In Vitro and in Vivo Reversal of P-Glycoprotein-mediated Multidrug Resistance by a Novel Potent Modulator, XR9576

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ABSTRACT

The overexpression of P-glycoprotein (P-gp) on the surface of tumor cells causes multidrug resistance (MDR). This protein acts as an energy-dependent drug efflux pump reducing the intracellular concentration of structurally unrelated drugs. Modulators of P-gp function can restore the sensitivity of MDR cells to such drugs. XR9576 is a novel anthranilic acid derivative developed as a potent and specific inhibitor of P-gp, and in this study we evaluate the in vitro and in vivo modulatory activity of this compound. The in vitro activity of XR9576 was evaluated using a panel of human (H69/LX4, 2780/AD) and murine (EMT6 AR1.0, MC26) MDR cell lines. XR9576 potentiated the cytotoxicity of several drugs including doxorubicin, paclitaxel, etoposide, vincristine, and vinblastine; complete reversal of resistance was achieved in the presence of 25–80 nM XR9576. Direct comparative studies with other modulators indicated that XR9576 was one of the most potent modulators described to date. Accumulation and efflux studies with the P-gp substrates, [3H]dauorubicin and rhodamine 123, demonstrated that XR9576 inhibited P-gp-mediated drug efflux. The inhibition of P-gp function was reversible, but the effects persisted for >22 h after removal of the modulator from the incubation medium. This is in contrast to P-gp substrates such as cyclosporin A and verapamil, which lose their activity within 60 min, suggesting that XR9576 is not transported by P-gp. Also, XR9576 was a potent inhibitor of photoaffinity labeling of P-gp by [3H]zidodipine implying a direct interaction with the protein. In mice bearing the intrinsically resistant MC26 colon tumors, coadministration of XR9576 potentiated the antitumor activity of doxorubicin without a significant increase in toxicity; maximum potential potency was observed at 2.5–4.0 mg/kg dosed either i.v. or p.o. In addition, coadministration of XR9576 (6–12 mg/kg p.o.) fully restored the antitumor activity of paclitaxel, etoposide, and vincristine against two highly resistant MDR human tumor xenografts (2780/AD, H69/LX4) in nude mice. Importantly all of the efficacious combination schedules appeared to be well tolerated. Furthermore, i.v. coadministration of XR9576 did not alter the plasma pharmacokinetics of paclitaxel. These results demonstrate that XR9576 is an extremely potent, selective, and effective modulator with a long duration of action. It exhibits potent i.v. and p.o. activity without apparently enhancing the plasma pharmacokinetics of paclitaxel or the toxicity of coadministered drugs. Hence, XR9576 holds great promise for the treatment of P-gp-mediated MDR cancers.

INTRODUCTION

The treatment of cancer with chemotherapeutic drugs is frequently impaired or ineffective as a result of either de novo or acquired resistance of tumor cells. In both cases, tumors can be refractory to a variety of antineoplastic drugs with different structures and mechanism of action. This phenomenon is termed MDR2 (1). Although there are several different mechanisms associated with the development of MDR, a common cause is believed to be overexpression of a M, 170,000 plasma membrane glycoprotein (P-gp). This MDRI gene product belongs to the ABC superfamily of transporter proteins, and it acts as an energy-dependent drug efflux pump, preventing adequate intracellular accumulation of a broad range of cytotoxic drugs including anthracyclines, Vinca alkaloids, epipodophyllotoxins, and taxanes for cell kill (1–3). P-gp is expressed in man in a cell- and tissue-specific manner, with high levels detectable in the kidney, liver, and intestine (4). In rodents, two genes, mdrla and mdrlb, have been reported to play a similar role in drug resistance to that of the MDRI gene in humans (5). Studies in the mdrla and mdrlb knockout mice as well as the P-gp tissue distribution studies in humans have suggested several physiological roles for P-gp including protection against toxic xenobiotics by excretion into bile, urine, or the intestinal lumen; maintenance of the blood-brain barrier; and transport of steroid hormones and cholesterol (2, 5, 6). In addition to P-gp, overexpression of the MRPs, a family of ABC transporter proteins, has also been reported to contribute to the development of MDR. For example, MRP1 confers resistance to certain anthracyclins, epipodophyllotoxins, and Vinca alkaloids but not to taxanes (7, 8). The MRP proteins are widely distributed in the body and appear to have a number of physiological functions including protection against toxic compounds, transport of cytokinin leukotriene LTC4, and the transport of organic anions into bile (reviewed in Refs. 7 and 8). Despite being members of the ABC superfamily of transporter proteins, human MDRI P-gp and MRP1 share only 15% amino acid homology (9). Thus, compounds that inhibit both proteins are likely to be less potent than specific inhibitors and may exhibit greater toxicity as a result of inhibition of other related or unrelated proteins. In addition, depending on the relative contribution of P-gp and MRP to the clearance of a particular cytotoxic drug, nonspecific modulators may alter the pharmacokinetics and enhance the toxicity of the cytotoxic drug to a greater extent than a specific modulator. This effect would be further exacerbated if the modulator was also metabolized by or inhibited enzymes involved in the metabolism of the cytotoxic drug such as P450 CYP3A4 or CYP2C8 (10, 11). In fact, there appears to be an overlap in substrate specificities and the tissue distribution of P450 3A and P-gp and several modulators are known to be metabolized by these enzymes (12).

A broad range of compounds that interact with P-gp and block drug efflux have been reported to reverse the MDR phenotype. The first generation modulators consisted of calcium channel blockers, calmodulin inhibitors, hormonal/steroidal derivatives, antibiotics, cardiovascular drugs, the cyclosporins, and other miscellaneous compounds (13). These compounds were developed for pharmacological uses other than reversal of MDR and were relatively nonspecific and weak inhibitors that were also substrates for P-gp. With the majority of these compounds, deleterious toxicities associated with their use at the

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2 The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; MRP, multidrug resistance; Vpm, verapamil; CsA, cyclosporin A; ABC, ATP-binding cassette; SCLC, small cell lung carcinoma; MS/MS, tandem mass spectrometry; LC-MS/MS, liquid chromatographic MS/MS.
required concentrations to inhibit P-gp function have precluded their widespread clinical use (14–16). The requirement for more selective and potent agents as resistance modifiers has led to the development of several “second-generation” modulators such as the nonimmunosuppressive cyclosporin D analogues, PSC 833 (17), VX-710 (18), the acridone carboxamide derivative GF120918 (GG918; Ref. 19), the substituted dibenzosuberane molecule LY335979 (20), and the diketopiperazine derivative XR9051 (21, 22). Clinical trials with some of the second-generation modulators are currently in progress, and initial studies have demonstrated some clinical benefit from the use of modulators such as PSC833 (23). However, to date, the nonspecific modulators dextrerapamil, VX-710, and PSC833 have shown significant enhancement of pharmacokinetics and toxicity of cytotoxics such as paclitaxel, which has necessitated the reduction of the cytotoxic drug dose when administered with these modulators (24, 25). In contrast the more potent and specific modulators (GG918, LY335979) have exhibited no significant pharmacokinetic interaction with doxorubicin, etoposide, and paclitaxel in animal studies (19, 20). These studies clearly indicate that the development of potent and selective P-gp inhibitors is an important approach to reversing MDR in the clinic.

A chemical program aimed at improving the potency and physicochemical properties of our MDR modulator XR9051, resulted in the discovery of XR9576 (Fig. 1), a potent and specific inhibitor of P-gp (26). The present studies were undertaken to characterize this novel anthranilamide derivative. The ability of XR9576 to sensitize a panel of MDR cell lines in vitro and in vivo to various cytotoxic drugs was evaluated along with its ability to inhibit drug efflux and interact with P-gp. The pharmacokinetic interaction between XR9576 and paclitaxel was also examined because this commonly used drug has shown significant interaction with other modulators including dexteroxerapamil (27), VX-710 (24), and PSC833 (25). The characteristics of XR9576 indicate that it holds great promise for the treatment P-gp-mediated MDR cancers.

MATERIALS AND METHODS

Cell Lines and Cell Culture

A panel of parental tumor cell lines and their resistant sublines, displaying characteristics of MDR attributable to expression of P-gp, were used. Drug-sensitive parental EMT6/P murine mammary carcinoma and the H69/P human small cell lung carcinoma cell lines and their MDR EMT6/AR1.0 and H69/ LX4 sublines were obtained from Dr. P. R. Twentyman (MRC Clinical Oncology and Therapeutics Unit, Cambridge, United Kingdom). Our in vitro studies showed that the EMT6/AR1.0 cells were 33-fold resistant to doxorubicin relative to the parental cells, and the H69/LX4 cells were about 20-, 290-, and 900-fold resistant to doxorubicin, etoposide, and paclitaxel, respectively. The human ovarian carcinoma cells, A2780 and their resistant variants, 2780AD (28), were provided by Dr. T. C. Hamilton (Fox Chase Center, Philadelphia, PA). The 2780AD cells were about 50- and 540-fold resistant to doxorubicin and paclitaxel, respectively. The intrinsically resistant MC26 murine colon carcinoma cell line, in which resistance is at least partly attributable to P-gp expression (5-fold to doxorubicin; 29), was obtained from Dr. A. S. Watson (University Hospital, Nottingham, United Kingdom). All of the cell lines were cultured as described previously (21).

Cytotoxics and Reagents

For in vitro use, daunorubicin, doxorubicin, vincristine, paclitaxel, etoposide, colchicine, and actinomycin D were obtained from Sigma. Stock solutions (5–100 mM) of all drugs were prepared in DMSO with the exception of daunorubicin (1 mM) and doxorubicin (500 μg/ml), which were prepared in sterile deionized H2O; the stock solutions were stored as aliquots at −20°C. For cell treatments, cytotoxics were further diluted in culture medium with the final DMSO concentration <1%. The resistance modifying agents XR9576 (free base and mesylate salt) and GG918 (free base and hydrochloride salt) were synthesized at Xenova. Stock solutions (5 mM) of these resistance modifying agents were prepared in DMSO. Vpm (Sigma, Poole, United Kingdom) and CsA (Calbiochem, Nottingham, United Kingdom) were dissolved in ethanol to give a stock concentration of 10 mM.

For in vivo use epirubicin and doxorubicin were obtained from Pharmastalia (St Albans, United Kingdom); etoposide from Bristol-Myers Squibb Pharmaceuticals Ltd (Middlesex, United Kingdom) and vincristine from David Bull Laboratories (Warwick, United Kingdom). XR9576 (mesylate salt) was dissolved in 5% (w/v) α-(+)-glucose (dextrose) solution at appropriate concentrations for i.v. and p.o. administration. GG918 (hydrochloride salt) was prepared in propylene-glycol:5% dextrose (3:2 v/v) at 10 mg/ml and diluted appropriately in 5% dextrose immediately prior to use.

The radiolabeled compounds [3H]daunorubicin (1–5 Ci/mmol) and [3H]lacidipine (48 Ci/mmol) were purchased from DuPont and Amersham Life Sciences, respectively.

Drug Potentiation Assays

The ability of modulators to potentiate the cytotoxicity of various drugs was evaluated in several cell lines as outlined previously (21) with minor modifications. Briefly, cells were seeded into 96-well plates (Falcon) at 6 × 102 to 2 × 105/well, depending on the cell line, in 100 μl of medium and incubated for 4 h at 37°C. Varying concentrations of modulator or solvent (50 μl/well) were subsequently added and incubated for an additional 1 h before the addition of the cytotoxic drug. The cytotoxic drug (50 μl) was added to give a range of final concentrations in quadruplicate wells. After incubation for an additional 4–6 days, cell proliferation of adherent cells was assessed using the sulforhodamine B assay and the proliferation of suspension cells by AlamarBlue. IC50 values for cytotoxic drugs (concentration resulting in 50% inhibition of cell growth) were calculated from plotted results using untreated wells as 100%. EC50 values for modulators (concentration required to give 50% of full reversal) were obtained from graphs of potentiation index (ratio of IC50 of cytotoxic drug alone:IC50 of cytotoxic drug in the presence of modulator) plotted against concentration of modulator. Full reversal was defined as the potentiation index obtained in the presence of 100 μM GG918.

Intrinsic Cytotoxicity

Cells (EMT6 AR1.0 8 × 102/well; A2780 5 × 103/well; 2780AD 6 × 102/well) were seeded into 96-well plates. After ~4 h, varying concentrations of XR9576 were added, and cells were incubated for an additional 4 days (EMT6 AR1.0) or 6 days (2780AD) before quantification of cell growth and calculation of IC50 values (concentration resulting in 10% inhibition of cell growth) as described above.

Accumulation of [3H]Daunorubicin in the EMT6/AR1.0 Cells

The ability of modulators to reverse the P-gp-dependent accumulation deficit in the resistant EMT6 AR1.0 cells was investigated as described previously (21).

Inhibition of P-gp-mediated Efflux

The ability of modulators to maintain inhibition of P-gp-mediated efflux after their removal from the incubation medium was assessed using two P-gp substrates, Rh123 and [3H]Daunorubicin. For the studies with Rh123, A2780 and 2780AD human ovarian cells were trypsinized and resuspended at a
density of 10^5/ml of culture medium. Modulators were added at fixed concentrations immediately before the addition of Rh-123 (0.8 μg/ml), and the cells were incubated for 1 h at 37°C (substrate-loading phase). The cells were then washed and resuspended in normal growth medium without modulator or Rh123. During the following “efflux phase,” samples were incubated at 37°C, and aliquots (~10^6 cells) removed at various time intervals, centrifuged, and washed in ice-cold PBS. The cells were then resuspended in 1 ml of ice-cold PBS and analyzed by flow cytometry. Fluorescence was measured from 10^4 cells and cell-associated fluorescence as a percentage of T0 was plotted against time. Similar experiments extended over a 22-h period using [3H]daunorubicin as the P-gp substrate, and EMT6/AR1.0 cells were performed as described previously (21).

Persistence of Activity after Removal of Modulator

EMT6 AR1.0 cells were plated as for the accumulation assay and allowed to attach for 48 h. Cells were then incubated with modulators for 1 h at 37°C, washed with culture medium, and further incubated in normal growth medium. At subsequent time points (up to 22 h) as indicated, the ability of the cells to accumulate [3H]daunorubicin was assessed as described for the accumulation assay. Graphs were plotted of cell-associated radioactivity/10^5 cells against time, where T0 represents the end of the modulator incubation phase.

Photoaffinity Labeling of P-gp

The photoaffinity labeling of P-gp was evaluated using a modified method of Ferry et al. (30). Cell membrane-enriched fractions of H69/P and H69/LX4 cells were adjusted to 1.0 mg/ml in “predilution buffer” [final concentration, 50 mM Tris-HCl (pH 7.4), 0.1 mM AEBSF, 0.25 mM sucrose, and 5 mM MgCl]. Samples of membrane (30 μl) were incubated in wells of a V-well polyvinyl chloride microtiter plate, with increasing concentrations of modulator (10 μl) prediluted in labeling buffer [50 mM Tris-HCl (pH 7.4), 0.1 mM p-aminoethylbenzenesulfonyl fluoride, 0.25 mM sucrose, and 5 mM MgCl] and 10 μl of [3H]azidopine prediluted in labeling buffer. The final concentration of [3H]azidopine in the incubation mixture was 1 μM. Incubation was for 1 h in darkness on ice. Samples were then exposed to UV light (366 nm) for 20 min on ice. Each incubation mixture was then diluted 1:1 with sample buffer [100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromphenol blue, and 20% glycerol] and electrophoresed on a 7.5% SDS-PAGE gel. Gels were then fixed in 5% glacial acetic acid and 5% isopropyl alcohol and rinsed in distilled water, and the signal was amplified by bathing in Autoroof (National Diagnostics) for 1 h. Dried gels were then exposed to XAR-5 film with an intensifying screen for 7 days at ~70°C. After development of film, bands corresponding to P-gp at M, 170,000 were quantified by densitometry (Pharmacia Gel Scanner).

In Vivo Efficacy Evaluation

MC26 Murine Colon Carcinoma Model. All of the animal experimentation was performed to United Kingdom Home Office regulations and the United Kingdom CCCR guidelines were adhered to at all times. The in vivo efficacy of XR9576 was evaluated using the intrinsically resistant MC26 colon carcinoma tumors that exhibited low levels of P-gp-mediated drug resistance as outlined previously (22). MC26 tumor slurry was implanted s.c. in female nude mice (~2780AD cells grown in vitro) and evaluated using MDR human carcinoma xenografts. Studies in parental (A2780) and the four resistant human ovarian carcinoma cell lines to doxorubicin is shown in Table 1. The four resistant cell lines used exhibited either acquired (EMT6/AR1.0, 2780AD, and H69/LX4) or intrinsic (MC26) MDR phenotype as a result of P-gp overexpression and were described previously (21). XR9576 was highly active across the panel of cell lines and gave significant reversal of resistance to doxorubicin at concentrations as low as 10 nm and almost complete reversal at 30 nm. The potency of XR9576 was comparable with that of GG918 and between 10- and 30-fold greater than that of PSC833 in these assays. XR9576 at 100 nm, caused a weak sensitization effect in the parental cell line to doxorubicin, presumably as a result of the basal level of P-gp expression (21). No effect was observed in the other parental cell lines examined.

The ability of XR9576 to potentiate the cytotoxicity of several clinically relevant chemotherapeutic drugs was also examined in the acquired resistant murine (EMT6/AR1.0) and human (2780AD and H69/LX4) cell lines (Table 2). The activity is expressed as EC50 values, the concentration of modulator required to reverse resistance to the various cytotoxic agents by 50% of the maximal level observed in the presence of 100 nm GG918 (see “Materials and Methods”). The results showed that XR9576 was very potent at fully reversing resistance to various agents associated with the MDR phenotype in all three...
Inhibition of P-gp-mediated Efflux and Duration of Modulatory Activity. The ability of XR9576 to inhibit P-gp-mediated efflux and its duration of action was investigated using two MDR substrates, Rh-123 and [3H]daunorubicin, and two cell lines, 2780AD and EMT6AR1.0. Fig. 3 illustrates clearly that XR9576 inhibited the efflux of Rh-123 from the 2780AD cells and that the modulator of the cell lines, with EC50 in the range of 12–38 nM. In these assays, XR9576 was up to 2-fold more potent than GG918 (EC50 range, 14–63 nM; Table 2). Moreover, the presence of 100 nM XR9576 had no effect on the activity of these cytotoxic agents in the parental cell lines (A2780 and H69/P) lacking P-gp. Importantly, XR9576 did not affect the activity of non-MDR cytotoxics, cisplatin and methotrexate, in the resistant cells (data not shown). These results indicate that reversal of resistance was attributable to the inhibition of P-gp function.

Intrinsic Cytotoxicity of XR9576. The intrinsic in vitro toxicity of XR9576 was evaluated to obtain an indication of the potential therapeutic window, i.e., the ability of the modulator to reverse resistance at nontoxic concentrations. This was evaluated in murine (EMT6/AR1.0) and human (2780AD) cell lines using an exposure period equivalent to that used in the cytotoxicity potentiation assays. The mean IC10 (concentration resulting in 10% inhibition of cell growth) for XR9576 in the EMT6/AR1.0 and the 2780AD cell lines was 570 and 2680 nM, respectively, whereas the mean concentration of modulator required for full reversal of resistance to various cytotoxic drugs was 29 and 56 nM, respectively. Thus, XR9576 began to show toxicity to murine and human cell lines only at concentrations 20- to 48-fold greater than those required for complete reversal of drug resistance.

Effect on Accumulation of Daunorubicin. The ability of XR9576 to inhibit P-gp-mediated transport was evaluated by measuring reversal of [3H]daunorubicin accumulation deficit in the MDR EMT6/AR1.0 cells. XR9576 had a significant effect on the accumulation of [3H]daunorubicin in these cells at concentrations as low as 10 nM (Fig. 2). Half-maximal reversal of accumulation deficit was observed at 38 ± 18 nM (n = 4) and near maximal at 300 nM. The potency of XR9576 was comparable with that observed for GG918 (EC50, 48 ± 26 nM; n = 4) but was significantly greater than that observed for CsA (EC50, 440 ± 230 nM) and Vpm (EC50, 580 ± 220 nM; data not shown).

Inhibition of P-gp-mediated Efflux and Duration of Modulatory Activity. The ability of XR9576 to inhibit P-gp-mediated efflux and its duration of action was investigated using two MDR substrates, Rh-123 and [3H]daunorubicin, and two cell lines, 2780AD and EMT6AR1.0. Fig. 3 illustrates clearly that XR9576 inhibited the efflux of Rh-123 from the 2780AD cells and that the modulator
remained effective even when it was excluded from the efflux medium. Maximal level of inhibition was retained up to the end of the efflux period (2 h) when XR9576 was present at 300 and 200 nM concentration during the loading phase only. Even at 100 and 50 nM XR9576, 80 and 50% of the cellular Rh-123 was retained at 2 h. GG918 displayed a similar concentration-dependent effect on efflux as that observed with XR9576 (data not shown). In contrast, 90% of the Rh-123 was effluxed by 1 h after incubation with CsA at 20 μM, a supramaximal concentration (Fig. 3).

Additional efflux studies with [3H]daunorubicin and the EMT6AR1.0 cell line, in which the cells were exposed to 100 nM XR9576, showed that there was no difference in the amount of radiolabel retained in the presence and absence of the modulator during the 22-h efflux phase (Fig. 4). This suggested that the modulator was effective at inhibiting P-gp-mediated transport for at least 22 h after its removal from the medium. At lower concentrations (50 and 30 nM), XR9576 still showed inhibitory activity over an extended period of time, although activity was lost more rapidly as the concentration of XR9576 was decreased (Fig. 4). Again, CsA, even after exposure to very high concentrations (20 μM), displayed only a short duration of inhibition (<60 min) when omitted from the efflux phase (data not shown).

To further evaluate the duration of action of XR9576, EMT6 AR1.0 cells were incubated with XR9576 or various other modulators for 1 h, and then washed and incubated in the absence of modulators for various periods before determining their ability to accumulate [3H]daunorubicin. The results indicated that exposure to 300 nM XR9576 for 1 h was able to fully reverse the accumulation deficit for at least 22 h (Fig. 5). At 50-nM concentration, XR9576 produced slightly less initial accumulation of [3H]daunorubicin; however, 62% of this was retained at 22 h. This long duration of action of XR9576 was in contrast to that observed with 1 μM PSC833 and 20 μM CsA, which lost the majority of their activity (70 and 90%, respectively) within 5 h of removal from the medium (Fig. 5).

**Inhibition of [3H]Azidopine Labeling of P-gp by XR9576.** The ability of XR9576 to interact with P-gp was examined by determining its ability to inhibit the binding of [3H]azidopine, a photoaffinity label.

![Cell associated Rh123 (% T0)](image)

**Fig. 3.** Inhibition of Rh123 efflux from 2780AD cells by XR9576 and CsA. After a 1-h loading period in the presence of Rh123 and XR9576 at 300 nM (●), 200 nM (▲), 100 nM (▼), and 50 nM (○) or CsA at 20 μM (●), the cells were washed and incubated in fresh medium for the indicated times (efflux period). At the end of the efflux periods, the cell-associated Rh123 was measured as described in “Materials and Methods.”

![Cell associated radiodinance (cpm/cell)](image)

**Fig. 4.** Effect of XR9576 on efflux of [3H]daunorubicin from EMT6/AR1.0 cells. EMT6/AR1.0 cells were loaded with [3H]daunorubicin in the presence or absence of the indicated concentrations of XR9576. Cells were then washed and allowed to efflux in the presence (——) or absence (·····) of the modulator for the indicated time periods. Data are plotted relative to cells in the presence of XR9576 at T0. Each point, the mean of quadruplicate measurements.

![Persistence of activity in EMT6/AR1.0 cells after removal of modulators from the medium.](image)

**Fig. 5.** Persistence of activity in EMT6/AR1.0 cells after removal of modulators from the medium. EMT6/AR1.0 cells were exposed to the modulator for 1 h, washed, and incubated in modulator-free medium for the indicated periods before the addition of [3H]daunorubicin and further incubation for 1 h. Cell-associated [3H]daunorubicin was measured along with the cell number as detailed in the “Materials and Methods” section. *Time points* incubation period between removal of the modulator and the addition of [3H]daunorubicin for the accumulation assay. The values represent the mean of triplicate determinations.

![Inhibition of [3H]azidopine labeling of P-gp by XR9576, CsA, and Vpm.](image)

**Fig. 6.** Inhibition of [3H]azidopine labeling of P-gp by XR9576, CsA, and Vpm. Crude H69/LX4 membrane extracts were incubated with modulator and [3H]azidopine prior to UV cross-linking as described in “Materials and Methods.” [3H]Azidopine binding to P-gp was visualized by SDS-PAGE followed by autoradiography.
to this protein. The labeling of cell membranes from H69/LX4 cells with [3H]azidopine revealed a Mr 170,000 P-gp band on polyacrylamide gels, which was absent in membranes from the parental, H69/P cells (Fig. 6). The identity of this band was confirmed by Western blot analysis using C219 P-gp antibody (data not shown). Fig. 6 shows that XR9576 inhibited the labeling of P-gp by [3H]azidopine in a dose-dependent manner. Densitometric evaluation indicated that XR9576 was more effective than CsA and Vpm at inhibiting [3H]azidopine binding. The IC50 values were 0.55, 1.17, and 20 μM for XR9576, CsA, and Vpm, respectively, which reflects the potency order obtained in accumulation and cytotoxic potentiation studies.

**In Vivo Efficacy of XR9576: MC26 Murine Colon Carcinoma Studies.** In vitro in the intrinsically resistant MC26 colon carcinoma cells, exposure to 30 nM XR9576 achieved the maximum potentiation of doxorubicin cytotoxicity (~5-fold; Table 1). This confirms that the MC26 cells have low levels of P-gp-mediated MDR that may be present in many human tumors (19). XR9576 was found to also significantly potentiate the antitumor activity of doxorubicin against s.c. MC26 tumors (Fig. 7). Treatment of MC26 tumor-bearing BALB/c mice with doxorubicin (5 mg/kg, i.v.) or XR9576 (p.o. or i.v.) alone had little or no effect on the growth rate of the tumors (Fig. 7). However, coadministration of XR9576 at 2–8 mg/kg p.o. (Fig. 7, A and B) with doxorubicin reduced the growth rate of the tumors significantly (P < 0.001). The enhancement in antitumor activity of doxorubicin was related to the dose of the modulator, and results from two separate experiments showed that maximum modulatory activity was obtained between a 2- and 4-mg/kg p.o. dose of XR9576. In comparison, coadministration of 9.5 mg/kg GG918 p.o. was required to produce a similar reduction in tumor growth (Fig. 7, A and B). Importantly, there was no substantial or reproducible increase in body weight loss in animals treated with doxorubicin plus XR9576 compared with drug alone groups (Fig. 7, C and D). The observed reduction in body weights in all groups on day 14 may be attributable to tumor-induced cachexia. Similar results were obtained when XR9576 was coadministered i.v. at 2 and 4 mg/kg with doxorubicin (data not shown). The similar potency observed after i.v. and p.o. administration of XR9576 correlates well with the observed high (80%) p.o. bioavailability of the modulator in BALB/c mice (32).

**Efficacy in Human Carcinoma Xenografts.** The ability of XR9576 to reverse P-gp-mediated MDR in vivo was also evaluated using human ovarian (A2780 and 2780AD) and small cell lung (H69/P and H69/LX4) carcinoma xenografts, in combination with various cytotoxic drugs. In vitro, the 2780AD-resistant cell line was found to be about 540-fold less sensitive to paclitaxel than the parental A2780 cell line was. Fig. 8 demonstrates that i.v. paclitaxel (15 mg/kg) alone significantly reduces the growth rate of the parental A2780 xenografts and that coadministration of XR9576 p.o. (12 mg/kg) did not enhance the activity of paclitaxel. Similar results were
obtained after i.v. coadministration of XR9576 (10 mg/kg) with paclitaxel (data not shown). In contrast, paclitaxel had no effect on the growth rate of the resistant 2780AD tumors, and coadministration of XR9576 p.o. or i.v. restored the antitumor activity of the cytotoxic drug (Fig. 9, A and B). Furthermore, this effect was related to the dose of XR9576. Comparison of growth delay observed in the parental and resistant tumor models indicated that coadministration of XR9576 at 12 mg/kg p.o. fully reversed resistance against paclitaxel in the highly resistant 2780AD tumors. Importantly, the effective combination schedules were well tolerated as indicated by changes in body weights compared with those in drug-alone groups. The enhancement in antitumor activity of paclitaxel by i.v. XR9576 was comparable with that observed with i.v. GG918, thus confirming the in vitro results (data not shown).

The H69/LX4 SCLC cell line in vitro exhibited 290- and 900-fold resistance to etoposide and vincristine, respectively. Studies with the H69/LX4 xenografts showed that the administration of XR9576 i.v. or p.o. with etoposide i.v. (30 mg/kg) significantly reduced tumor growth rate compared with that with either compound alone (Fig. 10). The potentiation of etoposide antitumor activity was related to the p.o. dose of XR9576, and comparison with the response of the parental xenografts to etoposide alone suggested that maximum reversal of resistance was achieved by the administration of 6 and 12 mg/kg of the modulator. The efficacious combination schedules were well tolerated as indicated by changes in body weights compared with those in drug-alone groups (data not shown). Similarly, the administration of XR9576 p.o. (12 mg/kg) significantly potentiated the antitumor activity of vincristine (0.5 mg/kg, i.v.) against the H69/LX4 xenografts (Fig. 11), and, again, the combination schedules were well tolerated.

Lack of Effect on Pharmacokinetics of Paclitaxel. Administration of XR9576 at an efficacious dose (10 mg/kg, i.v.) 15 min prior to paclitaxel (10 mg/kg, i.v.) in rats had no significant effect on plasma pharmacokinetic parameters of paclitaxel (Fig. 12). The $AUC_{0-48h}$ over 48 h for paclitaxel was similar in the groups treated with or without XR9576 (6500 and 5681 ng·h/ml, respectively). Also, no significant differences were observed in the elimination half-life (13.5 and 13.2 h) or the $C_{max}$ values (8208 and 8642 ng/ml) for paclitaxel in the two groups, respectively.

**DISCUSSION**

The present in vitro and in vivo studies demonstrate that XR9576 is a selective and very potent modulator of P-gp-mediated MDR. The in vitro potency was evaluated by several assays (including potentiation of cytotoxic drug activity, enhancement of drug uptake, and inhibition of drug efflux) using a panel of murine and human cell lines with different degrees of P-gp expression. In the potentiation assay, full reversal of resistance in all of the cell lines to all of the major classes of MDR drugs was achieved in the presence of 25–80 nm XR9576. Direct comparison with several modulators in this and other assays demonstrated that XR9576 is one of the most potent modulators described to date. It was up to 2-fold more potent than GG918, >15-fold more potent than PSC833, and several logs more potent than the first generation modulators, CsA and Vpm. In addition, these studies clearly indicated that reversal of MDR by XR9576 was through selective and potent inhibition of P-gp function. For example, in contrast to the modulatory activity in the resistant cell lines, XR9576 had no significant effect on cytotoxic drug activity in non-P-gp-expressing parental cell lines. Neither did it affect the cytotoxicity of non-P-gp substrates such as cisplatin and methotrexate. Moreover, the concentration of XR9576 required to fully reverse drug resistance in vitro was 20- to 48-fold lower than the concentration at
which any toxicity (IC\textsubscript{10}) was observed in the cell lines. Further confirmation of selectivity of XR9576 for P-gp was provided by the fact that the modulator, even at very high concentrations (50 \textmu M), did not inhibit MRP function, unlike CsA and Vpm (33).

Several studies were performed to examine the inhibition of P-gp-mediated transport by XR9576 and to determine the interaction of the modulator with this protein. XR9576 was very potent at reversing the accumulation deficit and at blocking the efflux of P-gp substrates, daunorubicin and Rh123, from P-gp-overexpressing cell lines. The finding that drug efflux and accumulation was not affected in the parental cell lines that lacked P-gp, indicated that reversal of drug resistance by XR9576 was probably attributable to the inhibition of P-gp-mediated efflux. The inhibition of drug efflux as a result of direct interaction of XR9576 with P-gp was implied by the potent displacement of a photoaffinity label, [\textsuperscript{3}H]azidopine. The direct interaction with P-gp was confirmed by equilibrium-binding studies with [\textsuperscript{3}H]XR9576 and membranes isolated from P-gp overexpressing MDR Chinese hamster ovary cells, which demonstrated that XR9576 binds to P-gp with a very high affinity, \(K_d\), 5.1 nM (34). The displacement and binding studies confirmed the superior potency of XR9576 compared with CsA and Vpm. These results suggest that XR9576 binds tightly to a site(s) distinct from, but linked to, those of the cytotoxics and modulators that are P-gp substrates. The efflux and persistence assays also showed that XR9576 has a long duration of action; the modulator inhibited P-gp function in cells in excess of 22 h after a short exposure to \(\geq 100\) nM XR9576 and subsequent removal from the incubation medium. In contrast, PSC 833 (1 \mu M), CsA (20 \mu M) and Vpm (50 \mu M) lost the majority of their P-gp inhibition activity within 1–5 h after removal of the incubation medium. These data along with the observations that accumulation of [\textsuperscript{3}H]XR9576 in sensitive (Aux B1) and MDR (CH B30) Chinese hamster ovary cells was the same and was unaffected by coexposure to GG918 (34) suggest that XR9576 is not a substrate of P-gp. This property may give XR9576 significant advantage for clinical administration because it should be able to reverse P-gp-mediated drug resistance in tumors over prolonged periods after exposure to low nM concentrations. Studies in \textit{mdr1a} and \textit{mdr1b} knockout mice have suggested that P-gp is not essential for normal function but is required for protection against xenobiotics because it can influence their pharmacokinetics/pharmacodynamics (35). Therefore, prolonged inhibition of P-gp by XR9576 may affect the pharmacokinetics and toxicity of anticancer agents. However, XR9576 had no substantial effect on plasma pharmacokinetics of paclitaxel or on the toxicity of various cytotoxic drugs at efficacious doses. Similar results have been reported for other specific modulators such as GG918 (19) and LY335979 (20), whereas
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REFERENCES


**In Vitro and in Vivo** Reversal of P-Glycoprotein-mediated Multidrug Resistance by a Novel Potent Modulator, XR9576


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