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Chemosensitization and drug accumulation effects of VX-710, verapamil, cyclosporin A, MS-209 and GF120918 in multidrug resistant HL60/ADR cells expressing the multidrug resistance-associated protein MRP

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Overexpression of the multidrug resistance *MDR*1 gene product P-glycoprotein and/or the multidrug resistance-associated protein MRP confers multidrug resistance to cancer cells. The pipecolinate derivative VX-710 has previously been demonstrated to reverse *MDR*1-mediated multidrug resistance at concentrations of 0.5–2.5 μM by direct interaction with P-glycoprotein and inhibition of its drug efflux activity. In this study we investigated whether VX-710 as well as four other known *MDR*1 modulators could also reverse multidrug resistance mediated by MRP. VX-710 at 0.5–5 μM restored senstivity of MRP-expressing HL60/ADR promyelocytic leukemia cells to the cytotoxic action of doxorubicin, etoposide and vincristine. VX-710 was approximately 2-fold more effective than verapamil, MS-209 and CsA in modulating MRP-mediated multidrug resistance, whereas GF120918 had no significant effect. VX-710 was also more effective than verapamil, MS-209 and CsA in restoring the daunorubicin accumulation deficit in HL60/ADR cells and in increasing calcein uptake. A photoaffinity analog of VX-710, [^{3}H]VF-13,159, specifically photolabeled the MRP protein and unlabeled VX-710 inhibited this binding in a concentration-dependent manner. These data suggest that VX-710 is not only a potent modulator of P-glycoprotein-mediated multidrug resistance, but also affects multidrug resistance in MRP-expressing cells and may exert its action, at least in part, by binding directly to MRP.

Key words: Chemosensitizer, cyclosporin A, GF120918, MS-209, multidrug resistance-associated protein, P-glycoprotein, verapamil, VX-710.

Introduction

The emergence of multidrug resistance (MDR), defined as broad spectrum resistance to clinical cytotoxic agents, is a major obstacle to successful chemotherapy of cancer. Studies of drug-selected cultured tumor cell lines have revealed that a number of proteins are overexpressed in multidrug resistant cells when compared with their drug-sensitive counterparts.[1] These include two different integral membrane proteins, the 170 kDa product of the human *MDR*1 gene, P-glycoprotein,[2] and the 190 kDa MDR-associated protein MRP.[3] Both of these proteins are members of a superfamily, known as the ATP-binding cassette (ABC) superfamily of transport proteins[4] or traffic ATPases[5] and confer resistance to a broad and partially overlapping spectrum of anticancer drugs, including doxorubicin, vincristine and etoposide.[6,7] In contrast to P-glycoprotein, MRP confers only low levels of resistance to paclitaxel and colchicine, but has also been associated with resistance to heavy metals such as sodium arsenite, sodium arsenate and potassium antimonate.[8–10] What determines whether a cell will acquire drug resistance through increased expression of P-glycoprotein or MRP is not yet known. Overexpression of these two proteins, however, is not mutually exclusive and increased levels of P-glycoprotein and MRP have been found in the same tumor cell line.[11–13]

Drug-selected cells which overexpress P-glycoprotein are generally defective in the cellular accumulation of cytotoxic drugs.[14,15] Reduced drug accumulation is also a characteristic of many MRP-overexpressing cell lines[7,16] The plasma membrane-associated P-glycoprotein confers MDR by functioning as an ATP-dependent drug efflux pump which is able to transport anticancer drugs against a substrate concentration gradient,[17] and causes both decreased drug uptake and increased drug efflux.[6] The basis for MRP-mediated drug resistance is less well defined. In addition to drug accumulation defects, differences in subcellular drug distribution have been observed in a number of drug-selected MRP-expressing cell lines or MRP cDNA-transfected

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cells.[7,12,18–20] Moreover, differences in the subcellular distribution of the MRP protein itself have been noted with predominant locations including the endoplasmic reticulum,[21] the plasma membrane[9,22] or post-Golgi vesicles.[23,24] It has been suggested that in some cells MRP participates in sequestering anticancer drugs away from their cellular target,[3,10] while plasma membrane-associated MRP may contribute to drug efflux.[8–10]

Elevated levels of *MDR*1 mRNA and/or P-glycoprotein have often been detected in hematological malignancies, mainly after failure of combination chemotherapy, and the MDR phenotype as a cause of clinical resistance is well documented in acute myeloid leukemia and myeloma.[25] Increased *MDR*1 gene expression has also been observed in different types of refractory solid tumor samples from relapsed or untreated patients, substantiating the clinical importance of the *MDR*1 gene product.[26] Because of its relatively recent discovery, the clinical significance of MRP is not well established. Thus far, elevated levels of MRP mRNA (and in some instances also MRP protein) have been detected in malignant cells derived from tumors that are known to respond poorly to chemotherapy, including non-small cell lung carcinoma, thyroid carcinoma and neuroblastoma.[7,27–29] Moreover, MRP mRNA is frequently expressed in acute myeloid, acute lymphoblastic, acute lymphocytic, prolymphocytic and chronic lymphocytic leukemia suggesting that the MRP gene product may contribute to the clinical chemoresistance of these hematological malignancies.[7,30–32]

To date, strategies aimed at reversing MDR in clinical oncology have mainly focused on inhibition or modulation of P-glycoprotein activity. A wide variety of MDR reversing agents have been reported which restore intracellular drug accumulation in *MDR*1-overexpressing multidrug resistant cells by inhibiting the drug efflux activity of P-glyco-protein.[33] These *MDR*1 modulators include calcium channel blockers, calmodulin antagonists, cyclic peptides, steroidal agents and many other classes of compounds.[33] These agents share only broad structural similarities, but all are amphiphilic and many are heterocyclic, positively charged molecules. A few compounds have been reported to restore the sensitivity of MRP-overexpressing cells to anti-cancer drugs to varying degree by increasing drug accumulation and/or altering drug distribution.[7,34] These compounds include some effective *MDR*1 modulators, e.g. the phenylalkylamine calcium channel blocker verapamil,[35–39] the dihydropyridines nicardipine[36] and NIK250,[38,40] the pyridine analog PAK-104P,[41] the cyclic peptide cyclosporin A,[19,42] the diiodinated benzofuran amiodarone[42] and the bisindoylmaleimide protein kinase C inhibitor GF109203X.[43]

In a previous study, we have investigated the pipecolinate derivative VX-710 (or (*S*)-*N*-[2-Oxo-2-(3, 4-trimethoxyphenyl)acetyl]piperidine-2-carboxylic acid 1,7-bis(3-pyridyl-4-heptyl ester) as a reversal agent for P-glycoprotein-mediated MDR.[44] VX-710 is a non-macrocyclic ligand of the FK506-binding protein FKBP12 which was found to interact directly with P-glycoprotein and block its drug efflux function. Concentrations of 0.5–5 μM VX-710 are sufficient to restore sensitivity of *MDR*1-expressing cells to the cytotoxic action of anthracyclines, Vinca alkaloids, epipodophyllotoxins and taxanes.

In the present study we investigated the effects of VX-710 on the sensitivity of the Adriamycin (doxorubicin)-selected human promyelocytic leukemic cell line HL60/ADR,[45] which overexpresses MRP,[16,46–49] to doxorubicin, etoposide and vincristine. The MDR reversing activity of VX-710 was compared with that of other known *MDR*1 modulators, including verapamil,[50] cyclosporin A (CsA),[51] the quinoline derivative MS-209[52] and the acridone carboxamide derivative GF120918.[53] Uptake and efflux studies involving the fluorescent drug daunorubicin and the fluorescent dye calcein were performed to show that the mechanism of action of these MDR reversing agents in MRP-expressing cells involves restoration of drug accumulation. A tritiated photoaffinity analog of VX-710, [^{3}H]VF-13,159, was used in photolabeling experiments to demonstrate that VX-710 interacts directly with the MRP protein.

Materials and methods

Compounds

VX-710 or (*S*)-*N*-[2-Oxo-2-(3,4,5-trimethoxyphenyl) acetyl]piperidine-2-carboxylic acid 1,7-bis (3-pyridyl)-4-heptyl ester) was used either as a free base or as a dicitrate salt. Verapamil was purchased from Sigma (St Louis, MO). CsA was obtained from a pharmacy. Chemical synthesis of MS-209[52] and GF120918[53] was performed at Vertex Pharmaceuticals Inc. Compounds were stored as 25 mM stock solutions in dimethylsulfoxide (DMSO) at −20°C and were freshly diluted with cell culture medium or buffer for each experiment. A radiolabeled photoaffinity analog of VX-710, [^{3}H]VF-13,159 ([^{3}H]-(*S*)-*N*-[2-Oxo-2-(4-azidophenyl)acetyl]piperidine-2-carboxylic acid 1,7-bis(3-pyridyl)-4-heptyl ester; 85.86 Ci/mmol) was stored as a stock solution of 1 mCi/ml in ethanol in the dark at −80°C until use.

Cell lines

The human promyelocytic leukemia cell line HL60 was obtained from ATCC (Rockville, MD). Adriamycin-selected HL60/ADR cells,[45,46] vincristine-selected HL60/Vinc cells[16,46] and HL60/S parental cells were a kind gift from Dr Melvin Center (Kansas State University, Manhattan, KS). All cell lines were maintained at 37°C, 5% CO_2 in drug-free culture medium consisting of RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (BioWhittaker), 2 mM L-glutamine (BioWhittaker), 50 U/ml penicillin and 50 μg/ml streptomycin (BioWhittaker).

MRP and P-glycoprotein detection

For MRP detection, a polyclonal rabbit anti-human MRP antiserum designated MRP 1032 was raised against a C-terminal MRP peptide containing amino acids 1517–1531 (NH_2-Gln-Arg-Gly-Leu-Phe-Tyr-Ser-Met-Ala-Lys-Asp-Ala-Gly-Leu-Val-COOH). The MRP peptide carrying an additional N-terminal Cys residue was cross-linked to maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL) prior to immunization of rabbits. The monoclonal antibodies C219 (Signet Laboratories, Dedhman, MA) and MRK16 (Kamiya, Thousand Oaks, CA) were used for detection of P-glycoprotein. Whole cell lysates were prepared in 1 × RIPA buffer [20 mM Tris HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton-X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) aprotinin]. For Western blot analyses, 20 μg total protein was resolved on SDS–8% polyacrylamide gels (Novex, San Diego, CA) and transferred to nitrocellulose. Immunodetection was performed according to previously published protocols[54] using the MRP antiserum at a dilution of 1:1000 or the P-glycoprotein-specific monoclonal antibody C219 at 0.2 μg/ml. Immunofluorescence detection of P-glycoprotein with the monoclonal antibody MRK16 (Kamiya) was performed as previously described.[55]

In vitro cytotoxicity assays

The MRP-specific MDR reversal potency of VX-710 and other MDR modulators was determined in cytotoxicity assays. Cells (4×10^4 cells per sample, $n = 4$ per condition) were seeded in 100 μl phenol red-free growth medium in 96-well microtiter plates and titrations of cytotoxic drugs in the absence or presence of VX-710 (or another MDR modulator) were performed by 50 μl additions of agents (or media). The cells were incubated at 37°C, 5% CO_2 for 3 days, and cell growth and viability were determined by sodium 3′-[1′[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) dye reduction assay.[56] Cytoxicity plots were generated for extrapolation of IC_{50} values which were used for calculating the relative drug resistance (IC_{50} resistant cells)/(IC_{50} sensitive cells) and the resistance modifying factor (IC_{50} drug alone)/(IC_{50} drug + MDR modulator).

Daunorubicin uptake experiments

Parental and MRP-expressing multidrug resistant cells (1×10^6 cells per condition) were pretreated with a titration (1, 2.5 and 5 μM) of MDR modulator in 1 ml growth medium for 60 min at 37°C and pelleted by centrifugation. After aspiration of the supernatant, the cells were resuspended in 1 ml growth medium containing the MDR modulator at the same concentration, as well as 2 μg/ml daunorubicin (Sigma, St Louis, MO). Cells were co-incubated with MDR modulator and daunorubicin for 60 min at 37°C. After 3 washes with ice-cold DPBS, cellular uptake of daunorubicin was determined in 10 000 cells by fluorescence-activated cells sorting (FACS) analysis using a FACSort flow cytometer and Lysis II software (Becton Dickinson Medical Systems, San Jose, CA).

Calcein uptake and efflux experiments

Cells at a concentration of 1×10^6 cells/ml in growth medium were incubated for 15 min at 37°C with 0.5 μM calcein acetoxy-methyl ester (calcein-AM) (Molecular Probes, Eugene, OR) alone or in the presence of a titration (0.25, 0.5, 1 and 2.5 μM) of MDR modulator. Then the cells were pelleted by centrifugation, washed twice with ice-cold medium containing 20 mM HEPES, pH 7.0, resuspended at 1×10^6 cells/ml in growth medium containing the MDR modulator at the same concentration and incubated at 37°C. At specified times between 0 and 120 min, the cells were washed twice with ice-cold medium supplemented with 20 mM HEPES, pH 7.0, and the cellular calcein content of 10 000 cells was analyzed with a FACSort flow cytometer using Lysis II software (Becton Dickinson Medical Systems).

[^{3}H]VF-13-13,159 photoaffinity labeling

For photoaffinity labeling with [^{3}H]VF-13,159 ([^{3}H]-(*S*)-*N*-[2-Oxo-2-4-azidophenyl)acetyl]piperidine-2-carboxylic acid 1,7-bis(3-pyridyl)-4-heptyl ester), 1×10^6 cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS), resuspended in 0.1 ml in DPBS and incubated with 2 μCi (0.232 μM) [^{3}H]VF-13,159 in the absence or presence of a dilution series (0.025–25 μM) of VX-710 for 60 min at 37°C with gentle agitation. Then the cells were placed on ice and irradiated at a distance of 5 cm for 20 min using a UV lamp with two self-filtering longwave UV tubes (Blak-Ray UV lamp, UVP; VWR Scientific, Boston, MA). The photolabeled cells were collected by centrifugation and cell extracts were prepared in $1 \times$ RIPA buffer (see above) supplemented with 1% (v/v) Benzonase (EM Industries, Gibbstown, NJ). Immunoprecipitations were performed according to previously described protocols[52] using 4 μl of polyclonal rabbit anti-MRP antiserum per 1×10^6 labeled cells. Photolabeled MRP was detected by SDS–polyacrylamide gel electrophoresis and subsequent fluorography (Amplify; Amersham, Arlington Heights, IL).

Results

Characterization of multidrug resistant and drug-sensitive HL60 promyelocytic leukemia cell lines

A polyclonal antiserum MRP-1032 directed against the C-terminus (amino acids 1517–1531) of the MRP gene product was used to corroborate overexpression of MRP protein in doxorubicin-selected HL60/ADR cells[45,46] (Figure 1). Western blot analyses of whole cell lysates revealed the presence of a high molecular weight 190 kDa protein which cross-reacted with the MRP antiserum. The 170 kDa P-glycoprotein was not detected in HL60/ADR cells, neither by Western blot analysis with the monoclonal antibody C219 (Figure 1) nor by immunofluorescence staining using the monoclonal antibody MRK16 (data not shown). P-glycoprotein was also not detected in drug-sensitive HL60/S cells[45,46] and HL60 cells from ATCC, but was overexpressed in HL60/Vinc cells[16,41] (Figure 1). Low basal levels of MRP protein were observed in the parental HL60/S cells and the P-glycoprotein-expressing HL60/Vinc cells, but not in drug-sensitive HL60 cells obtained from ATCC (Figure 1). Consistently, the IC_{50} for doxorubicin in HL60/S cells (0.20 ± 0.02 μM) was approximately 3-fold higher than the IC_{50} for doxorubicin in HL60 cells from ATCC (0.067 ± 0.027). Hence, we have used the HL60 cell line from ATCC as our control in this study.

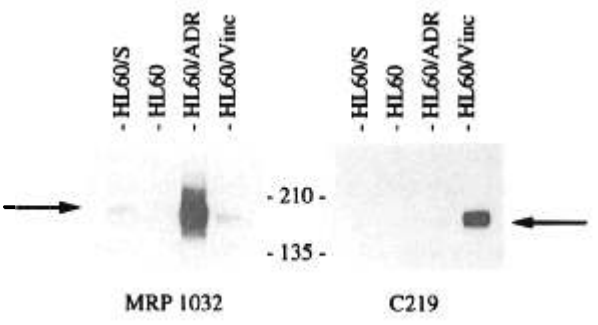

Figure 1. Detection of MRP and P-glycoprotein expression in multidrug resistant HL60/ADR and HL60/Vinc cell lines. Western blot analyses were performed after resolving whole cell extracts (20 μg protein per lane) isolated from drug-sensitive HL60/S cells, HL60 cells from ATCC (HL60), Adriamycin-selected HL60/ADR cells and vincristine-selected HL60/Vinc cells on an SDS–8% polyacrylamide gel. An MRP-specific polyclonal antiserum MRP 1032 (left panel) and the P-glycoprotein-specific monoclonal antibody C219 (right panel) were used for immunodetection. The arrows on the left points to MRP and the one on the right to P-glycoprotein signals. In between the two panels, the sizes of prestained protein standards are indicated in kDa.

Comparison of VX-710, CsA, verapamil, MS-209 and GF120918 as MDR modulators in P-glycoprotein-expressing multidrug resistant HL60/Vinc cells

In a previous study we found that VX-710 at concentrations of 0.5–5 μM fully restored drug-sensitivity to doxorubicin, vincristine, etoposide and paclitaxel in a variety of P-glycoprotein expressing cell lines.[44] VX-710 interacts directly with the *MDR*1 gene product and blocks its drug efflux function. A series of cytotoxicity assays were performed with HL60/Vinc cells to compare the potency of VX-710 (Figure 2A) with four other known *MDR*1 modulators: CsA[51] (Figure 2B), verapamil[50] (Figure 2C), MS-209[52] (Figure 2D) and GF120918[53] (Figure 2E). The IC_{50} of doxorubicin for HL60/Vinc cells was 1.02 μM ($n = 2$) and the IC_{50} for HL60 control cells was 0.067 ± 0.027 μM ($n = 10$), indicating a relative resistance of approximately 15 for the HL60/Vinc cell line. We found that all five MDR modulators

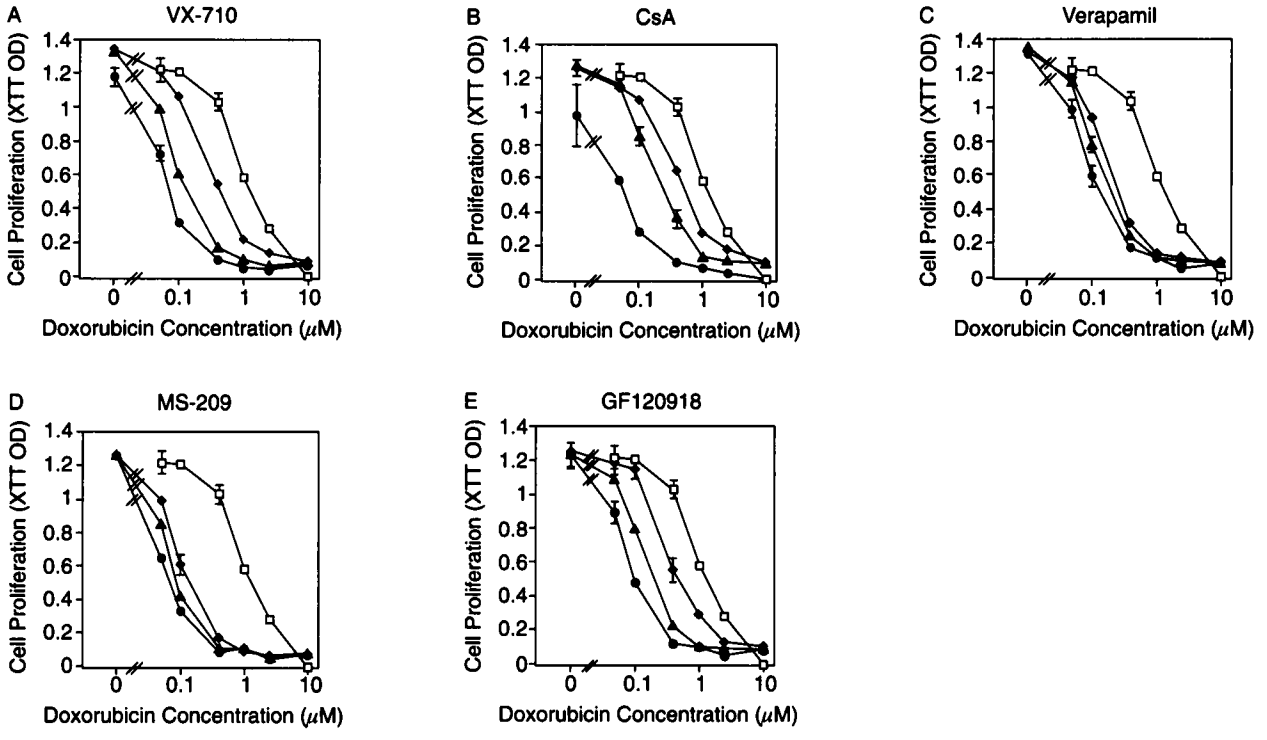

Figure 2. Effects of VX-710, CsA, verapamil, MS-209 and GF120918 on the cytotoxicity of doxorubicin in HL60/Vinc cells. The 3 day cytotoxicity assays involving titrations of doxorubicin in the absence (□) or presence of (A) 0.5 μM (◆), 1 μM (▲), and 2.5 μM (●) VX-710, (B) 0.5 μM (◆), 1 μM (▲), and 2.5 μM (●) CsA, (C) 2.5 μM (◆), 5 μM (▲), and 10 μM (●) verapamil, (D) 0.5 μM (◆), 1 μM (▲), and 2.5 μM (●) MS-209, and (E) 0.005 μM (◆), 0.01 μM (▲) and 0.025 μM (●) GF120918 were performed with P-glycoprotein-expressing HL60/Vinc cells as described in Materials and methods. Data represent means and SD from quadruplicates.

restored sensitivity of HL60/Vinc cells to the cytotoxic action of doxorubicin in a concentration-dependent manner (Figure 2). Complete reversal of doxorubicin resistance in HL60/Vinc cells to the levels in HL60 cells was achieved with 2.5 μM VX-710 (Figure 2A), 2.5 μM CsA (Figure 2B), 10 μM verapamil (Figure 2C), 2.5 μM MS-209 (Figure 2D) and 0.025 μM GF120918 (Figure 2E). Thus, all five compounds are very effective *MDR*1 modulators with GF120918 being the most potent.

Effects of VX-710, CsA, verapamil, MS-209 and GF120918 on sensitivity of HL60/ADR cells to doxorubicin

To examine the chemosensitizing activity of VX-710 in MRP expressing cells, HL60/ADR cells and HL60 control cells were cultured for 3 days in the presence or absence of a titration of doxorubicin, with or without different concentrations of VX-710 (0.5–5 μM), and cell viability was measured by XTT dye reduction (Figure 3). VX-710 increased the sensitivity of HL60/ADR cells to doxorubicin in a concentration-dependent manner (Figure 3A), but had no significant effect on the sensitivity of HL60 control cells to doxorubicin (Figure 3B). The cell viability of the HL60/ADR cells cultured in the presence of 5 μM or more VX-710 alone was greater than 95% compared to untreated control cells, indicating that VX-710 at these concentrations had no direct antiproliferative effect on HL60/ADR cells (Figure 3C).

The IC_{50} of doxorubicin for HL60 cells was 0.067 ± 0.027 μM and the IC_{50} for HL60/ADR cells was 7.53 ± 0.46 μM (Table 1), indicating a relative resistance of 112 for HL60/ADR cells. As summarized in Table 1, VX-710, as well as CsA, verapamil and MS-209 enhanced the sensitivity of HL60/ADR cells to the cytotoxic action of doxorubicin, whereas the potent *MDR*1 modulator GF120918 had no significant effect on the IC_{50} of doxorubicin in HL60/ADR cells. In comparison to the relative resistance of

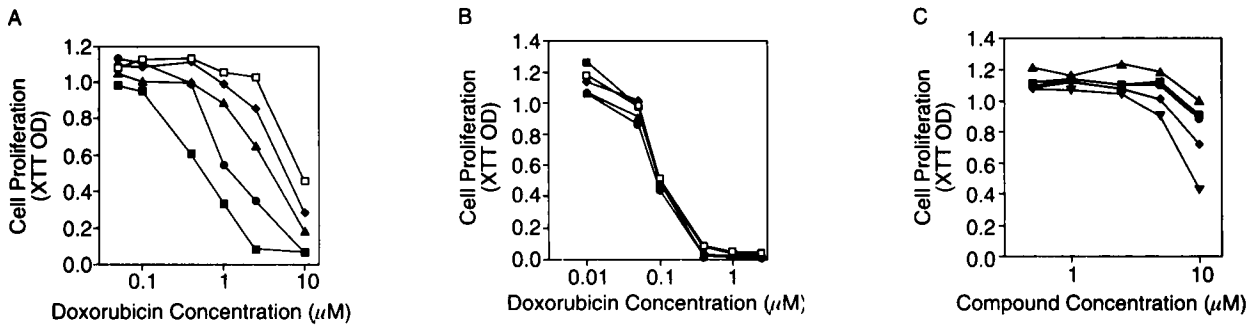

Figure 3. Effects of VX-710 on the cytotoxicity of doxorubicin in HL60/ADR cells and HL60 cells, and intrinsic cytotoxicity of MDR modulators in HL60/ADR cells. The 3-day cytotoxicity assays were performed as described in Materials and methods. For measuring reversal of doxorubicin resistance in HL60/ADR cells (A) and in HL60 cells (B), cells were incubated with doxorubicin in the absence (□) or presence of VX-710 at 0.5 μM (◆), 1 μM (▲), 2.5 μM (●) and 5 μM (■). For determining intrinsic cytotoxicity of MDR modulators (C), HL60/ADR cells were incubated in the presence of a titration (0.5, 1, 2.5, 5 and 10 μM) of VX-710 (◆), CsA (▼), verapamil (▲), MS-209 (●) and GF120918 (■). Data represent means from quadruplicates.

Table 1. Sensitization of multidrug resistant HL60/ADR cells to doxorubicin by VX-710, CsA, verapamil, MS-209 and GF120918

Compound	Doxorubicin + compound IC_{50} (μM) (resistance modifying factor)			
	0.5 μM	1.0 μM	2.5 μM	5.0 μM
VX-710	5.23 ± 0.25 (1.4)	3.37 ± 0.38 (2.2)	1.13 ± 0.23 (6.7)	0.48 ± 0.03 (15.7)
CsA	5.07 ± 0.80 (1.5)	5.03 ± 0.75 (1.5)	2.46 ± 0.90 (3.1)	1.00 ± 0.05 (7.5)
Verapamil	5.20 ± 0.98 (1.5)	3.47 ± 0.47 (2.0)	1.92 ± 0.19 (3.9)	1.03 ± 0.05 (7.3)
MS-209	6.03 ± 0.97 (1.2)	2.65 ± 0.45 (2.8)	1.80 ± 0.10 (4.2)	0.88 ± 0.12 (8.5)
GF120918	6.91 ± 0.32 (1.1)	7.20 ± 0.26 (1.0)	6.87 ± 0.32 (1.1)	6.63 ± 0.55 (1.1)

Dose-response curves were determined by cytotoxicity assays. Cultures contained no drug and/or reversing agent, or a cross-titration of doxorubicin and reversing agent (0.5, 1.0, 2.5 or 5.0 μM). IC_{50}(μM) values (means and SD from three independent experiment) were extrapolated from cytotoxicity plots. The IC_{50} values of HL60/ADR and HL60 cells for doxorubicin alone were 7.53 ± 0.46 and 0.067 ± 0.027 μM, respectively, and the relative resistance of the HL60/ADR cell line was 112-fold. The resistance modifying factor was calculated as the ratio (IC_{50} drug)/(IC_{50} drug + modulator).

HL60 cells, only partial reversal of doxorubicin resistance of HL60/ADR cells was achieved by all four MDR modulators. However, VX-710 was found to be approximately 2-fold more effective that MS-209, verapamil and CsA (Table 1). Control experiments in which the modulatory compounds were tested for their intrinsic cytotoxicity revealed a cell viability of greater than 95% for 5 μM VX-710, 5 μM verapamil, 5 μM MS-209 and 5 μM GF120918, but cell survival was reduced to 85% in the presence of 5 μM CsA (Figure 3C). The IC_{50} of CsA alone in HL60/ADR cells was approximately 7 μM, whereas the IC_{50} values for the other compounds were greater than 10 μM (Figure 3C). CsA was also noted to be more toxic than the other MDR modulators in experiments with HL60 and HL60/Vinc cells (data not shown).

Effects on sensitivity of HL60/ADR cells to etoposide

To assess whether the observed chemosensitizing activity of VX-710, MS-209, verapamil and CsA extends to other drugs associated with MRP-mediated MDR, we determined the effects of these compounds on the sensitivity of HL60/ADR cells to etoposide. Three independent cytotoxicity assays in the presence of drug alone indicated that HL60/ADR cells (IC_{50} 188 ± 18 μM) were 303-fold more resistant to etopo-

Table 2. Sensitization of multidrug resistant HL60/ADR cells to etoposide by VX-710, CsA, verapamil, MS-209 and GF120918

Compound	Etoposide + compound IC_{50} (μM) (resistance modifying factor)			
	0.5 μM	1.0 μM	2.5 μM	5.0 μM
VX-710	144 ± 40 (1.3)	71.4 ± 10.5 (2.6)	34.4 ± 8.5 (5.5)	15.2 ± 3.8 (12.4)
CsA	170 ± 42 (1.2)	138 ± 10.5 (1.4)	92.1 ± 15.8 (2.0)	49.0 ± 24.4 (3.8)
Verapamil	157 ± 33 (1.2)	91.4 ± 19.0 (2.1)	59.2 ± 10.3 (3.2)	29.9 ± 2.3 (6.3)
MS-209	151 ± 28 (1.2)	110 ± 5.0 (1.7)	69.1 ± 16.5 (2.7)	39.5 ± 14.1 (4.8)
GF120918	219 ± 78 (0.9)	218 ± 10 (0.9)	198 ± 42 (1.0)	188 ± 33 (1.0)

Dose–response curves were determined by cytotoxicity assays. Cultures contained no drug and/or reversing agent, or a cross-titration of vincristine and reversing agent (0.5, 1.0, 2.5 or 5.0 μM). IC_{50}(μM) values (means and SD from three independent experiment) were extrapolated from cytotoxicity plots. The IC_{50} values of HL60/ADR and HL60 cells for VP-16 alone were 188 ± 18 and 0.62 ± 0.25 μM, respectively, and the relative resistance of the HL60/ADR cell line was 303-fold. The resistance modifying factor was calculated as the ratio (IC_{50} drug)/(IC_{50} drug + modulator).

side than HL60 cells (IC_{50} 0.62 ± 0.25μM). As summarized in Table 2, VX-710, verapamil, MS-209 and CsA increased etoposide cytotoxicity to HL60/ADR cells in a concentration-dependent manner. VX-710 at 5 μM was approximately 2-fold more effective in reversing etoposide resistance of HL60/ADR cells than the other three MDR modulators (Table 2). Similar to the cytotoxicity assays performed in combination with doxorubicin, GF120918 had no significant effect on the IC_{50} values of etoposide in HL60/ADR cells.

Effects on vincristine resistance of HL60/ADR cells

The relative resistance of HL60/ADR cells (IC_{50} 12.49 ± 0.64 nM) in comparison with HL60 control cells (IC_{50} 2.9 ± 1.3 nM) to vincristine was found to be much lower (4.3-fold) than to etoposide and doxorubicin. Surprisingly, VX-710 at 1 μM fully restored sensitivity of HL60/ADR cells to vincristine, while 2.5–5 μM increased sensitivity 18- to 69-fold (Table 3). CsA, verapamil and MS-209 were also sensitizing HL60/ADR cells, but to a lesser degree (8- to 32-fold) (Table 3). All these compounds restored sensitivity of HL60/ADR cells to the cytotoxic action of vincristine beyond the level of the drug-sensitive HL60 cell line, while GF120918 only had a modest (less than 2-fold) effect on the vincristine resistance of HL60/ADR cells. Control experiments performed with the HL60 cell line showed that the cytotoxicity of vincristine was also slightly potentiated by VX-710, CsA verapamil and MS-209. Sensitization of HL60 cells to vincristine was approximately 5-fold at 5 μM VX-710, 6-fold at 5 μM CsA, 2- to 3-fold at 5 μM verapamil, 2- to 3-fold at 5 μM MS-209, whereas 5 μM GF120918 had no significant effect.

Table 3. Sensitization of multidrug resistant HL60/ADR cells to vincristine by VX-710, CsA, verapamil, MS-209 and GF120918

Compound	Vincristine + compound IC_{50} (nM) (resistance modifying factor)			
	0.5 μM	1.0 μM	2.5 μM	5.0 μM
VX-710	4.85 ± 1.73 (2.6)	1.66 ± 0.72 (7.5)	0.67 ± 0.36 (18.7)	0.18 ± 0.06 (69.4)
CsA	5.99 ± 1.20 (2.1)	3.71 ± 1.47 (3.4)	2.54 ± 1.65 (4.9)	0.39 ± 0.1 (32.0)
Verapamil	5.56 ± 0.64 (2.3)	2.93 ± 1.72 (2.6)	1.42 ± 0.34 (8.8)	0.71 ± 0.18 (17.6)
MS-209	6.28 ± 0.69 (2.0)	3.82 ± 1.21 (3.3)	1.78 ± 0.57 (7.0)	1.22 ± 0.57 (10.2)
GF120918	11.96 ± 4.23 (1.0)	11.62 ± 4.22 (1.1)	7.67 ± 2.29 (1.6)	6.73 ± 1.98 (1.9)

Dose–response curves were determined by cytotoxicity assays. Cultures contained no drug and/or MDR modulator, or a cross-titration of vincristine and reversing agent (0.5, 1.0, 2.5 or 5.0 μM). IC_{50}(nM) values (means and SD deviations from three independent experiment) were extrapolated from cytotoxicity plots. The IC_{50} values of HL60/ADR and HL60 cells for vincristine alone were 12.49 ± 0.64 and 2.91 ± 1.30 nM, respectively. The relative resistance of the HL60/ADR cell line was 4.3-fold. The resistance modifying factor was calculated as the ratio (IC_{50} drug)/(IC_{50} drug + modulator).

Effects on daunorubicin accumulation in HL60/ADR cells

Since VX-710, MS-209, verapamil and CsA all enhanced the sensitivity of HL60/ADR cells to doxorubicin, etoposide and vincristine, the effects of these compounds on intracellular drug accumulation were studied. Flow cytometry was used to analyze uptake of daunorubicin in HL60/ADR and HL60 cells in the absence or presence of a titration (1–5 μM) of MDR modulator. During these short-term assays, no reduction in cell viability was noted for HL60/ADR and HL60 cells in the presence of 5 μM or less CsA or any other MDR modulator tested. The cells excluded Trypan blue before and after the experiment and no change in size or granularity of the cells was detected with the flow cytometer, suggesting that the observed effects of the compounds are a direct measure for their modulatory action. As shown in Figure 4, HL60/ADR cells exhibited a significant drug accumulation defect when compared with HL60 cells and VX-710 at 5 μM restored daunorubicin accumulation in HL60/ADR cells to the level of the drug-sensitive HL60 cell line (Figure 4). VX-710 at 5 μM had only a modest effect on the daunorubicin accumulation in HL60 control cells reflected by a less than 10% increase in the mean of fluorescence (Figure 4). The increase in daunorubicin accumulation in HL60/ADR cells by VX-710 was concentration-dependent as summarized in Table 4. In contrast, GF120918 did not enhance daunorubicin accumulation in HL60/ADR cells (Figure 4 and Table 4). Verapamil, MS-209 and CsA also increased accumulation of daunorubicin in HL60/ADR cells in a concentration-dependent manner, but were less effective than VX-710 (Table 4).

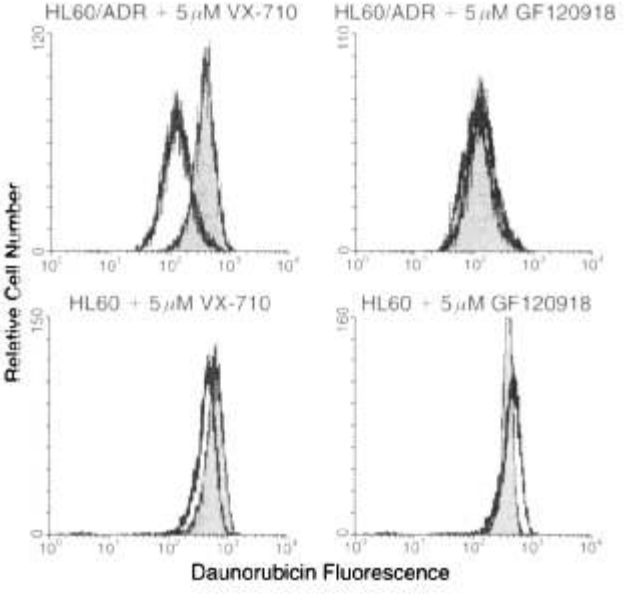

Figure 4. Effects of VX-710 and GF120918 on daunorubicin accumulation in HL60/ADR and HL60 cells. Compound-pretreated HL60/ADR cells (top panels) and HL60 cells (bottom panels) were incubated with daunorubicin in the absence (white peaks) or presence (gray peaks) of 5 μM VX-710 (left panels) or 5 μM GF120918 (right panels), and intracellular daunorubicin accumulation was analyzed by flow cytometry as described in Materials and methods.

Effects on calcein accumulation and retention in HL60/ADR cells

The non-fluorescent acetoxymethylester derivative of calcein, calcein-AM, is highly lipophilic and rapidly penetrates the plasma membranes of cells. Upon entry into the cytosol, esterases quickly and irreversibly convert calcein-AM into the hydrophilic free acid form of calcein which is intensely fluorescent and generally non-membrane permeable. Recently, the MRP protein has been reported to actively transport calcein,[57] which led us to investigate the effects of VX-710 and other MDR modulators on uptake and efflux of calcein.

Experiments were performed by loading HL60/ADR cells and HL60 cells in the absence or presence of a titration (0.25–2.5 μM) of MDR modulator with calcein-AM and accumulation of cytosolic calcein was determined to assess the effects of VX-710 on uptake of this MRP substrate [Figure 5A (time 0) and B] Then intracellular calcein retention was analyzed during a time course of up to 2 h in the presence of MDR modulator alone to evaluate the effects on calcein efflux (Figure 5A). Compared with drug-sensitive HL60 cells, the multidrug resistant HL60/ADR cells exhibited a significant calcein accumulation defect (Figures 5A and B). Some efflux of calcein from HL60/ADR cells was detectable, but the rate of efflux was very low and similar to the rate of efflux observed in control cells (Figure 5A). VX-710 increased uptake of calcein in HL60/ADR cells in a concentration-dependent manner (data not shown). VX-710 at 2.5 μM almost completely restored calcein uptake to the levels of drug-sensitive HL60 control cells (Figure 5A and B), but had no significant effect on uptake of calcein in HL60 cells (Figure 5A). VX-710 at 2.5 μM also appeared to have an inhibitory effect on efflux of calcein from both HL60/ADR and HL60 cells (Figure 5A), but this was difficult to determine exactly because of the low rate of calcein efflux observed in these cells. In contrast to VX-710,

Table 4. Effects of VX-710, CsA, verapamil, MS-209 and GF120918 on daunorubicin accumulation in HL60/ADR cells

Compound	Effects of compounds on daunorubicin accumulation		
	1.0 μM	2.5 μM	5.0 μM
VX-710	1.46 ± 0.40	2.21 ± 0.26	2.68 ± 0.44
CsA	1.08 ± 0.10	1.42 ± 0.17	1.68 ± 0.21
Verapamil	1.32 ± 0.06	1.87 ± 0.10	2.32 ± 0.19
MS-209	1.43 ± 0.15	1.72 ± 0.19	2.00 ± 0.20
GF120918	0.71 ± 0.16	0.88 ± 0.23	0.89 ± 0.16

Daunorubicin accumulation experiments were performed by incubating compound-pretreated HL60/ADR and HL60 cells with or without the indicated concentrations of MDR modulator in the presence of 2 μg/ml daunorubicin as described in Materials and methods. Intracellular daunorubicin accumulation was analyzed by flow cytometry. Data are means and SD from three independent experiments. Effects of compounds were determined as the ratio (mean of fluorescence daunorubicin + modulator)/(mean of fluorescence daunorubicin). The daunorubicin accumulation ratio for HL60/ADR cells (mean of fluorescence of HL60/ADR cells)/(mean of fluorescence of HL60ATCC cells) was 0.36 ± 0.02. Thus a value of 2.78 or more for effects of a compound indicates complete restoration of daunorubicin accumulation in HL60/ADR cells to the levels attained in HL60 cells.

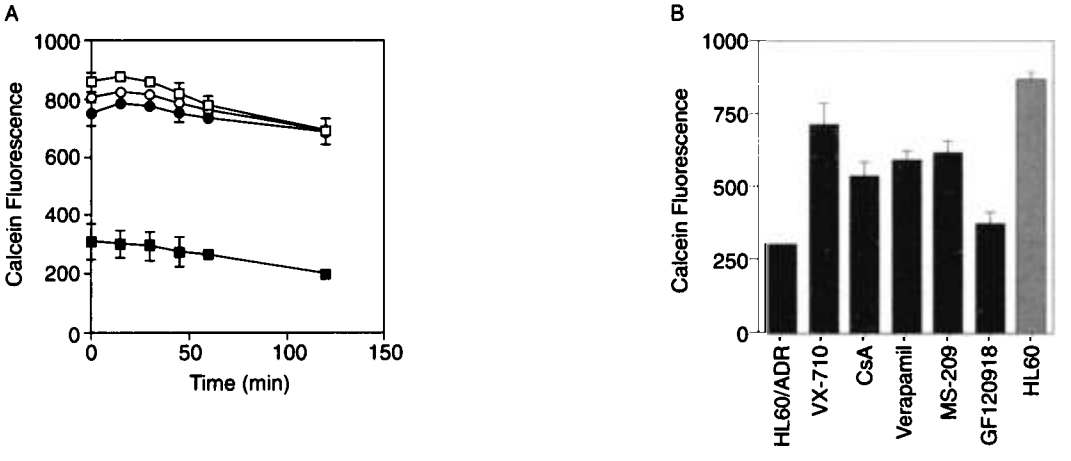

Figure 5. Effects of VX-710, CsA, verapamil, MS-209, and GF120918 on calcein retention. (A) The effects of VX-710 on uptake and efflux of calcein were determined by incubating HL60/ADR cells (filled symbols) and HL60 cells (open symbols) in the absence (squares) or presence (circles) of 2.5 μM VX-710 with calcein-AM as described in Materials and methods until steady-state levels of intracellular calcein were reached (time 0). Then the cells were incubated with MDR modulator alone and retention of calcein was determined at different times up to 120 min. Data represent means and standard determinations from three independent experiments. (B) The effects of VX-710, CsA, verapamil, MS-209 and GF120918 at a concentration of 2.5 μM on uptake of calcein in HL60/ADR cells (black bars) were determined in comparison to uptake of calcein in HL60 cells (gray bar) in the absence of MDR modulator. Data represent means and SD from three independent experiments.

GF120918 did not markedly enhance uptake of calcein in HL60/ADR cells (Figure 5B) and also had no detectable effects on efflux of calcein (data not shown). Similar to VX-710, the MDR modulators MS-209, verapamil and CsA also increased calcein uptake in HL60/ADR cells in a concentration-dependent manner (data not shown), but were generally less effective than VX-710 (Figure 5B).

Labeling of MRP by [^{3}H]VF-13,159 a photoaffinity analog of VX-710

A tritiated photoaffinity analog of VX-710, [^{3}H]VF-13,159, was found to be a very useful tool for demonstrating that VX-710 exerts its MDR modulating effects in P-glycoprotein expressing cells by directly interacting with the *MDR*1 gene product.[44]

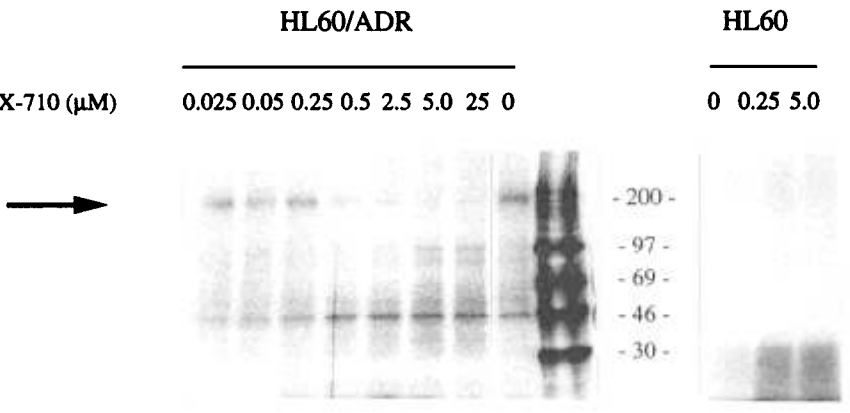

Figure 6. Photoaffinity labeling of MRP by [^{3}H]VF-13,159, a radiolabeled photoaffinity analog of VX-710. HL60/ADR cells (left panel) and HL60 cells (right panel) were incubated with the VX-710 photoaffinity analog [^{3}H]VF-13,159 and UV cross-linking was performed as described in Materials and methods. HL60/ADR cells were also photolabeled in the presence of increasing concentrations of VX-710 (0.025, 0.05, 0.25, 0.5, 2.5, 5 and 25 μM). Cell extracts were prepared and labeled MRP was visualized by SDS-4–20% polyacrylamide gel electrophoresis and subsequent fluorography. Each lane contains proteins extracted from approximately 75 000 photolabeled cells. The arrow on the left points to MRP signals. In between the two panels, the sizes of [^{14}C]methylated protein standards are indicated in kDa.

We therefore used this photoprobe to examine whether the MRP protein was also a target for direct interaction with VX-710. For photoaffinity experiments, HL60/ADR cells and HL60 control cells were labeled with [^{3}H]VF-13,159 in the absence or presence of a titration (0.025–25 μM) of VX-710. Figure 6 demonstrates that [^{3}H]VF-13,159 specifically photolabeled a 190 kDa protein in HL60/ADR cells which was not detected in drug-sensitive HL60 cells. The labeled protein was immunoprecipitated by an antiserum raised against a C-terminal MRP peptide (data not shown) confirming its identity as MRP. Photoaffinity labeling of MRP by [^{3}H]VF-13,159 was inhibited by an excess of VX-710 in a concentration-dependent manner (Figure 6). Incorporation of [^{3}H]VF-13,159 into several low molecular weight proteins was also observed, most predominantly into a 45 kDa protein. Photolabeling of these proteins, however, was not blocked by increasing concentrations of VX-710.

Discussion

P-glycoprotein and MRP can be simultaneously expressed in the same tumor cell line and confer MDR.[11–13] This prompted us to investigate MDR modulators that may interfere with both of these mechanisms of MDR. In the present study, five compounds which are known to reverse P-glycoprotein-mediated MDR were evaluated for their ability to antagonize MRP-mediated MDR *in vitro* in the doxorubicin-selected HL60/ADR promyelocytic leukemia cell line.[16,45–49] These MDR reversing agents include VX-710,[44] verapamil,[48] CsA,[51] MS-209[52] and GF120918.[53]

Experiments involving the vincristine-selected P-glycoprotein-expressing HL60/Vinc promyelocytic leukemia cell line[16,46] (Figure 1) corroborated that these five compounds are potent *MDR*1 modulators. Full reversal of the doxorubicin resistance in HL60/Vinc cells to the level of drug-sensitive HL60 cells was achieved with 0.025 μM GF120918, 2.5 μM VX-710, 2.5 μM MS-209, 2.5 μM CsA and 10 μM verapamil (Figure 2). Thus, GF120918 was up to 100-fold more potent than VX-710, MS-209 and CsA, and approximately 400-fold more potent than verapamil as an MDR reversal agent in P-glycoprotein-expressing cells. Generally, the relative potencies of these compounds observed in the HL60/Vinc cell line agree well with literature values determined using a variety of *MDR*1 expressing cell lines.[33]

The doxorubicin-selected HL60/ADR promyelocytic leukemia cell line was one of the first cell lines described to exhibit an MDR phenotype and drug accumulation defect in the absence of P-glycoprotein expression.[45,46] Evidence for the presence of an ATP-dependent drug efflux pump in HL60/ADR cells was gained and this transporter was presumed to contribute to decreased intracellular accumulation of cytotoxic drugs, although its identity was not known at the time.[16] A glycosylated, ATP-binding 190 kDa protein was found to be overexpressed in membranes isolated from HL60/ADR cells which, after

the cloning of an MRP cDNA from H69/AR cells,[3] was identified as MRP.[47–49] Thus, overexpression of MRP is at least one major mechanism responsible for drug resistance in HL60/ADR cells (Figure 1). In support of this hypothesis and in agreement with previously reported studies,[9,58,59] recent transfection experiments performed with drug-sensitive NIH 3T3 host cells have clearly demonstrated that expression of an MRP cDNA isolated from HL60/ADR cells is sufficient to confer resistance to etoposide, doxorubicin, daunorubicin and vincristine.[10]

We found that four of five MDR reversing agents examined in our study, i.e. VX-710, MS-209, verapamil and CsA, increased the sensitivity of HL60/ADR cells to the cytotoxic action of doxorubicin, etoposide and vincristine, and that the very potent *MDR*1 modulator GF120918 had no significant effects on MRP-mediated MDR (Figure 3A and Tables 1–3) and appears to be highly specific for P-glycoprotein.

In comparison with a drug-sensitive HL60 control cell line, HL60/ADR cells exhibited a 112-fold relative resistance to doxorubicin, a 303-fold resistance to etoposide and a 4.3-fold resistance to vincristine. VX-710 at 5 μM reversed the resistance of HL60/ADR cells to doxorubicin and etoposide partially (15.7- and 12.4-fold, respectively), while 1 μM VX-710 completely reversed the vincristine resistance of HL60/ADR cells. Generally, VX-710 was at least 2-fold more effective than MS-209, verapamil and CsA in restoring sensitivity of HL60/ADR cells to the above mentioned cytotoxic agents. All compounds were tested at concentrations of 5 μM or lower which were non-toxic concentrations for HL60/ADR (Figure 3C), indicating that the effects reflect direct action on the MDR mechanism. However, 5 μM CsA reduced the viability of HL60/ADR cells, suggesting that its observed MDR reversing activity may represent a combination of the antiproliferative and modulatory effects of CsA on these cells (Figure 3C).

To our knowledge, there are no other reports so far describing the potential of MS-209 in reversing MDR in MRP-expressing cells. However, for verapamil and/or CsA similar studies have been performed in several different MRP-expressing cell lines. Data obtained in H69AR small cell lung cancer cells,[36] HT1080/DR4 fibrosarcoma cells[36] and COR-L23/R large cell lung cancer cells[19] suggested that both verapamil and CsA had modest effects as chemosensitizers and that verapamil was somewhat more effective than CsA.[19,36] Also in the epirubicin-selected E1000 subline of CCRF-CEM leukemia cells, which exhibited a relative resistance of 51, only 3-fold reversal of daunorubicin resistance was achieved with 4 μg/ml verapamil.[39] In the 62-fold etoposide-resistant P/VP20 prostatic cancer cell line, 6- to 11-fold reversal was achieved with verapamil at 10 and 20 μg/ml.[40] Verapamil at 10 μM also caused a 3- to 4-fold reversal in NIH 3T3 cells transfected with an MRP cDNA, which were 8- to 10-fold drug-resistant.[10] Our data for reversal of drug resistance in the MRP-expressing HL60/ADR cell line by verapamil and CsA are highly consistent with the data reported by other laboratories, at least for doxorubicin and etoposide. Taken together these studies suggest that CsA and verapamil are less effective for MRP-mediated resistance to anthracyclines and epipodophyllotoxins than for P-glycoprotein-mediated drug resistance. However, some variability of chemosensitization in various MRP-overexpressing cell lines has been observed, which may depend on the cell or tumor type involved, or perhaps on the intracellular location of MRP.[8]

The MDR modulating effects of VX-710, CsA, verapamil and MS-209 in HL60/ADR cells appear to be related to the particular drug under study and/or its cellular target(s). Only partial reversal of the high resistance of HL60/ADR cells to cytotoxics with nuclear targets (doxorubicin and etoposide) was observed. In contrast, oversensitization to vincristine, an anti-microtubule agent, was observed with these compounds (69-fold for 5 μM VX-710, 10- to 32-fold for 5 μM MS-209, verapamil and CsA, Table 3). Since the HL60/ADR cell line was selected by growing drug-sensitive parental HL60/S cells in increasing concentrations of doxorubicin for a prolonged period of time,[45,46] it is possible that HL60/ADR cells harbor multiple drug resistance mechanisms, as has been observed for other drug-selected MDR isolates.[60] Indeed, enhanced expression of proteins other than MRP (e.g. P180, P150 and P130) in HL60/ADR cells has been reported[47,61] and, although no clear experimental evidence has been provided, it is possible that some of these proteins contribute to the MDR phenotype of HL60/ADR cells. Based on our photoaffinity experiments (Figure 6), however, one would not expect VX-710 to target these other high molecular weight protein, at least not via a direct interaction.

Originally the HL60/ADR cell line was described to be 80-fold resistant to the selective agent doxorubicin and 20-fold cross-resistant to vincristine,[45,61] but we found HL60/ADR cells to be more resistant to doxorubicin (112-fold) and etoposide (303-fold), and less resistant to vincristine (4.3-fold). Some of these differences may be explained by use of another and, at least with respect to MRP expression (Figure 1), more stringent drug-sensitive HL60 con-

trol cell line in our study. However, our observed relative resistance to doxorubicin and etoposide in HL60/ADR cells was 26- and 70-fold higher than the relative resistance to vincristine, whereas only a 4-fold difference for doxorubicin resistance versus vincristine resistance was previously reported.[44,61] A recent study involving NIH 3T3 cells which were stably transfected with an MRP cDNA isolated from HL60/ADR cells[10] indicated only a 2- to 3-fold difference between relative resistance levels to doxorubicin and etoposide versus vincristine that was attributable to MRP expression. Taken together, these data may suggest that other biochemical mechanisms responsible for anthracycline- and podophyllotoxin-specific resistance (e.g. topoisomerase II alteration[62]) are operational in the HL60/ADR cell line utilized in our studies, which are not affected by VX-710, CsA, verapamil or MS-209. However, no data are available at present to support this hypothesis.

The more pronounced effects of CsA, verapamil, MS-209 and VX-710 in particular on the resistance of HL60/ADR cells to vincristine are not well understood at present. VX-710 also sensitized HL60 control cells to vincristine to a greater degree (5-fold) than to doxorubicin (less than 2-fold), indicating that part of the vincristine reversal activity observed in HL60/ADR cells may involve mechanisms independent of MRP. However, sensitization of HL60/ADR cells to vincristine by VX-710 exceeded that of HL60 cells, suggesting that VX-710 preferentially targets the resistant isolate. We have previously noted a selective effect of VX-710 on vincristine cytotoxicity in drug-sensitive murine L1210 leukemia cells and NIH 3T3 fibroblasts,[44] and similar data have been reported for CsA and verapamil.[8] It remains to be elucidated whether interactions of the chemosensitizers with protein(s) other than P-glycoprotein and MRP are responsible for the dramatic vincristine sensitization by VX-710, verapamil and CsA in certain cells, or whether interaction of vincristine with its cellular target(s), e.g. tubulin, might increase the cytotoxicity of the chemosensitizing agents themselves.

Although the exact mechanism of reversal of MDR in HL60/ADR cells by VX-710, CsA, verapamil and MS-209 has not been identified, our data from daunorubicin and calcein uptake experiments suggest that potentiation of the cytotoxicity of anticancer drugs by these MDR modulators is associated with restored drug accumulation in HL60/ADR cells (Figures 4 and 5, Table 4). We found that VX-710, verapamil, MS-209 and CsA at 2.5–5 μM significantly increased daunorubicin and calcein accumulation in HL60/ADR cells. These agents had only a minimal effect (less than 10%) on the intracellular drug or dye accumulation in drug-sensitive HL60 control cells. Consistent with the data from the cytotoxicity assays, VX-710 was more potent than verapamil, MS-209 and CsA at restoring fluorescent drug or dye accumulation in HL60/ADR cells, while GF120918 had no significant effects. Generally, our findings for verapamil and/or CsA are in agreement with previously reported daunorubicin and/or calcein accumulation studies performed with HL60/ADR cells,[57,61,63] COR-L23/R cells[19] the E1000 subline of CCRF-CEM leukemia cells[39] or S1MRP cells.[63]

Feller *et al.*[55] recently reported a relatively rapid, ATP-dependent efflux of accumulated free calcein from the cytosol of MRP-expressing cells. Although we performed our experiments using similar conditions, we only detected a low rate of calcein efflux from HL60/ADR cells, comparable to the rate of calcein efflux observed in drug-sensitive HL60 control cells. Thus, we were not able to use calcein flow cytometry to determine if VX-710, verapamil, CsA and MS-209 cause a decrease in MRP-specific drug efflux from HL60/ADR cells.

Interestingly, VX-710 at 2.5–5 μM almost fully restored intracellular accumulation of daunorubicin and calcein in HL60/ADR cells to the levels attained in HL60 control cells (Figures 4 and 5, Table 4). Yet, the same concentration of VX-710 was not nearly as effective in restoring sensitivity of the resistant cell line to doxorubicin and etoposide (Tables 1 and 2). Similar discrepancies have also been observed for the chemosensitizing effects of verapamil, CsA and MS-209 in HL60/ADR cells and their modulatory action on accumulation of fluorescent drug or dye. Thus, it appears that the short-term drug accumulation effects of these agents are only partially related to their potentiation of drug cytotoxicity. As discussed above, this may suggest co-expression of atypical mechanisms of drug resistance (e.g. altered topoisomerase II[61]) in the HL60/ADR cell line utilized in our studies. On the other hand, the relationship between drug resistance and drug accumulation in MRP overexpressing cells is complex. In addition to drug efflux, compartmentalization of drug away from its subcellular target may be one component of drug resistance by some MRP-overexpressing cells.[3,7] Indeed, Center and colleagues have shown that in HL60/ADR cells high levels of MRP are localized in the endoplasmic reticulum[48,49] and that daunorubicin initially enters the nucleus of these cells, but is subsequently redistributed into cytoplasmic vesicles.[20] Flow cytometry studies visualize the total cellular retention, but not the intracellular distribution of fluorescent molecules. It is possible that this assay measures predominantly VX-

710 (or other modulator) activity on MRP molecules in the plasma membranes and not on MRP within vesicular membranes.

Our labeling studies using [^{3}H]VF-13,159, a tritiated photoaffinity analog of VX-710, provide evidence to suggest that VX-710 may exert its action at least in part by direct binding to MRP (Figure 6). This is the first demonstration of a direct interaction of a P-glycoprotein inhibitory compound with the MRP protein. Similar experiments performed with photoaffinity analogs of verapamil as well as cytotoxic drugs (vinblastine and doxorubicin) have thus far failed to reveal detectable signals.[8,61] We noted, however, that the efficiency of [^{3}H]VF-13,159 labeling of MRP in HL60/ADR cells was markedly reduced in comparison to [^{3}H]VF-13,159 labeling of P-glycoprotein in *MDR*1-transfected N3V2400 cells. Although experimental evidence is lacking at present, it is tempting to speculate that these differences may relate to the accessibility of the photoprobe to MRP in plasma membranes versus intracellular membranes, or perhaps a lowered affinity for MRP binding. Preliminary mapping of the [^{3}H]VF-13,159 binding site(s) within P-glycoprotein has revealed a major labeling site in the transmembrane segments 5 and 6 adjacent to the N-terminal nucleotide binding domain (Germann UA *et al.*, in preparation). Since most of the sequence similarity between P-glycoprotein and MRP is found within the nucleotide binding domains, it will be of interest to determine whether a [^{3}H]VF-13,159 binding site is present within an analogous region of MRP. Such information would be useful to fully elucidate the mechanism of MDR reversal by VX-710 in MRP expressing cell lines.

Conclusions

Among five known *MDR*1 modulators tested, VX-710 potentiated sensitization of MRP-expressing HL60/ADR promyelocytic leukemia cells to doxorubicin, etoposide and vincristine more effectively than verapamil, CsA and MS-209. GF120918 had no effect and appears highly specific for P-glycoprotein. One component of the mechanism of action of VX-710, as well as of verapamil, CsA and MS-209 for reversal of MRP-mediated MDR includes restoration of the drug accumulation deficit in HL60/ADR cells by increasing drug uptake. VX-710 may exert its action at least in part by binding directly to MRP. The combined reversal activity of VX-710 for both *MDR*1- and MRP-mediated mechanisms of MDR at 2.5–5 μM concentrations suggests an enhanced clinical potential of this compound for chemotherapeutical treatment of patients with multidrug resistant cancer.

Acknowledgments

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