548) (Figure 2). These modifications alter the conformation, restrict rotation, and direct the tail portion of these compounds into different regions (Figure 4). While these close-in analogs are not as structurally distinct as the previous set of compounds, they are useful to determine whether or not P-gp isoforms can distinguish compounds

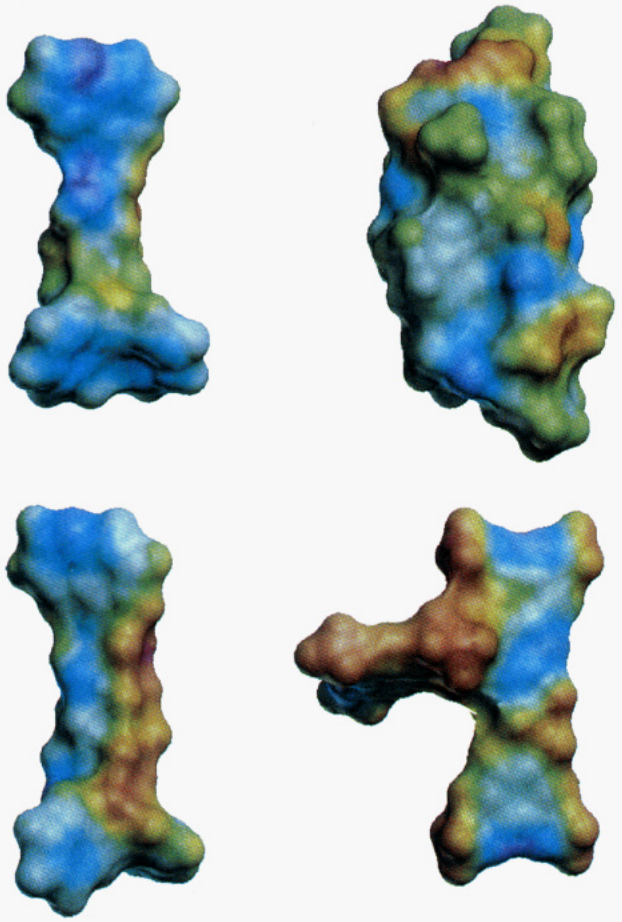


FIGURE 3: Electrostatic potential map of P-gp modulators. Conolly surfaces of (clockwise from upper right corner) cyclosporin A, CP100356, CP147478, and CP162398. The electrostatic potentials are mapped onto the surfaces; blue denotes negatively charged regions and red denotes regions of positive charges.

FIGURE 4: Molecular overlap of the hydroxy (CP162398, blue), methoxy (CP215545, red), and cyano (CP215548, green) analogs.

containing subtle changes. We have previously observed that these compounds are potent inhibitors of P-gp function and are active in the submicromolar range *in vitro* (except CsA and CP215548). *In vivo*, coadministration of CP100356, CP162398, and CsA in conjunction with adriamycin or etoposide results in growth inhibition of KBV1 xenografts implanted in nude mice and increases mean survival of P388/ADR leukemia-bearing mice, respectively (Kajiji et al., 1994b). Since the three P-gp isoforms display strong differences in their capacity to confer resistance to ADM, COL, and ACT, analyzing the capacity of modulators to reverse drug resistance expressed by the three isoforms against either ADM, COL, or ACT would be difficult (comparing effects on high vs low levels of initial resistance). Therefore, we chose to measure the modulating activity of the various compounds on the level of cellular resistance to VBL expressed by each clone. VBL was selected because the three isoforms expressed robust resistance to this drug and showed the least divergent initial base line of comparison. For this, we determined the IC_{50} for VBL of mouse *mdr1* and *mdr3* and human *MDR1* transfectants in medium containing increasing concentrations of each modulator over a 0.01−1 μM concentration range (complete data set is shown in Figure 5). Since the IC_{50} for VBL determined for the *mdr* transfectants in modulator free medium was similar but not identical (see Table 1), a more accurate relative modulating index was calculated. This index (MC_{90}) corresponds to the modulator dose required to reduce the VBL IC_{50} of each *mdr* transfectant by 90% (Table 2). Each of the modulators tested had very little effect on the IC_{50} for control LR73 cells in the concentration range analyzed, except at the highest concentration where a small decrease in IC_{50} was noted (Figure 5). Striking differences were detected in the capacity of the various modulators to reverse VBL resistance encoded by the three P-gp isoforms. The seven compounds tested fell into three groups. The first type of compounds, which included CP215548 and cyclosporin A, showed equal efficacy against the three isoforms (Table 2). The second group of compounds, exemplified by CP215545, and CP100356, again showed similar efficacy for mouse *mdr1* and *mdr3* but were more active against human *MDR1* by a factor of 3−10-fold (Table 2). The third group, which included CP147795 and CP162398, were most efficacious against human *MDR1* and least efficacious against mouse *mdr1* , with *mdr3* showing levels of sensitivity intermediate to the two other isoforms (Table 2). This was most obvious for CP162398, where a 64-fold difference was noted between the dose required to reduce by 90% the VBL IC_{50} of human *MDR1* compared to mouse *mdr1* (Table 2). The distinct effect of individual modulators against each P-gp isoform appeared highly specific, since minor chemical modifications of the linker region of the modulator produced dramatic effects on the noted differential effect of these compounds for the three isoforms. For example, the substitution of the hydroxyl group at position C11 of CP162398 by a CN group in CP215548 caused a 60-fold decrease in the efficacy of this compound against human *MDR1* while causing only a 2-fold decrease in its efficacy against mouse *mdr1* (Table 2). Taken together, these results indicate that the mouse *mdr1* and *mdr3* and human *MDR1* isoforms can also be distinguished on the basis of their relative sensitivity to modulators.

DISCUSSION

We wanted to determine if functional differences, in particular with respect to substrate specificity, could be detected between the human *MDR1* and mouse *mdr1/mdr3* P-glycoproteins. Such differences may provide important

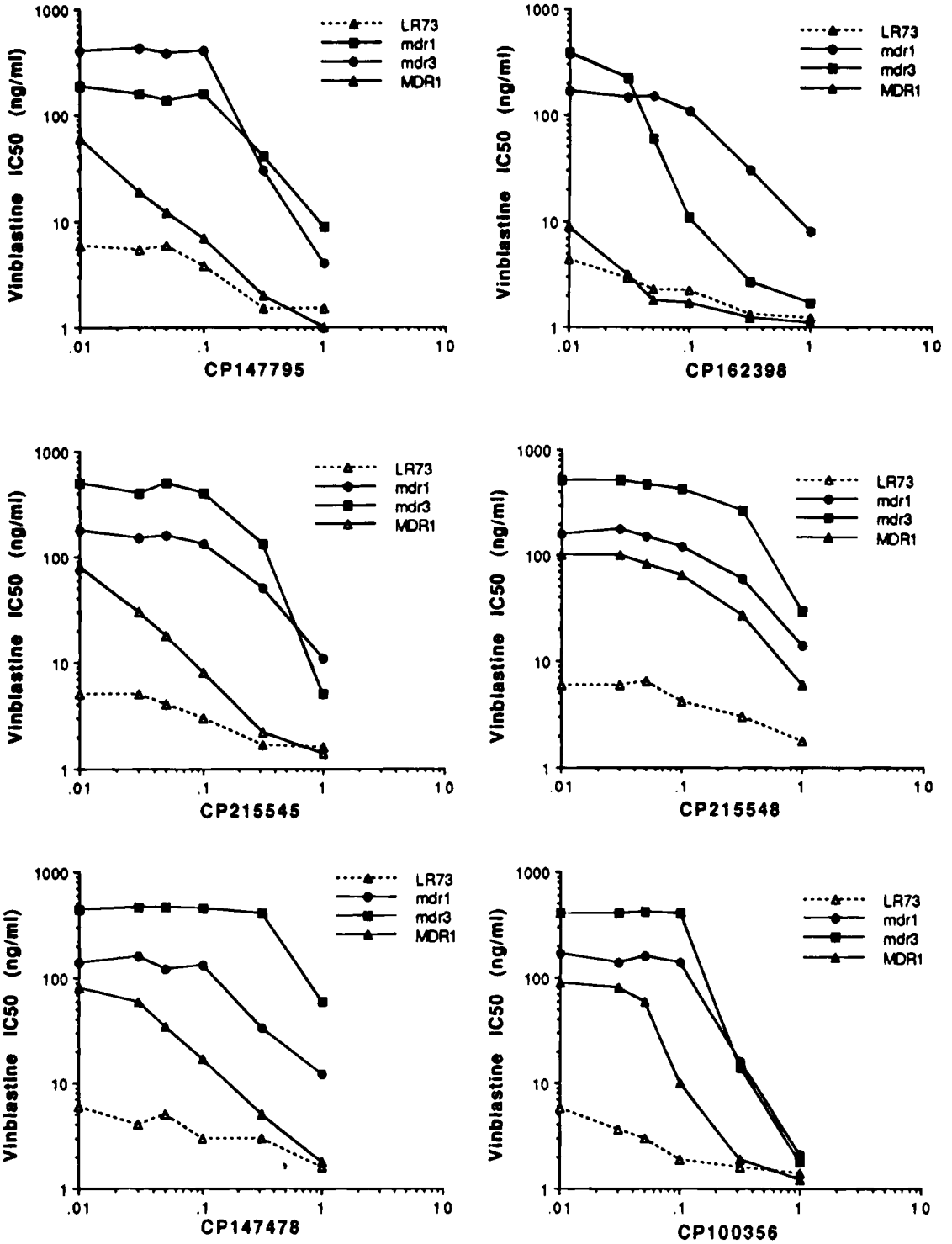


FIGURE 5: Reversal of vinblastine resistance of control LR73 cells and multidrug-resistant cell clones expressing either mouse *mdr1* or *mdr3* or human *MDR1* cDNAs. The effect of individual modulators on the activity of each P-glycoprotein isoform was determined by calculating the IC_{50} for vinblastine of each cell clone in increasing concentrations of individual modulators. The vinblastine IC_{50} (ng/mL) and the dose of modulator (in μM) are plotted as log values. The cell clones used are those described in Figure 1, and the structures of the modulators are shown in Figure 2.

clues on structure/activity relationships of these proteins, including the identification of putative amino acid residues implicated in substrate interactions. Although a large number of multidrug-resistant cell lines overexpressing P-gp have been described, their specific drug resistance profiles are quite heterogeneous (Beck & Danks, 1991), precluding simple comparison with those associated with mouse P-gps. To detect possible functional differences between the mouse and humans P-gps, we have expressed them on the same cellular background and isolated clones expressing comparable levels of each protein by the same drug selection protocol. Analysis of the drug resistance profiles of these transfected cell clones revealed that although all three isoforms conferred overlapping patterns of resistance to a defined group of drugs, each isoform encoded a unique drug resistance phenotype (Table 1). The most striking differences were observed for actinomycin D and colchicine where the specific pattern of resistance to these two drugs was unique and quite distinct for each P-gp. The identity of protein segments and amino acid residues responsible for these

Table 2: Doses of Modulators Required To Reduce by 90% (MC_{90}) the Level of Vinblastine Resistance (IC_{50}) in LR73 Cell Clones Transfected with Mouse *mdr1* and *mdr3* and Human *MDR1* cDNAs

	cell lines[a]		
	mdr1	*mdr3*	*MDR1*
CsA[b]	ND[c]	0.7	1.5
CP147795	0.8	0.3	0.06
CP162398	0.64	0.07	0.01
CP215545	0.82	0.82	0.08
CP215548	1.0	1.1	0.64
CP147478	0.8	>1.0	0.16
CP100356	0.31	0.24	0.09

[a] Stably transfected CHO cells expressing similar amounts of P-glycoproteins encoded by wild-type mouse *mdr1* and *mdr3* and human *MDR1* genes were used in these experiments. Their level of vinblastine resistance was determined by measuring the plating efficiency of these cells in increasing concentrations of vinblastine and was calculated as an IC_{50} value or the dose required to reduce the plating efficiency of each clone by 50%. [b] The effect of individual modulators on the activity of each P-glycoprotein isoform was determined by calculating the IC_{50} for vinblastine of each cell clone in increasing concentrations of the modulator and is expressed as the dose of modulator (in μM) required to reduce the vinblastine IC_{50} of each clone by 90% (MC_{90}). [c] ND, could not be determined even at the maximum dose tested.

differences in substrate specificity can now be studied in chimeric or mutant proteins.

P-gp function can be blocked or reversed by a large group of structurally unrelated so-called "modulators" like the calcium channel blocker verapamil (Tsuruo et al., 1981), the immunosuppressive antifungal cyclosporin A (Slater et al., 1986) and its analogs (Twentyman, 1988), the natural hormone progesterone (Yang et al., 1989), and novel compounds like CP100356 and CP162398 (Kajiji et al., 1994b). Although the mechanism of action of these compounds is not completely understood, they all seem to compete for drug binding sites on P-gp (Safa, 1993). While some of these inhibitors can be transported by P-gp, others apparently cannot (Yusa & Tsuruo, 1989; Ueda et al., 1992; Saeki et al., 1993). Mutations, such as those at serine position 939 within the predicted TM11 of mouse Mdr3 that affect substrate specificity by modulating initial drug binding to the protein (Kajiji et al., 1993), also affect the capacity of structurally unrelated inhibitors to block P-gp action (Kajiji et al., 1994a), suggesting that interactions with drug molecules and modulators involve common P-gp determinants or secondary structure. Therefore, differences in drug resistance profiles detected between the mouse and human P-gps raised the interesting possibility that the sensitivity of the three P-gp isoforms to known modulators may be quite distinct. A major effort is currently underway to design novel P-gp modulators to be used for the circumvention of MDR in humans. The *in vivo* testing of these compounds has relied on the mouse as an animal model, where a transplantable tumor cell line and its multidrug-resistant P-gp positive derivative are used to assess activity and potency of these compounds against P-gp. Although some studies have relied on human transplantable tumors grown in nude mice such as KB carcinoma, the vast majority of studies have relied on murine tumors such as Ehrlich ascites carcinoma, L1210 leukemia, and in particular the P388 murine leukemia grown in an histocompatible host [for review, see Tew et al. (1993)]. Since potential modulators evaluated in these assays would be used ultimately to revert MDR in human tumors positive for human *MDR1* expression, it seemed appropriate to determine if functional differences with respect to interaction with modulators could be detected between the human and mouse isoforms. In addition, since we (Raymond et al., 1990) and others (Lothstein et al., 1989) have previously shown that the emergence of MDR in mouse cells (LTA, J774A, P388) can be asociated with the independent or simultaneous expression of *mdr1* and/or *mdr3*, it also seemed important to ask the same question for the two mouse isoforms in view of the previously reported differences in the modulating activity of progesterone (Yang et al., 1990) and other agents (Kajiji et al., 1993, 1994a) against the two mouse proteins.

Analysis of the sensitivity of the three P-gp isoforms to structurally related and unrelated modulators revealed major differences in the range of 5−60-fold in the modulator dose required to reduce by 90% the VBL IC_{50} of clones expressing either the human or the mouse proteins (Table 2). These differences were most obvious for compounds CP162398 and CP215548, where the human P-gp isoform was much more sensitive to the modulator than either mouse isoform. Since these compounds are close-in analogs of each other, they must be accommodated by similarly shaped binding pockets on the P-gp isoforms. Differences in potency can be attributed to changes within amino acids forming this pocket, altering the nature of this pocket by introducing small changes such as hydrogen bond donors/acceptors. As a result, minor alterations in the modulators can translate into large changes in binding affinity, because while the overall shape and size may be sufficient for binding, smaller unfavorable contacts may reduce optimal interaction. Since the trends in potency differ among the three different proteins, their binding sites must also be correspondingly distinct. Most of the modulators described show improved potency and specificity when compared to CsA, a compound currently undergoing clinical evaluation. For example, CP162398 is 100-fold more potent that CsA against the human isoform. Moreover, it is 10-fold more specific for *MDR1* than *mdr3*.

Although human *MDR1* was more sensitive to the modulators tested than its mouse counterparts, this is unlikely to be a general characteristic, and novel modulators may prove to be more active against the mouse than human proteins. These results suggest that efficacy data for modulators estimated *in vivo* with murine tumors must be interpreted with caution when extrapolated to human *MDR1*. Utilizing transplantable human tumors such as KB carcinoma cells (Tew et al., 1993), 8226 myeloma cells (Bellamy et al., 1993), or MDR1 transfectants (Schinkle et al., 1991) in immunocompromised mice may be important to verify pharmacological data obtained with drug-sensitive and P-gp positive P388 cells. However, human tumor xenografts are generally grown as subcutaneous implants in immunocompromised mice, and the substantial difference in drug delivery to subcutaneous site vs highly perfused normal tissues often prevents accurate determination of the therapeutic index to identify improved MDR modulators. Generation of mutant mice bearing null alleles at the *mdr* locus (Schinkel et al., 1994) expressing a transgenic human *MDR1* in well-vascularized normal tissues would provide useful preclinical *in vivo* model systems for systematic identification of clinically useful novel modulators. In addition, we observed that small alterations in the structure of the modulator had major effects on the differences detected between each

isoform for a given compound. For example, the substitution of the hydroxyl group at position C11 of CP162398 by a CN group in CP215548 caused a 60-fold decrease in the efficacy of this compound against human *MDR1* while causing only a 2-fold decrease in its efficacy against mouse *mdr1* (Table 2). Therefore, results from structure/activity relationship studies of congeners of the same compound carried out in murine tumors should also be verified in human cells overexpressing *MDR1*.

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