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Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance *in vitro*

Ursula A Germann, Dina Shiyakhter, Valerie S Mason, Robert E Zelle, John P Duffy, Vincent Galullo, David M Armistead, Jeffrey O Saunders, Joshua Boger and Matthew W Harding

Vertex Pharmaceuticals Inc., 130 Waverly Street, Cambridge, MA 02139-4242, USA.
Tel: (+1) 617 577-6000; Fax: (+1) 617 577-6437.

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, a novel non-macrocyclic ligand of the FK506-binding protein FKBP12, was evaluated for its ability to reverse P-glycoprotein-mediated multidrug resistance *in vitro*. VX-710 at 0.5–5 μM restored sensitivity of a variety of multidrug resistant cells to the cytotoxic action of doxorubicin, vincristine, etoposide or paclitaxel, including drug-selected human myeloma and epithelial carcinoma cells, and human *MDR*1 cDNA-transfected mouse leukemia and fibroblast cells. Uptake experiments showed that VX-710 at 0.5–2.5 μM fully restored intracellular accumulation of [^{14}C]doxorubicin in multidrug resistant cells, suggesting that VX-710 inhibits the drug efflux activity of P-glycoprotein. VX-710 effectively inhibited photoaffinity labeling of P-glycoprotein by [^{3}H]azidopine or [^{125}I]iodoaryl azidoprazosin with EC_{50} values of 0.75 and 0.55 μM. Moreover, P-glycoprotein was specifically labeled by a tritiated photoaffinity analog of VX-710 and unlabeled VX-710 inhibited analog binding with an EC_{50} of 0.75 μM. VX-710 also stimulated the vanadate-inhibitable P-glycoprotein ATPase activity 2- to 3-fold in a concentration-dependent manner with an apparent k_a of 0.1 μM. These data indicate that a direct, high-affinity interaction of VX-710 with P-glycoprotein prevents efflux of cytotoxic drugs by the *MDR*1 gene product in multidrug resistant tumor cells.

Key words: Chemosensitizer, chemotherapy, FKBP12, MDR modulator, *MDR*1 gene product.

Introduction

Broad-spectrum resistance of tumor cells to anticancer drugs, termed multidrug resistance (MDR), is a principal reason for failure of chemotherapy in patients with cancer. Studies of drug-selected cultured tumor cell lines as *in vitro* MDR models have revealed that multidrug resistant cells differ from their drug-sensitive counterparts by decreased accumulation of cytotoxic agents due to reduced drug influx and/or enhanced drug efflux, altered expression and/or activity of specific proteins, as well as physiological changes that alter the intracellular milieu.[1] MDR may occur due to intrinsic or acquired mechanisms and is frequently associated with overexpression of the 170 kDa human *MDR*1 gene product P-glycoprotein.[2,3] Elevated levels of *MDR*1 mRNA and/or P-glycoprotein have been detected in different types of refractory tumor samples from relapsed and untreated patients, substantiating the clinical importance of the *MDR*1 gene product.[4,5] Several other proteins have been associated with drug resistance *in vitro*, including the MDR-associated protein MRP,[6] the p110 lung resistance-related protein LRP which has recently been identified as the major vault protein,[7] enzymes in glutathione metabolic pathways[8] as well as DNA topoisomerases.[9] The clinical significance of these alternative resistance mechanisms is currently being investigated.

P-glycoprotein is a polytopic plasma membrane protein which confers cross-resistance to several cytotoxic agents that differ in structure, function and intracellular target(s). These include anthracyclines (doxorubicin, daunorubicin, idarubicin), Vinca alkaloids (vincristine, vinblastine), epipodophyllotoxins (etoposide, teniposide), taxanes (paclitaxel, taxotere), actinomycin D, colchicine and others. The basic mechanism of action of P-glycoprotein consists of an energy-dependent drug efflux activity that leads to reduced intracellular drug accumulation in multidrug resistant cells.[2,3] P-glycoprotein interacts with both drug substrates and ATP to fulfill its

Correspondence to UA Germann

energy-dependent drug efflux function.[2,3] Biochemical analyses have revealed that P-glycoprotein exhibits a vanadate-inhibitable ATPase activity that is stimulated by drugs or non-cytotoxic substrates in a dose-dependent manner.[10–16] Moreover, reconstitution of drug-stimulatable ATPase and energy-dependent drug transport activities of purified P-glycoprotein have been demonstrated in proteoliposomes.[17–20]

Efforts directed at circumventing MDR in clinical oncology are focused on inhibition or modulation of P-glycoprotein function and/or *MDR*1 gene expression. A wide range of P-glycoprotein inhibitory compounds, so-called MDR reversing agents, MDR modulators or chemosensitizers have been identified, including calcium antagonists, calmodulin inhibitors and cyclosporins.[21,22] Most of these MDR reversing agents restore intracellular drug accumulation in multidrug resistant cells by interacting with P-glycoprotein directly. In general, MDR modulators interfere with drug binding to P-glycoprotein and some chemosensitizers may be substrates for P-glycoprotein-mediated transport.[23–25] In the clinic, it is hoped that co-administration of MDR-reversing agents with cytotoxic drugs will enhance the efficacy of chemotherapeutics by blocking the drug efflux function of P-glycoprotein in multidrug resistant tumor cells. Phase I and phase I/II studies with several first generation MDR modulators including verapamil, cyclosporin A (CsA), quinidine and others have indicated some intolerable side effects of the MDR-reversing agents themselves at therapeutic doses.[21,26,27] For example, verapamil at 6–10 μM reverses MDR *in vitro*, but cardiovascular toxicity is dose-limiting at serum concentrations of 1–2 μM.[27] Analogs of the prototype chemosensitizers with reduced toxicity, such as R-verapamil and the cyclosporin analog SDZ PSC 833,[28] are currently being tested in clinical studies.[27] Alternatively, screening and rational design of novel types of MDR modulators that lack other pharmacological activities may identify more suitable candidates for clinical evaluation. Recently, several novel agents that are very effective in reversing P-glycoprotein-mediated MDR at micromolar or sub-micromolar concentrations have been described, including GF120918,[29] MS-209,[30] dexniguldipine,[31] PAK-104P[32] and RS-33295-198.[33]

The structurally related macrolides FK506 and rapamycin have also been identifed as modulators of P-glycoprotein-mediated MDR *in vitro*.[34] Like verapamil and CsA, FK506 is a substrate for P-glycoprotein-mediated transport.[25] FK506 and rapamycin, however, are potent immunosuppressants and their clinical utility as MDR modulators is questionable. Both FK506 and rapamycin are known to bind to the cytosolic immunophilin FKBP12.[35,36] We have designed a series of novel non-macrocyclic high-affinity ligands to FKBP12 which are potent inhibitors of its peptidyl prolyl *cis–trans* isomerase (PPIase) activity.[37,38] In contrast to complexes of FKBP12 with the immunosuppressants FK506 and rapamycin,[39,40] these synthetic compounds do not block calcineurin phosphatase activity upon binding to FKBP12.[41] X-ray structure analyses of FKBP12 complexed with synthetic compounds or FK506 and modeling studies revealed strong aromatic–aromatic ligand–protein interactions with a largely hydrophobic binding pocket of FKBP12.[37,41] Hydrophobic interactions also appear to be important for P-glycoprotein–compound interactions.[2,3] Moreover our FKBP12-binding ligands are lipophilic, heterocyclic, positively charged molecules and share common features with many MDR modulators.[22] This prompted us to evaluate some of these compounds for their ability to reverse P-glycoprotein-mediated MDR, which led to the design of novel analogs, including the pipecolinate derivative VX-710 (Figure 1). In the present study we investigated the cellular and biochemical mechanisms of VX-710 for reversing P-glycoprotein-mediated MDR *in vitro*. We characterized the effects of VX-710 on resistance to doxorubicin, vincristine, etoposide and paclitaxel in a series of drug-selected, P-glycoprotein-expressing cell lines derived from hematological cancers

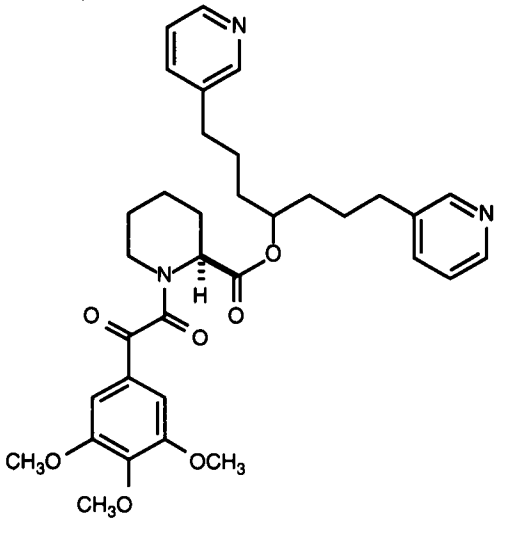

Figure 1. Chemical structure of VX-710 [(*S*)-*N*-[2-Oxo-2-(3,4,5-trimethoxyphenyl)acetyl]piperidine-2-carboxylic acid 1,7-bis(3-pyridyl)-4-heptyl ester], a non-macrocyclic ligand for FKBP12.

and solid tumors, as well as in *MDR*1 cDNA-transfected cell lines and drug-sensitive parental cell lines. Drug accumulation studies, photoaffinity labeling experiments and ATPase assays were performed to demonstrate that VX-710 restores drug accumulation in *MDR*1-expressing cells and that VX-710 interacts directly with P-glycoprotein.

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 (Figure 1) was used either as the free base (M_r 603) or as the dicitrate salt (M_r 987). The free base was stored as a 25 or 50 mM stock solution in dimethylsulfoxide at −20°C and, depending on the experiment, freshly diluted with dimethylsulfoxide, cell culture medium or buffer. The dicitrate salt of VX-710 was stored at −20°C as a 50 mM solution in water and freshly diluted with buffer or cell culture medium. A radiolabeled photoaffinity analog of VX-710, [^{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), designated [^{3}H]VF-13,159, was stored as a stock solution of 1 mCi/ml in ethanol in the dark at −80°C until use.

Cell lines

Human multiple myeloma cell lines (the drug-sensitive parental 8226/S cell line, and doxorubicin-selected 8226/Dox6 and 8226/Dox40 sublines[42]) were a gift from Dr William S Dalton (University of Arizona, Arizona Cancer Center, Tucson, AZ). A series of human epithelial adenocarcinoma cell lines (drug-sensitive parental KB-3-1 cells, colchicine-selected KB-8-5 cells and vinblastine-selected KB-V1 cells[43]), L1210 mouse leukemia cells and a colchicine-selected L1210/VMDRC.06 subclone transduced with a recombinant retrovirus that carries a human *MDR*1 cDNA with a mutation that changes the amino acid at position 185 of P-glycoprotein from glycine to valine,[44] as well as mouse NIH 3T3 and N3V2400 fibroblasts (a wild-type human *MDR*1 cDNA-transfected, vincristine-selected NIH 3T3 subpopulation[45]) were kindly provided by Dr Michael M Gottesman (National Cancer Institute, National Institutes of Health, Bethesda, MD). Bowes melanoma cells including the drug-sensitive, parental cell line, the vincristine-selected VCR 4.5, vinblastine-selected VBL 3.0 and colchicine-selected COL-1 sublines[46] were kindly provided by Dr Philippe Gros (McGill University, Montreal, Canada). Vincristine-selected GM3639 T cell acute lymphocytic leukemia cells[47] and doxorubicin-selected P388/ADR mouse leukemia cells[48] were a gift from Dr Lewis Slater (University of California, Irvine, CA). Intrinsically drug-resistant HCT15 human colon carcinoma cells, as well as U937 human histiocytic lymphoma cells, Jurkat clone E6-1 acute T cell leukemia cells, CTLL-2 mouse T cells and HUV-EC-C human umbilical cord vein endothelial cells were obtained from the ATCC (Rockville, MD). Culture conditions (media and additives, passage schedules) were as described previously for individual cell lines. Where applicable, cell lines were maintained in the appropriate concentration of the selecting cytotoxic drug (Sigma, St Louis, MO) to sustain the MDR phenotype. For all multidrug resistant cell lines, expression of P-glycoprotein was corroborated by fluorescence-activated cell sorting (FACS) analysis (MRK16 monoclonal antibody; Kamiya Biomedical Company, Thousand Oaks, CA) or Western blot analysis with 4007 polyclonal antiserum (a gift from Dr Michael M. Gottesman, National Cancer Institute, National Institutes of Health, Bethesda, MD) or C219 monoclonal antibody (Signet Laboratories, Dedham, MA) as described previously.[45]

In vitro cytotoxicity and colony formation assays

The potency of VX-710 as an MDR reversal agent was determined by measuring cell growth in cytotoxicity assays or by assessing cell survival in colony formation assays. For cytotoxicity assays, cells ($n = 4$ per condition) were seeded in phenol red-free growth medium in 96-well microtiter plates at optimized densities, which ranged from 1×10^4–4×10^5 cells in 0.1 ml/well depending on the doubling time of each cell line. Titrations of cytotoxic drugs in the absence or presence of VX-710 (usually concentrations of 0.1, 0.25, 0.5, 1, 2.5 and 5 μM) were performed by 50 μl additions of agents (or media) to a final volume of 200 μl/well. After incubation at 37°C, 5% CO_2 for 3 days, 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.[49] Cytotoxicity plots were generated for extrapolation of IC_{50} values and MDR reversal activity defined as a resistance modifying factor. The

resistance modifying factor was calculated as the ratio (IC_{50} drug)/(IC_{50} drug + modulator). The relative resistance of drug-resistant cell lines was calculated as the ratio (IC_{50} resistant cells)/(IC_{50} parental cells).

For colony formation assays, dose–response curves were determined as described previously.[43] Briefly, 300 KB KB-3-1, KB-8-5 or KB-V1 cells, 500 NIH3T3 or N3V2400 fibroblasts, or 250 HCT-15 cells were seeded per 60 mm tissue culture dish in 5 ml growth medium. Cross-titrations of doxorubicin (approximately 3-fold serial dilutions) and VX-710 (concentrations as described above) were added, and cells incubated at 37°C/5% CO_2 for 10–14 days, depending on the cell line. Surviving cell colonies were stained with 0.5% methylene blue in 50% ethanol, washed and counted. Values for IC_{50}, resistance modifying factor and relative resistance were derived as described above.

Drug accumulation experiments

Studies on the effects of VX-710 on accumulation of [^{14}C]doxorubicin were determined in drug uptake assays as described previously.[50] Briefly, L1210/VMDRC.06 drug-resistant or parental drug-sensitive L1210 cells (1×10^6 cells/ml) were incubated with 2 μM [^{14}C]doxorubicin in the absence or presence of VX-710. After a 45 min incubation at room temperature, cell aliquots were layered over oil cushions (silicon/paraffin oil 4:1) and centrifuged for 10 min at 12 000 *g*. The supernatant and oil layer were carefully aspirated and the cell pellet was dissolved in 0.1 ml 1 N NaOH for 16 h, neutralized and aliquots were removed for analysis of [^{14}C]doxorubicin content and protein concentration. Data were expressed as pmol [^{14}C]doxorubicin/mg cell protein. A similar protocol was used to perform uptake experiments with [^{14}C]VX-710 in drug-sensitive and multidrug resistant mouse fibroblasts.[50]

Photoaffinity labeling of P-glycoprotein

Photoaffinity labeling experiments with [^{3}H]azidopine, [^{125}I]iodoaryl azidoprazosin or a ^{3}H-labeled photoaffinity analog of VX-710 were performed according to a protocol adapted from Bruggemann *et al.*[51] Murine N3V2400 fibroblasts (5×10^5 per test condition) or NIH 3T3 cells as negative controls were harvested by trypsinization, washed three times with Dulbecco's phosphate-buffered saline (DPBS), resuspended in 100 μl DPBS and incubated with 1 μCi (0.2 μM) [^{3}H]azidopine (Amersham, Arlington Heights, IL), 1 μCi (0.004 μM) [^{125}I]iodoaryl azidoprazosin (ICN, Costa Mesa, CA) or 1 μCi (0.116 μM) radiolabeled VX-710 photoaffinity analog [^{3}H]VF-13,159 in the absence or presence of a dilution series of VX-710 for 60 min at room temperature with gentle agitation. The cells were pelleted by centrifugation at 1000 *g* for 1 min, supernatants were aspirated, and cells were resuspended in 100 μl DPBS and irradiated on ice 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, cell extracts were prepared and immunoprecipitation with 4007 polyclonal anti-P-glycoprotein antiserum performed as described previously.[51] P-glycoprotein was detected by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and subsequent fluorography (^{3}H-labeled photoprobes) or autoradiography ([^{125}I]iodoaryl azidoprazosin). The extent of P-glycoprotein photolabeling was quantitated by densitometer scanning (UltroScan XL; Pharmacia LKB, Piscataway, NJ).

Measurement of P-glycoprotein ATPase activity

Enriched membranes prepared from N3V2400 murine fibroblasts were used for ATPase assays. Control experiments were performed with crude membrane preparations from drug-sensitive NIH 3T3 parental cells. Briefly, cells from 60 confluent 150 mm culture dishes were harvested in prechilled DPBS containing 1% (v/v) aprotinin (DPBSAp), washed twice with DPBSAp, resuspended at $2-5 \times 10^7$ cells/ml in hypotonic lysis buffer [10 mM Tris HCl, pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) aprotinin], lysed on ice for 20 min and Dounce homogenized (20 strokes with a tight-fitting pestle). After centrifugation at 500 *g* for 10 min, the cell pellet was re-extracted with 0.5 volume of hypotonic lysis buffer as described above. The low speed supernatants were pooled and centrifuged at 100 000 *g* for 1 h. The crude membrane pellet was resuspended in membrane storage buffer [10 mM Tris–HCl, pH 7.5, 250 mM sucrose, 50 mM NaCl, 1 mM PMSF and 1% (v/v) aprotinin] and stored in aliquots at −80°C until use. Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

ATPase assays were carried out as described by Sarkadi *et al.*[10] with minor modifications. Crude membranes (100 μg per test condition) were suspended in 1 ml total volume of ATPase assay buffer [12.5 mM Tris–HCl, pH 7.5, 75 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 5 mM NaN_3, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 1 mM ouabain] in the absence or presence of 100 μM sodium orthovanadate, with or without a dilution series of VX-710, verapamil or CsA. Duplicate samples were prewarmed for 10 min at 37°C and reactions were initiated by addition of ATP to a final concentration of 5 mM. Aliquots (100 μl) were taken at various time points (usually within the linear range of the reaction at 0, 20, 40, 60 and 80 min). The reaction was stopped in the presence of 2.5% (w/v) SDS and liberated inorganic phosphate (P_i) was quantitated immediately in comparison to a series of P_i standards by use of a colorimetric method involving ammonium molybdate complexes.[10] Results are expressed either as ATPase activity (μmol P_i/mg membrane protein) attributable to P-glycoprotein (i.e. ATPase activity measured in the absence of vanadate – ATPase measured in the presence of vanadate) or as vanadate-inhibitable P-glycoprotein ATPase activity measured in comparison to control (samples without MDR modulator). Data presented are means calculated from time points within the linear range of the reaction (20, 40, 60 and 80 min).

Results

Characterization of VX-710 as a non-macrocyclic ligand for FKBP12

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 (Figure 1) is a member of a non-macrocyclic class of compounds designed to bind to the FK506 and rapamycin receptor protein FKBP12.[37] The interaction of VX-710 with FKBP12 was determined by measurement of its k_i as an inhibitor of FKBP12 PPIase activity as described by Park *et al.*[38] VX-710 was found to be a tight binder to FKBP12 and a nanomolar inhibitor of its PPIase activity (k_i of 3.7 nM), whereas FK506 and rapamycin were subnanomolar inhibitors (k_i values of 0.6 and 0.2 nM, respectively). FKBP12-VX-710 complexes at concentrations up to 30 μM did not inhibit calcineurin phosphatase activity or block interleukin (IL)-2-dependent proliferation of CTLL-2 cells. In contrast, inhibition of calcineurin phosphatase activity or IL-2 stimulation of CTLL-2 proliferation was accomplished with lower than 5 nM concentrations of FK506 or rapamycin, respectively.[39,40]

Effects of VX-710 on sensitivity of multidrug resistant cells to cytotoxic drugs

The activity of VX-710 as a reversing agent of P-glycoprotein-mediated MDR was characterized using a panel of multidrug resistant and drug-sensitive parental cell lines. Cytotoxicity experiments were performed with drug-selected representatives of hematological cancers and solid tumors with differing levels of P-glycoprotein expression to determine the effects of VX-710 on sensitization to four classes of drugs involved in the classical MDR phenotype (anthracyclines, Vinca alkaloids, epipodophyllotoxins and taxanes). Moreover, human *MDR*1-transfected mouse cell lines were analyzed, including L1210/VMDRC.06 leukemia cells expressing a P-glycoprotein mutant with a Gly185 → Val substitution and N3V2400 fibroblasts expressing wild-type *MDR*1 cDNA. Suspension cells were assayed by using XTT dye reduction to measure cell viability after 3 days of co-culture with cytotoxic drug and VX-710. Dose–responses of multidrug resistant cells growing as a monolayer were determined by colony formation assays.

Figure 2 documents the ability of VX-710 to restore doxorubicin cytolytic activity to L1210/VMDRC.06 cells and paclitaxel cytolytic activity to 8226/Dox6 cells. A dose-dependent increase in sensitization of these multidrug resistant lines was achieved with 0.25–2.5 μM VX-710. Additional data obtained with 8226/Dox6, L1210/VMDRC.06, KB-8-5, KB-V1 and N3V2400 cells are summarized in Tables 1 and 2. Both cytotoxicity and colony formation assay methods indicated similar *in vitro* MDR reversal activity of VX-710 for the selected multidrug resistant cell lines. In general, multidrug resistant cells were partially sensitized to cytotoxic drugs at VX-710 concentrations of 0.5–1.0 μM and concentrations of 1.0–2.5 μM VX-710 were sufficient to fully restore (and occasionally exceed) sensitivity (Tables 1 and 2). Similarly, VX-710 concentrations of 1.0–2.5 μM were also found to be effective in reversing MDR in the vincristine-selected VCR 4.5, the vinblastine-selected VBL 3.0 and the colchicine-selected COL-1 Bowes human melanoma cell lines, as well as in vincristine-selected GM3639 T cell

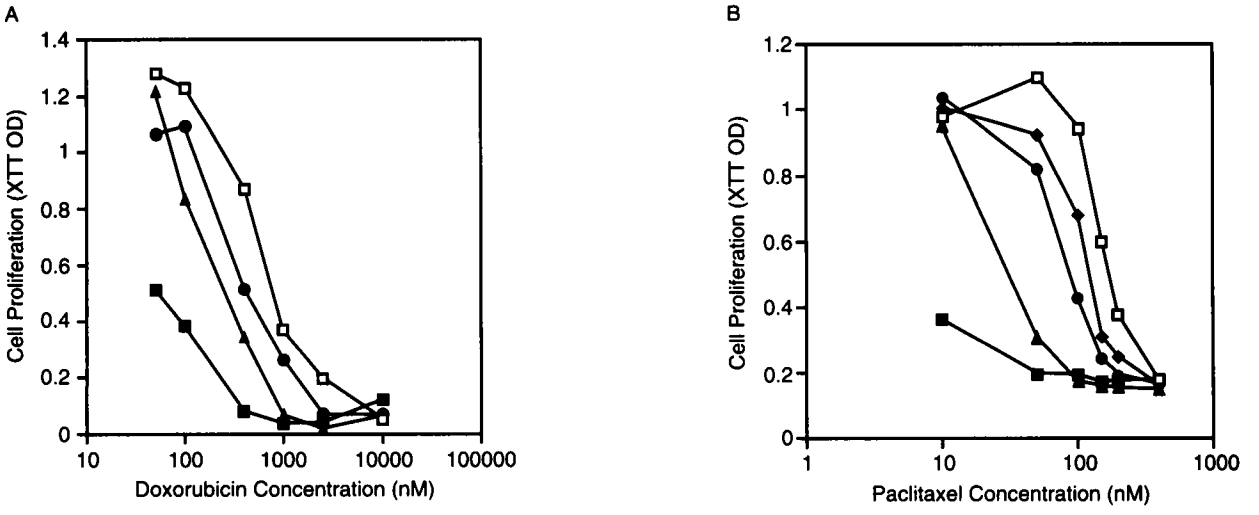

Figure 2. *In vitro* MDR reversing activity of VX-710. Dose-dependent reversal of doxorubicin resistance of L1210/VMDRC.06 leukemia cells (A) and of paclitaxel resistance of 8226/Dox6 multiple myeloma cells (B). Cytotoxicity assays involving titrations of cytotoxic drug in the absence (□) or presence of 0.25 μM (◆), 0.5 μM (●), 1 μM (▲) and 2.5 μM (■) VX-710 were performed as described in Materials and methods, and XTT dye reduction was used to determine cell viability. Data represent means from quadruplicates.

acute lymphocytic leukemia and doxorubicin-selected P388/ADR mouse leukemia cells when evaluated with vincristine, vinblastine, colchicine or doxorubicin, respectively (data not shown).

A correlation was noticed between levels of P-glycoprotein expression and the VX-710 concentration required for MDR reversal (Table 2). According to Shen *et al.*[43] the multidrug resistant KB-8-5 and KB-V1 cell lines, both derived from the KB-3-1 parental cell line, differ approximately 33- to 130-fold in relative drug resistance and levels of P-glycoprotein are significantly higher in KB-V1 cells. In our experiments, the KB-V1 cell line was approximately 30-fold more resistant to doxorubicin than the KB-8-5 cell line (Table 2) and a slightly higher concentration of VX-710 was required for complete chemosensitization of KB-V1 cells compared to KB-8-5 cells (Table 2). Similarly, a higher concentration of VX-710 (5 μM or above) was required for full chemosensitization of highly drug-resistant 8226/Dox40 multiple myeloma cells compared with 8226/Dox6 cells (Table 1) which express lower levels of P-glycoprotein.[52]

We have also observed that VX-710 at concentrations of 2.5–5.0 μM may exhibit a 2- to 3-fold chemosensitizing effect on 8226 parental drug-sensitive human myeloma cells (for doxorubicin, vincristine, etoposide or paclitaxel) and KB-3-1 epithelial adenocarcinoma cells (only doxorubicin was tested). A similar 2- to 3-fold sensitization of murine L1210 leukemia cells and NIH 3T3 fibroblasts was also observed with doxorubicin, etoposide or paclitaxel. However, sensitization of parental murine L1210 leukemia cells and murine NIH 3T3 fibroblasts to vincristine was more pronounced (greater than 10-fold) at 2.5 μM VX-710.

The antiproliferative activity of VX-710 itself was also investigated in a variety of both drug-sensitive and multidrug resistant cells to determine if intrinsic cytotoxicity of VX-710 contributes to its observed MDR reversing activity. To this end, cell viability was determined after a 3-day continuous exposure to VX-710 (Table 3). We found that VX-710 concentrations of 2.5–5.0 μM generally had no significant antiproliferative effect (i.e. less than 5% growth inhibition) in any of the cell lines tested. Some antiproliferative activity of 5 μM VX-710, however, was noted in colony formation assays performed with the KB-V1 cell line (but not the KB-8-5 and KB-3-1 cell lines) and is reflected by oversensitization of KB-V1 cells to doxorubicin (Table 2). As presented in Table 3, some differences in sensitivity to VX-710 was observed in a panel of more than 20 cell lines examined, but the IC_{50} values for VX-710 were all above 10 μM and ranged from 13 to 70 μM. Generally, the maximal non-toxic concentration was significantly higher for VX-710 than for CsA and IC_{50} values for VX-710 were approximately 2- to 9-fold higher than for CsA in parallel experiments conducted with some of the cell lines (Table 3).

Table 1. *In vitro* MDR reversing activity of VX-710 for multidrug resistant leukemia and multiple myeloma cells

MDR cell line	Drug	Drug IC_{50} (μM) (relative resistance)	Drug + VX-710 IC_{50} [μM] (resistance modifying factor)				
			0.1 μM	0.25 μM	0.5 μM	1.0 μM	2.5 μM
L1210/ VMDRC.06	doxorubicin	0.7 ± 0.1 (6.4)	0.67 ± 0.10 (1.0)	0.57 ± 0.12 (1.2)	0.50 ± 0.10 (1.5)	0.26 ± 0.02 (2.7)	0.047 ± 0.006 (14.8)
	vincristine	0.103 ± 0.011 (5.7)	0.103 ± 0.011 (1.0)	0.113 ± 0.032 (1.0)	0.082 ± 0.008 (1.3)	0.029 ± 0.022 (3.6)	0.005 ± 0.001 (20.6)
	etoposide	4.7 ± 0.6 (15.2)	4.3 ± 0.6 (1.1)	4.3 ± 0.6 (11)	3.4 ± 1.0 (1.4)	1.8 ± 0.2 (2.6)	0.23 ± 0.06 (20.4)
	paclitaxel	0.296 ± 0.036 (17.6)	0.207 ± 0.015 (1.4)	0.183 ± 0.015 (1.6)	0.138 ± 0.032 (2.1)	0.043 ± 0.006 (6.8)	0.004 ± 0.001 (74)
8226/Dox6	doxorubicin	1.46 ± 0.49 (18)	0.95 ± 0.13 (1.5)	0.81 ± 0.42 (1.8)	0.36 ± 0.15 (4.1)	0.19 ± 0.03 (7.7)	0.13 ± 0.05 (11.2)
	vincristine	0.126 ± 0.038 (29)	0.103 ± 0.006 (1.2)	0.093 ± 0.006 (1.3)	0.055 ± 0.015 (2.3)	0.029 ± 0.009 (4.7)	0.005 ± 0.001 (25.2)
	paclitaxel	0.16 ± 0.01 (32)	0.14 ± 0.02 (1.1)	0.11 ± 0.01 (1.5)	0.086 ± 0.015 (1.9)	0.04 ± 0.017 (4.0)	0.012 ± 0.006 (13.3)

Cytotoxicity assays involving cross-titrations of cytotoxic drugs and VX-710 (0.1, 0.25, 0.5, 1.0 and 2.5 μM as indicated) were performed as described in Materials and methods using XTT dye reduction to determine cell viability. Seeding densities were 1×10^4 for human *MDR*1-transfected L1210/vMDRC.06 and L1210 leukemia cells ($n = 4$ per condition) and 4×10^4 for doxorubicin-selected 8226/Dox6 and 8226 multiple myeloma cells ($n = 6$ per condition). Cytotoxicity plots were generated to extrapolate IC_{50} values and calculate MDR ratios. Data represent means and standard deviations from three independent experiments. The relative resistance was calculated by the ratio (IC_{50} resistant cells)/(IC_{50} parental cells) and the resistance modifying factor by the ratio (IC_{50} drug)/(IC_{50} drug + VX-710).

Table 2. Doxorubicin sensitization by VX-710 in multidrug resistant human epithelial adenocarcinoma and human *MDR*1-transfected murine NIH 3T3 fibroblasts

MDR cell line	Doxorubicin IC_{50} (μM) (relative resistance)	Doxorubicin + VX-710 IC_{50} (μM) (resistance modifying factor)			
		0.5 μM	1.0 μM	2.5 μM	5.0 μM
KB-8-5	0.022 ± 0.009 (2.2)	0.009 (2.4)	0.008 ± 0.003 (2.7)	0.004 ± 0.001 (5.5)	0.0031 ± 0.0003 (7.1)
KB-V1	0.667 ± 0.139 (67)	0.586 (1.4)	0.178 ± 0.070 (3.7)	0.012 ± 0.005 (55.6)	0.0026; 0.0017 (256; 392)
N3V2400	0.266 ± 0.050 (38)	0.224 (1.5)	0.052 ± 0.022 (5.1)	0.0063 ± 0.0010 (42.2)	0.0055 ± 0.0001 (48.4)

Dose–response curves were determined by colony formation assays as described in Materials and methods. Cultures contained no drug and/or reversing agent or a cross-titration of doxorubicin and VX-710 (1.0, 2.5 and 5.0 μM as indicated). After incubation at 37°C, 5% CO_2 for 10–14 days, the surviving cell colonies were stained with methylene blue and counted. IC_{50} values (means and SD from three to five independent experiment, with the exception of assays at 0.5 μM VX-710 for which $n = 1$, and experiments with KB-V1 and 5 μM VX-710 for which $n = 2$) were extrapolated from cytotoxicity plots. The relative resistance was calculated by the ratio (IC_{50} resistant cells)/(IC_{50} parental cells) and the resistance modifying factor by the ratio (IC_{50} drug)/(IC_{50} drug + VX-710).

Table 3. Evaluation of VX-710 and CsA for direct antiproliferative activity *in vitro*

Cell line	IC_{50} (μM)	
	VX-710	CsA
L1210	20	8
L1210/VMDRC.06	18	ND
U-937	19	5
Jurkat clone E6-1	11	6
THP-1	20	ND
8226/S	27	ND
8226/Dox6	19	4
8226/Dox40	17	ND
Bowes	35	8
COL-1	35	7.2
VCR 4.5	35	9
VBL 3.0	48	5.2
CTLL-2	12.5	1.5
HUV-EC-C	50	> 20
Bovine aortic endothelial cells	70	> 20
Human primary foreskin fibroblasts	70	> 20
NIH 3T3	35	10
N3V2400	30	ND
KB-3-1	21	ND
KB-8-5	18	ND
KB-V1	13	ND

Cells were seeded in 96-well microtiter plates ($n = 6$ per drug concentration) with varying seeding density depending on the growth rate of each line and cultured for 3 days in the presence of 1–100 μM VX-710 or 1–20 μM CsA. Cell viability was measured by XTT assay as described in Materials and methods, and IC_{50} values were calculated from the dose titration curves. ND = not determined.

Effects of VX-710 on drug accumulation in multidrug resistant cells

Multidrug resistant cells which overexpress P-glycoprotein are characterized by reduced intracellular drug accumulation.[2] Thus, the ability of VX-710 to restore [^{14}C]doxorubicin accumulation was evaluated in L1210/VMDRC.06 cells. Compared to parental L1210 cells, accumulation of [^{14}C]doxorubicin in human *MDR*1-transduced L1210/VMDRC.06 cells was decreased by approximately 50% due to drug efflux by P-glycoprotein (Figure 3). Increasing concentrations of VX-710 (0.5–1.0 μM) restored [^{14}C]doxorubicin accumulation in L1210/VMDRC.06 cells to the level of the drug-sensitive parental L1210 cell line, while 2.5μM or higher VX-710 significantly enhanced drug accumulation beyond the level of the parental cell line (Figure 3). CsA at concentrations of 0.25–2.0 μM had a similar effect on [^{14}C]doxorubicin accumulation by L1210/VMDRC.06 cells. In additional experiments with a fluorescent P-glycoprotein substrate,[53] VX-710 was found to increase rhodamine-123 accumulation in 8226/Dox40 multiple myeloma cells to levels of drug-sensitive parental cells with an EC_{50} of 2.5 μM (data not shown). Moreover, VX-710 at a concentration of 5.0 μM completely restored accumulation of the P-glycoprotein substrate [^{99}Tc]SESTAMIBI[54] in 8226/Dox6 cells to levels observed within drug-sensitive cells (data not shown). Taken together, these data suggest that increasing drug accumulation is an important component of the biochemical mechanism of MDR reversal by VX-710.

Effects of VX-710 on photoaffinity labeling of P-glycoprotein

Photoaffinity labeling reagents have been developed that label P-glycoprotein specifically.[55] These photoprobes are useful for investigating the interaction of MDR modulators with the *MDR*1 gene

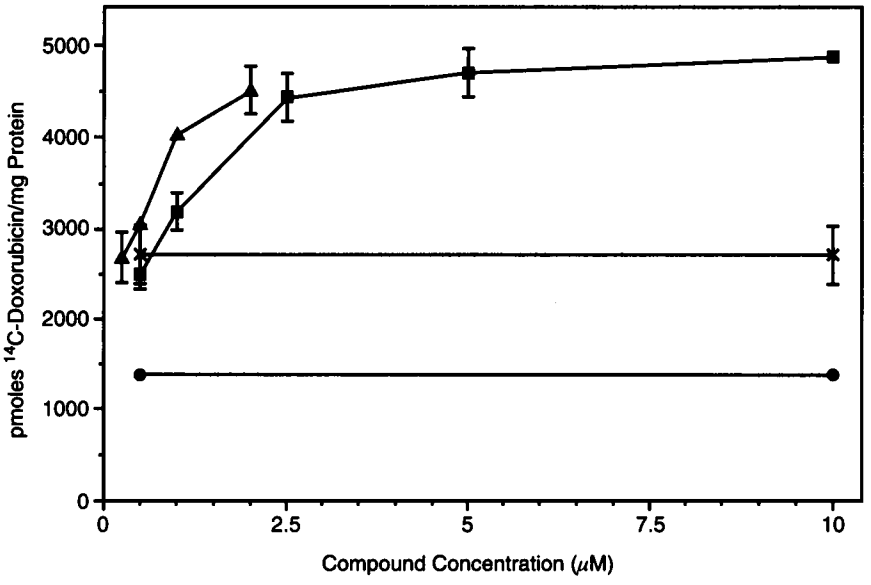

Figure 3. Comparison of VX-710 and CsA for restoring the [^{14}C]doxorubicin accumulation defect in multidrug resistant L1210/VMDRC.06 cells. L1210/VMDRC.06 cells were incubated at a concentration of 1×10^6 cells/ml with 2 μM [^{14}C]doxorubicin in the absence (●) or presence of VX-710 (■) or CsA (▲) for 45 min at 37°C and analyzed for [^{14}C]doxorubicin content and protein concentration as described in Materials and methods. [^{14}C]Doxorubicin accumulation in the absence of MDR reversing agent was also measured in the parental L1210 cell line (×). Data indicated are means and SD of three determinations.

product in competitive binding assays. We performed photoaffinity labeling experiments using the human *MDR*1-transfected N3V2400 cell line which expresses high levels of human P-glycoprotein.[45] P-glycoprotein was the most prominent protein in N3V2400 cells specifically labeled by [^{3}H]azidopine or [^{125}I]iodoaryl azidoprazosin (Figure 4). In some experiments (e.g. Figure 4B), photolabeled P-glycoprotein fragments of a molecular size of approximately 85 and 55 kDa were detected. The identity of photolabeled P-glycoprotein and fragments thereof was corroborated in immunoprecipitation experiments and Western blot analyses with P-glycoprotein-specific antibodies (monoclonal C219 antibody, 4007 and 4077 polyclonal antisera, data not shown). In view of biochemical analyses of the location of [^{3}H]azidopine or [^{125}I]iodoaryl azidoprazosin labeling sites within human P-glycoprotein reported by Bruggemann *et al.*[51] and Greenberger,[56] it may be speculated that the 85 and the 55 kDa P-glycoprotein fragments comprise the N- and C-terminal labeling sites, respectively.

We then analyzed displacement of [^{3}H]azidopine and [^{125}I]iodoaryl azidoprazosin from P-glycoprotein by increasing concentrations of VX-710 and found that photoaffinity labeling of P-glycoprotein (and fragments thereof) was blocked in a dose-dependent manner (Figure 4). VX-710 concentrations of 0.75 and 0.55 μM reduced [^{3}H]azidopine or [^{125}I]iodoaryl azidoprazosin labeling of P-glycoprotein by 50% (EC_{50}). These VX-710 EC_{50} concentrations were 4- to 8-fold lower than for CsA (EC_{50} of about 3.5 μM for both [^{3}H]azidopine and [^{125}I]iodoaryl azidoprazosin) and > 100-fold lower than for verapamil (EC_{50} greater than 100 μM for both [^{3}H]azidopine and [^{125}I]iodoaryl azidoprazosin) (data not shown). The data from these competitive displacement studies suggest a direct, high-affinity interaction of VX-710 with P-glycoprotein.

A tritiated photoaffinity analog of VX-710, [^{3}H]VF-13,159 or [^{3}H]-(*S*)-*N*-[2-Oxo-2-(4-azidophenyl)acetyl]-piperidine-2-carboxylic acid 1,7-bis(3-pyridyl)-4-heptyl ester, with a *p*-azidoaryl group in place of the trimethoxyaryl group, was used to corroborate direct binding of VX-710 to P-glycoprotein and to investigate the specificity of VX-710. As shown in Figure 4(C), [^{3}H]VF-13,159 specifically labeled the human *MDR*1 gene product and an 85 kDa fragment thereof. Consistent with the 3.7 nM k_i of VX-710 for FKBP12 PPIase activity, the 12 kDa immunophilin FKBP12 was also labeled by [^{3}H]VF-13,159 (data not shown). The identity of photolabeled P-glycoprotein and the P-glycoprotein fragment was corroborated by a series of immunoprecipitation experiments involving several different P-glycoprotein-specific antibodies and by the absence of this signal in

parental NIH 3T3 cells (data not shown). Unlabeled VX-710 inhibited photolabeling of P-glycoprotein in a dose-dependent manner with an EC_{50} of 0.75 μM (Figure 4C). These data indicate that VX-710 binds to P-glycoprotein directly and may act as a competitive ligand for binding site(s) of cytotoxics within P-glycoprotein.

Effects of VX-710 on vanadate-sensitive ATPase activity of P-glycoprotein

Since it has been shown that several P-glycoprotein substrates (both anti-cancer drug substrates, e.g. Vinca alkaloids, anthracyclines, epipophyllotoxins, as well as MDR modulators, e.g. verapamil) stimulate the vanadate-sensitive P-glycoprotein-specific ATPase activity in a dose-dependent manner,[10,14–16] we performed ATPase assays to analyze the nature of VX-710 interactions with P-glycoprotein. The effects of VX-710 on the vanadate-sensitive ATPase activity of P-glycoprotein were measured *in vitro* in enriched membrane preparations isolated from human *MDR*1-transfected N3V2400 cells,[45] which represent a novel model system for analysis of the human P-glycoprotein ATPase activity. Specific inhibitors for several membrane-associated ATPases (e.g. F_1-F_0-ATPase, Na^+,K^+-ATPase, calcium-dependent ATPase) which do not affect P-glycoprotein-dependent drug efflux were included in the assays.[10]

A shown in Figure 5(A), crude membranes from NIH 3T3 parental control cells expressed a vanadate-sensitive ATPase activity of approximately 0.004 μmol/mg protein·min which was not affected significantly by VX-710. The vanadate-sensitive ATPase activity measured in crude membranes from human *MDR*1-transfected N3V2400 cells was 3.5-fold higher (0.014 μmol/mg protein·min; Figure 5A), while the vanadate-insensitive ATPase activity was the same for both the parental and the human *MDR*1-transfected cell lines. Time-course experiments showed that the basal P-glycoprotein ATPase activity increased linearly for at least 60 min at 37°C (Figure 5A). VX-710 at concentrations above 0.01 μM enhanced the P-glycoprotein-specific ATPase activity (Figure 5A and B). A maximal increase (approximately 3-fold) was achieved with 1 μM VX-710 (Figure 5A and B) and an apparent k_a value of 0.1 μM (i.e. VX-710 concentration required for half-maximal stimulation of P-glycoprotein ATPase activity) was calculated from nine independent experiments performed with different preparations of crude N3V2400 cell membranes. VX-710 concentrations of 10 and 100 μM also stimulated P-glycopro-

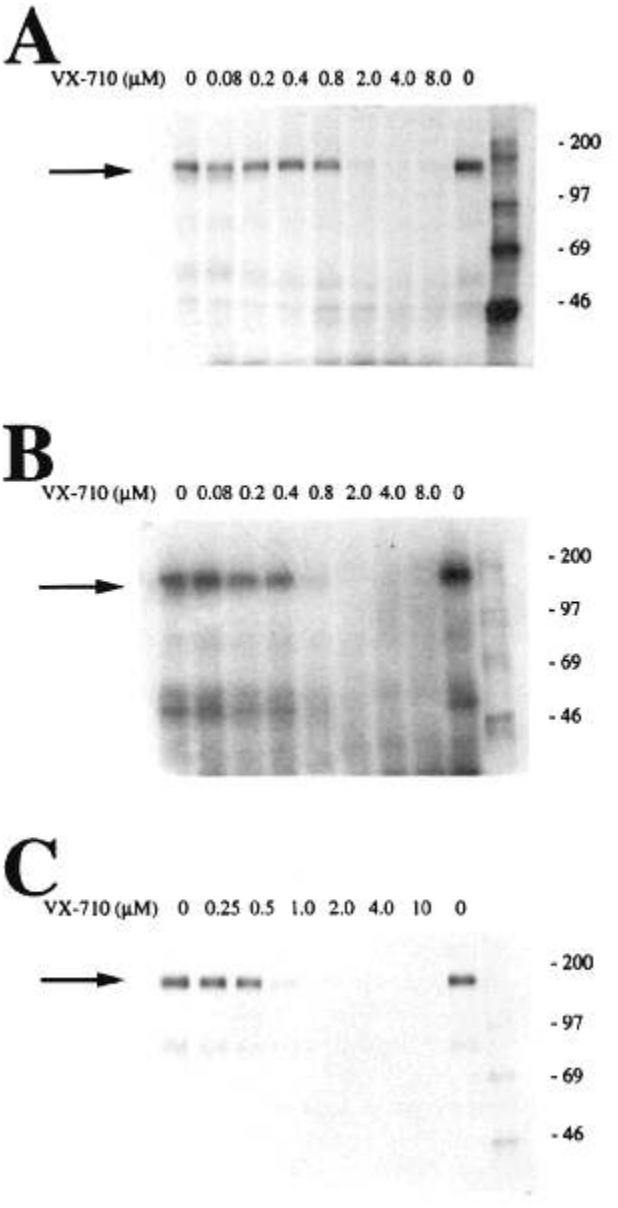

Figure 4. VX-710 competition for photoaffinity labeling of P-glycoprotein by [^{3}H]azidopine, [^{125}I]iodoaryl azidoprazosin and [^{3}H]VF-13,159. Murine N3V2400 cells expressing high levels of human P-glycoprotein were co-incubated with [^{3}H]azidopine (A), [^{125}I]iodoaryl azidoprazosin (B) or the VX-710 photoaffinity analog [^{3}H]VF-13,159 (C) and a dilution series of VX-710 for 60 min at room temperature with gentle agitation, and UV cross-linking was performed as described in Materials and methods. Cell extracts were prepared and labeled P-glycoprotein was visualized by SDS–8% polyacrylamide gel electrophoresis and subsequent fluorography (^{3}H-labeled photoprobes) or autoradiography ([^{125}I]iodoaryl azidoprazosin). Each lane contains proteins extracted from approximately 75 000 photolabeled N3V2400 cells. The arrow on the left points to P-glycoprotein signals. On the right, the sizes of [^{14}C]methylated protein standards are indicated in kDa.

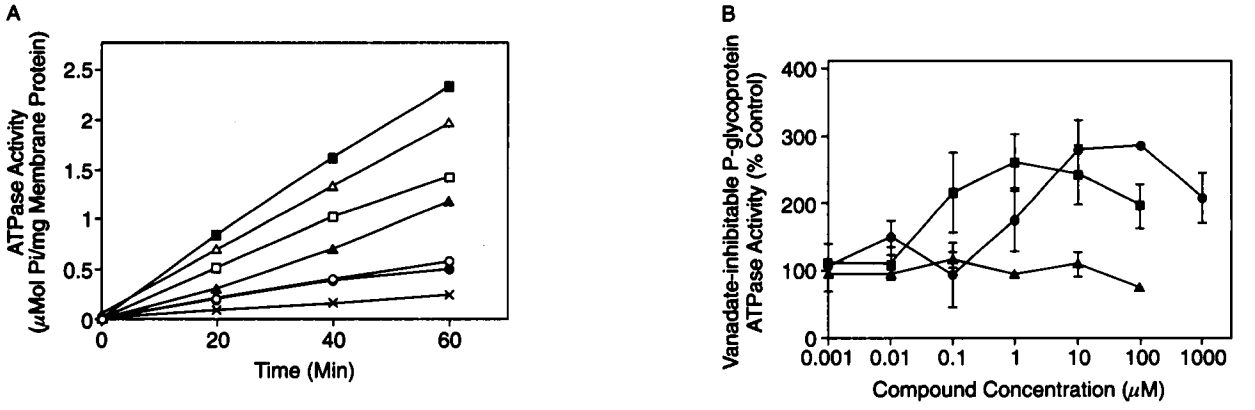

Figure 5. Modulation of the vanadate-sensitive P-glycoprotein ATPase activity in multidrug resistant N3V2400 cells by VX-710, CsA and verapamil. ATPase assays were performed with enriched membranes prepared from murine N3V2400 fibroblasts and NIH 3T3 cells as described in Materials and methods. The ATPase activity attributable to P-glycoprotein was calculated as (ATPase activity measured in the absence of vanadate) – (ATPase activity measured in the presence of vanadate). (A) The vanadate-sensitive P-glycoprotein ATPase activity was determined as a function of time using crude membranes from multidrug resistant N3V2400 fibroblasts in the absence of VX-710 (○) or at VX-710 concentrations of 0.01 μM (●), 0.1 μM (□), 1 μM (■), 10 μM (△) and 100 μM (▲). Control assays were performed with drug-sensitive NIH 3T3 cells (×). Data are means from duplicate samples. (B) Modulation of the vanadate-sensitive P-glycoprotein ATPase activity by VX-710 (■), CsA (▲) and verapamil (●). ATPase assays were performed with duplicate samples and P_i measured after 20, 40, 60 and 80 min. Data points reflect means of these four time points obtained from three independent experiments performed with different N3V2400 cell crude membrane preparations. Bars indicate SD.

tein ATPase activity, but were less effective than lower VX-710 concentrations (Figure 5A and B). This submaximal stimulation at high drug concentrations has been observed with other P-glycoprotein substrates, including both cytotoxic drugs and MDR reversing agents (e.g. verapamil; Figure 5B), and may reflect non-specific effects associated with membrane partitioning of hydrophobic compounds or reduced compound solubility.[10,14–16] Among other MDR reversing agents tested in ATPase assays with enriched N3V2400 membrane preparations, verapamil stimulated the vanadate-sensitive P-glycoprotein ATPase activity 3.5-fold, with an apparent k_a of approximately 1 μM (Figure 5B) while CsA alone had no statistically significant effect on P-glycoprotein ATPase activity (Figure 5B), consistent with data reported by others.[10,14]

Discussion

The emergence of resistance to multiple anti-cancer drugs is a major obstacle to successful cancer chemotherapy and an important goal of cancer research is to discover agents that are useful to reverse or circumvent MDR in the clinic. Numerous compounds have been demonstrated to antagonize P-glycoprotein-mediated MDR in a variety of tissue culture model systems when co-administered with anti-cancer drugs to which the cells are resistant.[21,22] Some of these MDR reversing agents were subsequently found to possess clinical pharmacological characteristics appropriate for evaluation as potential modulators of clinical MDR.[21,26,27] However, the most potent chemosensitizers that emerged from such *in vitro* studies have not yet been adequately tested in clinical trials. Thus, the continued identification of specific and potent chemosensitizers for clinical use remains critical to the possible success of experimental reversal of multidrug resistance in human cancer.

Prompted by reports that the immunophilin-binding immunosuppressants FK506 and rapamycin were MDR reversing agents,[34] we evaluated a series of novel non-macrocyclic high-affinity ligands to FKBP12[37] for their ability to restore the sensitivity of cells which overexpress P-glycoprotein to cytotoxic drugs. In the present study we show that the pipecolinate derivative VX-710 (Figure 1) is an effective *MDR*1 modulator.

We used a panel of drug-selected, P-glycoprotein-expressing cell lines derived from hematological cancers and solid tumors, as well as two different *MDR*1 cDNA-transfected cell lines for analyzing the effects of VX-710 on resistance to multiple anti-cancer drugs (Figure 2, Tables 1 and 2). VX-710 restored sensitivity of multidrug resistant cells inde-

pendent of the tissue origin of the cell line, the cytotoxicity assay method used and the MDR class cytotoxic drug tested. Moreover, the MDR reversal activity of VX-710 was similar for wild-type P-glycoprotein (e.g. KB-V1 or N3V2400 cells) and for a P-glycoprotein mutant in which the glycine at position 185 is substituted with valine (e.g. L1210/VMDRC.06 cells), at least for doxorubicin. Generally, VX-710 was active as an *MDR*1 modulator at 2.5 μM, a concentration 4- to 28-fold lower than its apparent IC_{50} against a variety of normal and tumor cell lines (Table 3).

A comparison of our VX-710 studies with literature values reported for verapamil and CsA[22] suggests that VX-710 is 2- to 4-fold more effective than *S*- or *R*-verapamil and similar to CsA in potency for MDR reversal *in vitro*. We corroborated this for the anticancer drug doxorubicin in the P-glycoprotein-expressing vincristine-selected HL60/Vinc cell line,[57] but found that 2.5 μM VX-710 was approximately 5-fold more effective than 5 μM CsA in restoring doxorubicin sensitivity to *MDR*1 cDNA-transfected vincristine-selected N3V2400 cells, whereas 2.5 μM VX-710 was equipotent with 1 μM CsA in restoring doxorubicin sensitivity to colchicine-selected L1210/VMDRC.06 and doxorubicin-selected 8226/Dox6 cells. Thus, the relative extent to which resistance is reversed by VX-710 and CsA appears to vary somewhat between different cell lines and may depend on other phenotypic characteristics of the cell lines tested. Similar to VX-710, CsA generally reversed MDR at 1–2.5 μM; however, in the *MDR*1 cDNA-transfected, vincristine-selected N3V2400 cell line only partial reversal of doxorubicin resistance was achieved at 5 μM, for reasons unknown at present.

Most agents described to date, which antagonize MDR *in vitro*, alter the drug accumulation defect present in P-glycoprotein-expressing cells via direct interaction with the *MDR*1 gene product[22] and VX-710 appears to function the same way. In studies involving various P-glycoprotein substrates ([^{14}C]doxorubicin, rhodamine-123 and [^{99}Tc]SESTAMIBI) we demonstrated that VX-710 at concentrations required for reversal of MDR *in vitro* (2.5–5 μM) was similarly effective as CsA in reversing the cellular accumulation defect in multidrug resistant cell lines to (or beyond) the levels of the drug-sensitive parental cell lines (Figure 3), suggesting that VX-710 acts by blocking P-glycoprotein-mediated drug efflux. Moreover, data from a series of photoaffinity labeling experiments involving the P-glycoprotein-specific photoprobes [^{3}H]azidopine, [^{125}I]iodoaryl azidoprazosin and, in particular, [^{3}H]VF-13,159, a radiolabeled photoaffinity analog of VX-710, indicate that VX-710 directly interacts with P-glycoprotein (Figure 4).

One possible mechanism for reversal of MDR is that VX-710 inhibits the drug transport activity of P-glycoprotein by acting as a competitive ligand for binding site(s) of cytotoxics. However, given the incomplete understanding of the mechanism of drug efflux by P-glycoprotein[2,3] and the conflicting evidence with regard to the exact mechanism of action of MDR modulators, alternative models will also have to be considered. To date, it remains unclear whether similar or different chemosensitizers function in the same way, and whether they function as competitive or as non-competitive inhibitors of drug efflux.[21,22] Also the number and location of binding site(s) for chemosensitizers within P-glycoprotein remain to be determined. Of particular interest is whether chemosensitizers share binding sites with cytotoxic drugs.[2,3] However, it has not been clearly established whether P-glycoprotein contains a common drug-binding pore, partially overlapping drug binding sites or multiple non-overlapping drug binding sites, which may have different affinities for different drugs, classes of drugs, chemosensitizers or classes of chemosensitizers.[2,3,22] The VX-710 photoaffinity analog selectively labels P-glycoprotein, is an excellent photoprobe and represents a very useful tool for further biochemical analyses of VX-710 binding site(s). Mapping of the VX-710 photoaffinity analog binding site(s) within the primary structure of human P-glycoprotein has been initiated, and preliminary data suggest that a major labeling site is located within a segment in the N-terminal half of the protein that includes the transmembrane regions TM5 and TM6 (Germann UA *et al.*, in preparation). Interestingly, this region appears to be of central importance for drug interactions, based on previously reported P-glycoprotein photoaffinity labeling studies and mutational analyses of the *MDR*1 gene product.[2,3]

In support of their possible role as competitive ligands for drug binding site(s), chemosensitizers themselves (e.g. verapamil and CsA) may be transported by P-glycoprotein.[23,25,58] We attempted to address the question whether VX-710 is a direct substrate for P-glycoprotein by measuring the accumulation of ^{14}C-labeled VX-710 in parental NIH 3T3 cells and P-glycoprotein-overexpressing N3V2400 cells according to experimental protocols[50] which we also used for our [^{14}C]doxorubicin accumulation studies. However, these experiments were technically not feasible because of a high background of [^{14}C]VX-710 accumulation observed at time 0 in both drug-sensitive and multidrug resistant cells

independent of the presence of ATP. It is possible that rapid membrane partitioning and/or a high-affinity interaction of VX-710 with FKBP12 interfered with these experiments.

In an alternative approach, we performed ATPase assays to analyze the nature of VX-710 interactions with P-glycoprotein, since it is known that P-glycoprotein substrates (e.g. anti-cancer drugs, MDR modulators, cellular indicators and bioactive peptides) stimulate the vanadate-sensitive P-glycoprotein-specific ATPase activity in a dose-dependent manner.[10–16] We observed a concentration-dependent stimulation of the vanadate-sensitive P-glycoprotein ATPase activity (Figure 5), providing further evidence for a direct, high-affinity interaction between VX-710 and the *MDR*1 gene product. With an apparent k_a of 100 nM, VX-710 was found to be a more effective stimulator of P-glycoprotein ATPase than cytotoxic drug substrates (vinblastine, vincristine, daunorubicin, doxorubicin, paclitaxel and colchicine) which had apparent k_a values of 0.5 μM or above, in agreement with data reported by Sarkadi *et al.*[10] Although a tight coupling of ATPase stimulation with drug efflux activity has not been proven, these data may infer that VX-710 blocks the drug efflux activity of P-glycoprotein by serving as a preferred substrate. Alternatively, VX-710 may inhibit cytotoxic drug interaction with P-glycoprotein via competition for a common drug binding site, or by a non-competitive allosteric mechanism, or by a coupled mechanism interfering with both drug binding and energy supply for drug transport. Clearly, additional experiments are required to further elucidate the nature of the molecular interactions of VX-710 with P-glycoprotein.

The interaction of VX-710 with P-glycoprotein suggests that FKBP12 binding is not required for its MDR reversing activity. We and others[9] have evaluated several other pipecolinate derivatives and did not observe a correlation between FKBP12 binding affinity and inhibition of the P-glycoprotein drug transport function. For example, the D-diastereomer of VX-710 (*R*)-*N*-[2-Oxo-2(3,4,5-trimethoxy-phenyl)-acetyl]piperidine-2-carboxylic acid 1,7-bis(3-pyridyl)-4-heptyl ester) had reduced affinity for FKBP12 (k_i 150 nM), but was equipotent with VX-710 as an MDR reversing agent. Similar results were obtained with other MDR reversing analogs that differ even more significantly in FKBP12 binding affinity. From our studies with VX-710 and other FKBP12 ligands, and the results reported by Hauske *et al.*,[59] it appears that the structural components required for the immunosuppressive activity of FK506 and rapamycin are not essential for P-glycoprotein interaction.

In general, most MDR modulators described to date quite selectively target P-glycoprotein-expressing cells and cause little or no changes in drug-sensitive cells.[22] We have noticed a modest (2- to 3-fold) chemosensitizing effect of VX-710 at 2.5 μM for several parental drug-sensitive cell lines. However, sensitization of two murine cell lines to vincristine, but not to doxorubicin, etoposide and paclitaxel, was quite dramatic (greater than 10-fold). These data suggest that VX-710 may also affect non-P-glycoprotein-associated mechanisms of drug resistance, e.g. MRP,[6] LRP[7] or another, so far unknown drug resistance protein. A continued characterization of the *in vitro* activities of VX-710 in HL60/ADR cells[60] revealed that VX-710 at concentrations of 2.5–5 μM also modulates MDR mediated by the multidrug resistance-associated protein MRP and restores drug accumulation in MRP-expressing cells.[57] Western blot analyses with an MRP-specific polyclonal antiserum showed low basal levels of expression of MRP protein in all the drug-sensitive, parental cell lines used in the present study; however, expression of MRP protein was not enhanced in the multidrug resistant cell lines analyzed (data not shown). Hence, part of the MDR reversing activity of VX-710 observed in parental drug-sensitive cell lines (and perhaps a minor part of the MDR reversing activity of VX-710 in the multidrug resistant cell lines) might reflect the inhibitory effect of VX-710 on MRP-mediated drug efflux. On the other hand, the more selective effect of VX-710 on vincristine cytotoxicity in the two drug-sensitive murine cell lines tested in this study is presently not understood, and may involve a biochemical mechanism unique to these murine cell lines and vincristine, or possibly other Vinca alkaloids.

We have described here the identification of VX-710 as an effective reversing agent for P-glycoprotein-mediated multidrug resistance *in vitro*. A subsequent pharmacokinetic analysis showed that VX-710 has properties that make it a suitable candidate for clinical studies.[61] VX-710 is presently under clinical evaluation for its efficacy in treatment of patients with multidrug resistant cancers.[62]

Conclusions

We have investigated the *in vitro* efficacy of VX-710, a novel non-macrocyclic ligand of the FK506-binding protein FKBP12, in reversing P-glycoprotein-mediated MDR and its interaction with the human *MDR*1 gene product. Our results show that VX-710 is a potent MDR reversing agent for multiple cell

lines representative of hematological cancers and solid tumors, and that VX-710 is effective for all MDR class cytotoxic drugs examined to date. VX-710 directly interacts with the human *MDR*1 gene product resulting in inhibition of the drug efflux function of P-glycoprotein, but the precise biochemical mechanism of MDR reversal by VX-710 is unclear. Preliminary data from parental cells suggest that VX-710 may also affect non-P-glycoprotein-associated mechanisms of drug resistance. A continued *in vitro* and *in vivo* analysis of VX-710 as a candidate for clinical reversal of MDR in human cancer is warranted.

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