

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, 2780AD) and murine (EMT6 AR1.0, MC26) MDR cell lines. XR9576 potentiated the cytotoxicity of several drugs including doxorubicin, paclitaxel, etoposide, and vincristine; 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]daunorubicin 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]azidopine 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 potentiation 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 (2780AD, 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_r 170,000 plasma membrane glycoprotein (P-gp). This *MDR1* 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, *mdr1a* and *mdr1b*, have been reported to play a similar role in drug resistance to that of the *MDR1* gene in humans (5). Studies in the *mdr1a* and *mdr1b* 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 cysteinyl 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 *MDR1* 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, MDR protein; Rh123, rhodamine 123; 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 analogue, PSC 833 (17), VX-710 (18), the acridone carboxamide derivative GF120918 (GG918; Ref. 19), the substituted dibenzosuberane molecule LY335979 (20), and the dike-topiperazine 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 dexverapamil, 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 to 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 dexverapamil (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 65- 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 attrib-

utable 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 Pharmatalia (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) D-(+)-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]azidopine (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 6×10^2 to 2×10^4 /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 control (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 cells 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 nM GG918.

Intrinsic Cytotoxicity

Cells (EMT6 AR1.0 8×10^2 /well; A2780 5×10^3 /well; 2780AD 6×10^3 /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 IC₁₀ 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

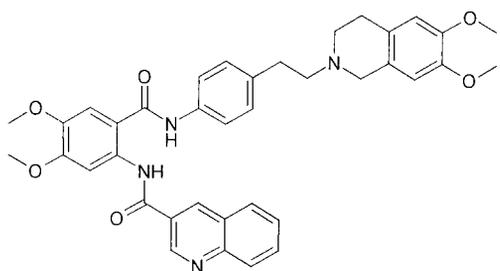


Fig. 1. Structure of XR9576.

density of 10^6 /ml of culture medium. Modulators were added at fixed concentrations immediately before the addition of Rh-123 (0.8 μ g/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 ($\sim 10^6$ 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 T_0 was plotted against time. Similar experiments extended over a 22-h period using [3 H]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 [3 H]daunorubicin was assessed as described for the accumulation assay. Graphs were plotted of cell-associated radioactivity/ 10^5 cells against time, where T_0 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 μ l) were incubated in wells of a V-well polyvinyl chloride microtiter plate, with increasing concentrations of modulator (10 μ l) 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 μ l of [3 H]azidopine prediluted in labeling buffer. The final concentration of [3 H]azidopine in the incubation mixture was 1 μ M. 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 Autofluor (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_r 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 BALB/c mice (day 0). The animals were then randomized, 24 h later, into groups of 15–18 and treated once with various regimens. XR9576 or vehicle was administered either i.v. via a lateral tail vein or p.o. with doxorubicin (5 mg/kg) or vehicle i.v. The modulator was administered either i.v. at 2–4 mg/kg (10 ml/kg) at the same time as doxorubicin or p.o. at 2–8 mg/kg (10 ml/kg) 1 h before the cytotoxic drug. GG918 was administered p.o. 1 h before doxorubicin. All of the animals were weighed twice weekly. The animals were killed by cervical dislocation on day 14, and the tumors were excised and weighed. The data were analyzed by Student's *t* test.

Human Carcinoma Xenografts. The efficacy of XR9576 was also evaluated using MDR human carcinoma xenografts. Studies in parental (A2780) and resistant (2780AD) human ovarian carcinoma xenografts were performed using a modification of the method of Plumb *et al.* (31). Briefly, A2780 and 2780AD cells grown *in vitro* were harvested by exposure to trypsin-EDTA, washed three times in PBS, and implanted s.c. in female nude mice (2×10^6 cells in 0.1 ml). When the tumors had reached a mean diameter of 0.5–1.0 cm,

the animals were randomized into groups of 6 and treated with various regimens on days 0 (start of treatment), 2, and 4. XR9576 or vehicle was administered i.v. 1 h before or p.o. 2 h before paclitaxel (15 mg/ml; formulated in 5% cremophor EL and 5% ethanol in (5% w/v) dextrose) or vehicle i.v. Administration of all of the i.v. compounds was via a lateral tail vein. The efficacy of GG918 was also evaluated after i.v. administration. Tumor volume and body weights were recorded as described previously (31), and an ANOVA model was used to assess significance between treatment groups.

The ability of XR9576 to potentiate the antitumor activity of etoposide and vincristine was evaluated using the H69/LX4 SCLC xenografts. The xenografts were established in female nude mice by s.c. implantation of $7\text{--}8 \times 10^6$ cells resuspended in PBS (0.1 ml) after harvesting from *in vitro* culture. When the tumors had reached a mean diameter of about 0.6 cm, the animals were randomized into groups and treated on days 0, 5, and 10 with XR9576 (i.v. or p.o.) and etoposide (30 mg/kg) or vincristine (0.5 mg/kg) i.v. The modulator and the cytotoxic drug were mixed together immediately prior to i.v. administration by the caudal vein. Control animals were treated with vehicle. Tumor volumes and body weights were recorded as described previously (22).

Pharmacokinetic Studies. Male CD rats (3 animals per time point) were dosed i.v. with paclitaxel alone [15 min infusion at 10 mg/kg in Tween 80:ethanol:5% dextrose (5:10:85% v/v/v)] or in combination with XR9576 (10 mg/kg). XR9576 was administered as a bolus (i.v.) dose 15 min before infusion of paclitaxel. Blood samples were collected by cardiac puncture using heparinized syringes at various times between 0.083 and 48 h and were centrifuged to prepare plasma, which was stored at -20°C until analysis. Paclitaxel concentration in plasma samples was measured by a LC-MS/MS assay. Paclitaxel and Baccatin III (internal standard) were extracted from 100 μ l of rat plasma using liquid-liquid extraction procedure with diethyl ether (7 ml). The plasma samples were mixed with diethyl ether and centrifuged, and the organic layer was evaporated to dryness under nitrogen. The residues were reconstituted in 100 μ l of mobile phase and aliquots (5–15 μ l) were analyzed using an isocratic LC-MS/MS. The chromatography was carried out using a Hypersil C_1 column [5 μ m \times 100 mm \times 2.1 mm (i.d.)] with acetonitrile:0.1% aqueous formic acid (50:50 v/v) mobile phase at a flow rate of 0.2 ml/min. Detection was via MS/MS in multiple reaction monitoring mode. The area under the concentration time curve (AUC) and half life of paclitaxel in plasma was calculated by noncompartmental analysis using WinNonlin software (Scientific Consulting Inc.). Statistical analysis was performed using an ANOVA model.

RESULTS

Modulation of Drug Resistance in MDR Cell Lines. The ability of XR9576, GG918, and PSC833 to reverse resistance of various 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 EMT6/P 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 EC_{50} 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

Table 1 Sensitization of MDR cell lines to doxorubicin by XR9576, GG918, and PSC 833^a

	Drug-sensitive cells			
	EMT6/P	H69/P	A2780	
Doxorubicin IC ₅₀ (nM)				
With no modulator	30.5	45.2	13	
With XR9576 100 nM	5	49.6	11	
	Drug-resistant cells			
	EMT6/AR1.0	MC26	H69/LX4	2780AD
Doxorubicin IC ₅₀ (nM)				
With no modulator	1019	35.8	972	927.4
With XR9576				
3 nM	598	25.6	523.6	1040
10 nM	24.7	17.5	117.4	73
30 nM	7.1	7.7	43.8	20
50 nM	7.4	7.2	42.4	14
100 nM	6.8	6.7	43.1	13
With GG918				
3 nM	484.2	ND ^b	282.1	647
10 nM	38	29.6	116.5	51
30 nM	9.2	17	45.5	18
50 nM	7.8	10.4	43.4	14
100 nM	7.1	7.6	43.1	14
With PSC 833				
50 nM	89.2	25.4	444.3	678
100 nM	40.2	20.4	233.8	627
300 nM	30.5	8.3	58	58
500 nM	28	7.9	36.8	28
1000 nM	26.9	ND	41.1	13 ^c

^aThe values represent results from a single potentiation assay performed in quadruplicate wells as described in "Materials and Methods" and is representative of at least three separate experiments.

^b ND, not done.

^c Toxic, modulator alone inhibited cell growth by >10% of control value.

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

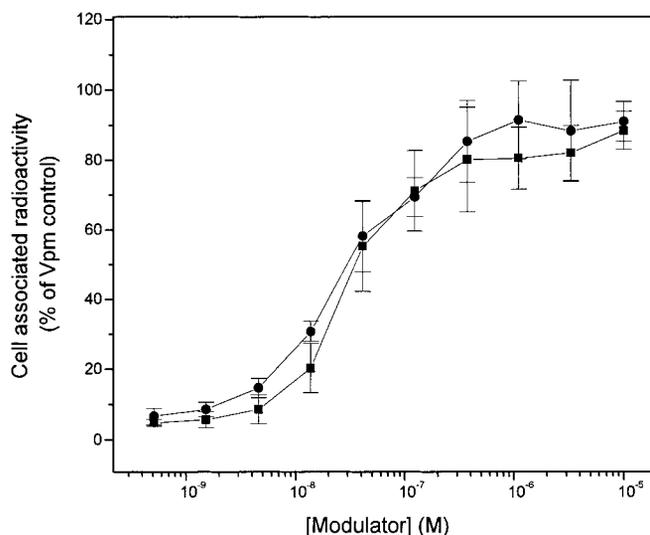


Fig. 2. The effect of modulators on the accumulation of [³H]daunorubicin by EMT6/AR1.0 cells. The cells were incubated with various concentrations of XR9576 (●), GG918 (■), or 100 μM Vpm plus [³H]daunorubicin for 1 h before measuring cell-associated radioactivity as described in "Materials and Methods." Results are expressed as a percentage of that seen in the presence of 100 μM Vpm. Data plotted, the mean ± SD of quadruplicate data points.

Table 2 Potency of XR9576 and GG918 in enhancing cytotoxicity of various drugs in MDR cell lines^a

Cytotoxic drug	Modulator EC ₅₀ ^b (nM) for potentiating cytotoxicity					
	EMT6/AR1.0		H69/LX4		2780AD	
	XR9576	GG918	XR9576	GG918	XR9576	GG918
Doxorubicin	15 ± 2	20 ± 6	16 ± 5	26 ± 21	20 ± 3	32 ± 18
Vincristine	22 ^c	36 ^c	38 ± 3	34 ^d	30 ± 10	49 ^d
Paclitaxel	12 ^d	35 ^d	22 ± 16	63 ^d	34 ± 10	51 ^d
Colchicine	17 ± 11	14 ± 10	18 ± 1	34 ± 5	ND ^e	ND
Actinomycin D	12 ± 9	28 ± 13	34 ± 16	34 ± 6	ND	ND
Etoposide	14 ^d	19 ^d	18 ± 10	44 ± 22	ND	ND

^a Values are mean ± SD, where *n* ≥ 3 separate experiments except where indicated.

^b Concentration of modulator required to reverse resistance to cytotoxic drug by 50%. For method see "Drug Potentiation Assay" section in "Material and Methods."

^c Value from a single experiment.

^d Mean values from two experiments.

^e ND, not determined.

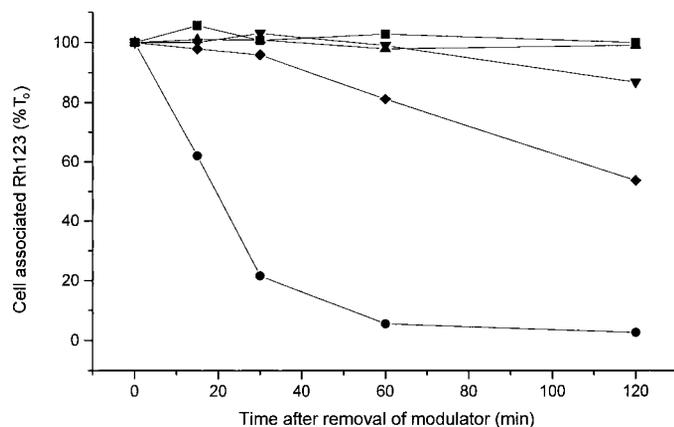


Fig. 3. Inhibition of Rh123 efflux from 2780AD cells by XR9576 and CsA. After a 1-h loading period in the presence of Rh123 and XR9576 at 300 nM (■), 200 nM (▲), 100 nM (●), and 50 nM (◆) or CsA at 20 μ M (●), the cells were washed and incubated in fresh medium for the indicated times (efflux period).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

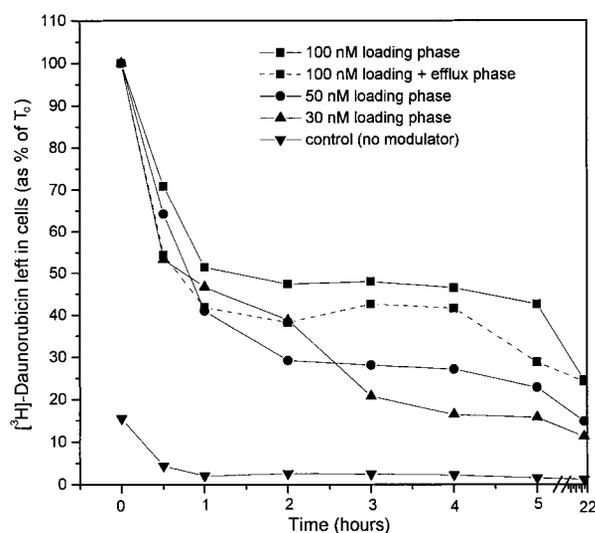


Fig. 4. Effect of XR9576 on efflux of [3 H]daunorubicin from EMT6/AR1.0 cells. EMT6/AR1.0 cells were loaded with [3 H]daunorubicin in the presence or absence of the indicated concentrations of XR9576. Cells were then washed and allowed to efflux in the presence (—) or absence (---) of the modulator for the indicated time periods. Data are plotted relative to cells in the presence of XR9576 at T_0 . Each point, the mean of quadruplicate measurements.

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 μ M, a supramaximal concentration (Fig. 3).

Additional efflux studies with [3 H]daunorubicin and the EMT6/AR1.0 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 concen-

tration of XR9576 was decreased (Fig. 4). Again, CsA, even after exposure to very high concentrations (20 μ M), 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 [3 H]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 [3 H]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 μ M PSC833 and 20 μ M CsA, which lost the majority of their activity (70 and 90%, respectively) within 5 h of removal from the medium (Fig. 5).

Inhibition of [3 H]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 [3 H]azidopine, a photoaffinity label,

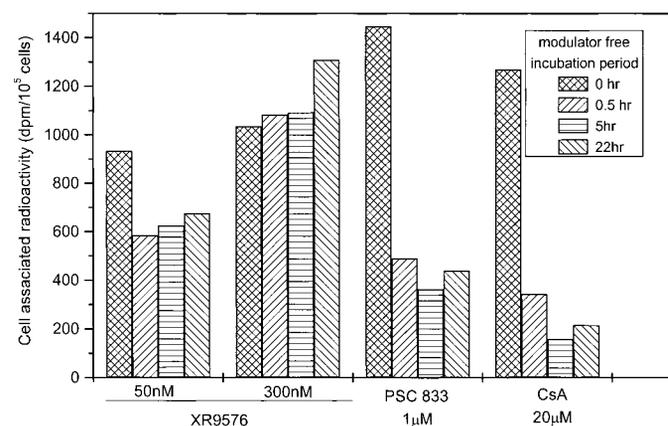


Fig. 5. Persistence of activity in EMT6/AR1.0 cells after removal of modulators from the medium. EMT6/AR1.0 cells were exposed to the modulator for 1 h, washed, and incubated in modulator-free medium for the indicated periods before the addition of [3 H]daunorubicin and further incubation for 1 h. Cell-associated [3 H]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 [3 H]daunorubicin for the accumulation assay. The values represent the mean of triplicate determinations.

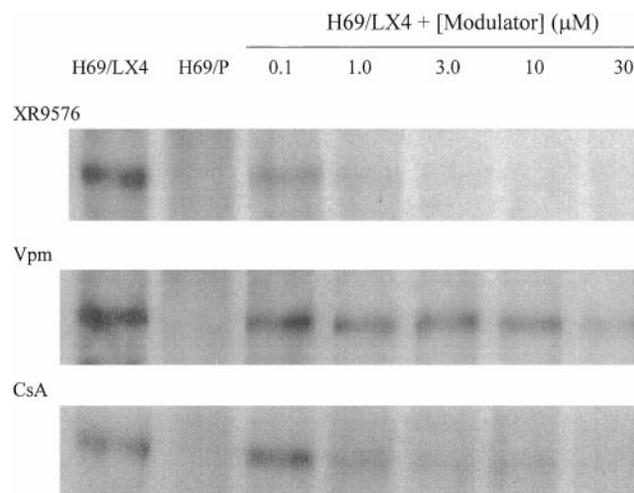


Fig. 6. Inhibition of [3 H]azidopine labeling of P-gp by XR9576, CsA, and Vpm. Crude H69/LX4 membrane extracts were incubated with modulator and [3 H]azidopine prior to UV cross-linking as described in "Materials and Methods." [3 H]azidopine binding to P-gp was visualized by SDS-PAGE followed by autoradiography.

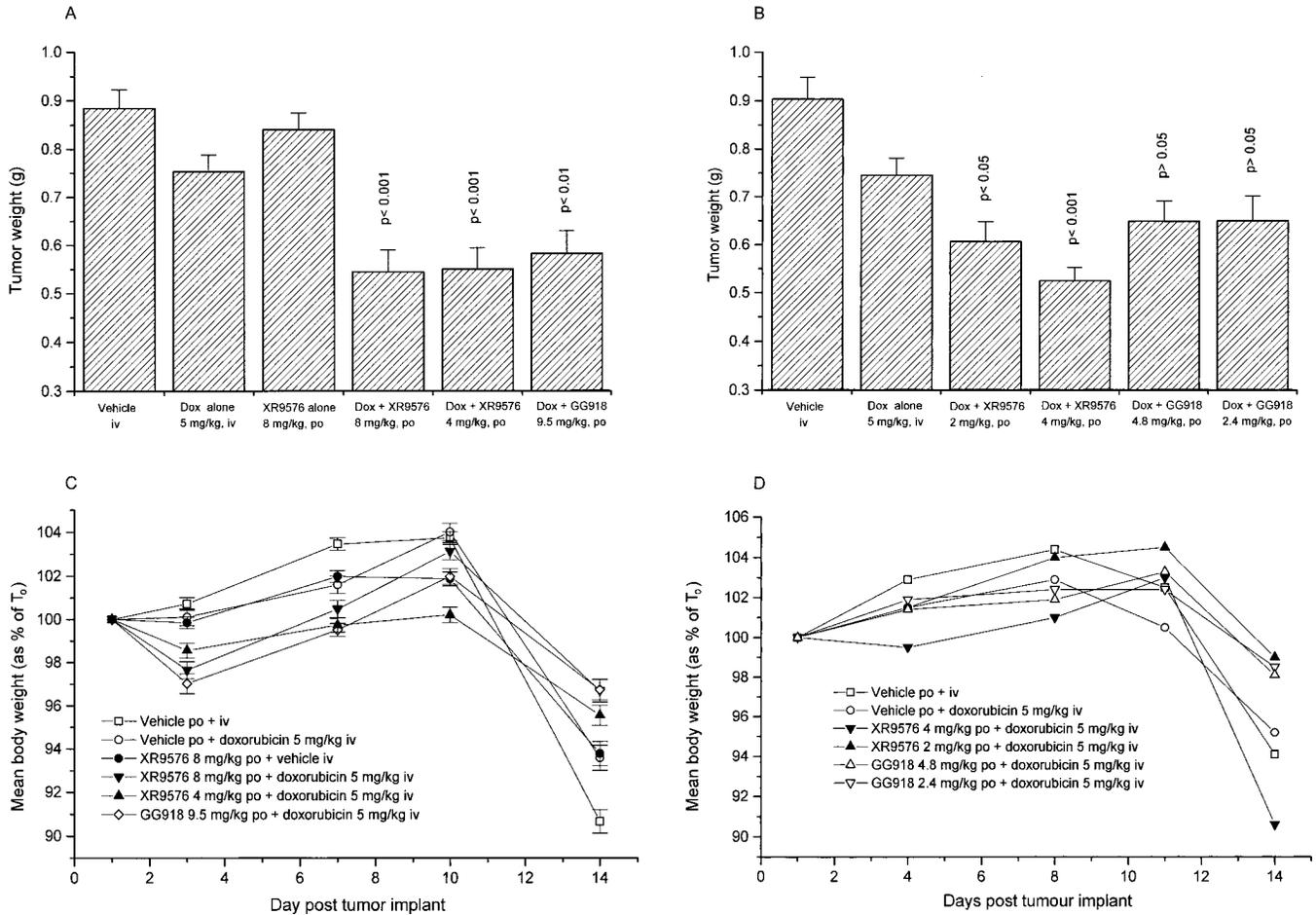


Fig. 7. A and B, effect of p.o. administration of XR9576 and GG918 on the antitumor activity of doxorubicin against the MC26 mouse colon carcinoma. Tumor slurry was implanted s.c., and 24 h later, the animals were randomized and treated once only. The modulator was administered p.o. 1 h before doxorubicin (5 mg/kg) i.v. Animals were weighed twice a week, and the tumor weight was determined on day 14 after implantation as detailed in "Materials and Methods." The mean tumor weights \pm SE, from groups of 15–18 animals are shown. The *P*s refer to comparison of doxorubicin-alone group with the combination-schedule groups. Vehicle, 5% dextrose; Dox, doxorubicin. C and D, body weights for the various treatment groups after tumor implantation.

to this protein. The labeling of cell membranes from H69/LX4 cells with [³H]azidopine revealed a M_r 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 IC_{50} values were 0.55, 1.17, and 20 μ M 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 *in vivo* (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

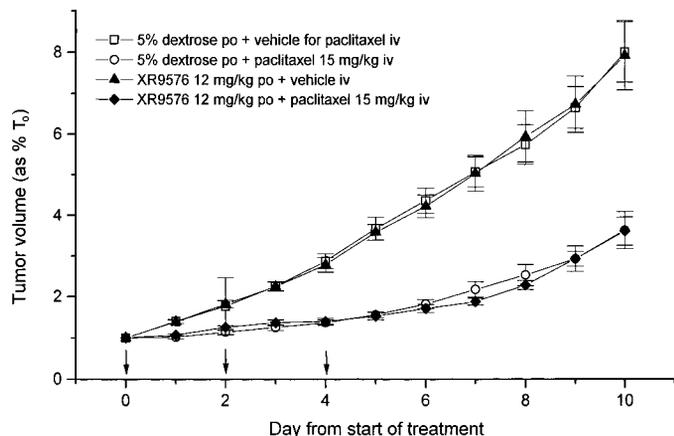


Fig. 8. Effect of p.o. administration of XR9576 on the antitumor activity of paclitaxel in mice bearing the sensitive A2780 ovarian carcinoma xenografts. Nude mice bearing established s.c. A2780 tumors were treated on day 0, 2, and 4 (arrows) as described in "Materials and Methods." The modulator was administered 2 h before the cytotoxic agent. The values represent mean \pm SE of six animals per group. The growth rate of the tumor was significantly reduced ($P < 0.01$) by administration of paclitaxel and by paclitaxel in combination with XR9576. There was no difference in the response between these two groups.

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_{0-1} 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

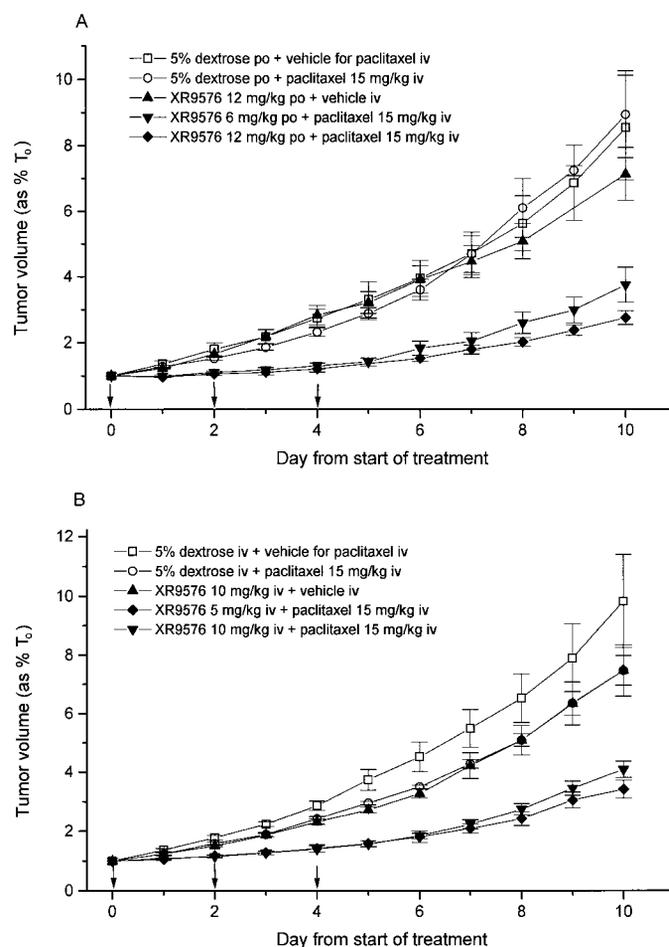


Fig. 9. Restoration of paclitaxel antitumor effect on MDR 2780AD ovarian carcinoma xenografts by XR9576. Effects of p.o. (A) and i.v. (B) administration of XR9576 in combination with paclitaxel on the growth rate of the tumors. Animals bearing s.c. tumors were treated three times at 2-day intervals. Arrows, days of treatment. The modulator was administered p.o. 2 h before and i.v. 1 h before paclitaxel. The values represent mean \pm SE of six animals per group. The growth rate of the tumor was significantly reduced ($P < 0.01$) by coadministration of XR9576 with paclitaxel compared with drug-alone or vehicle-treated groups.

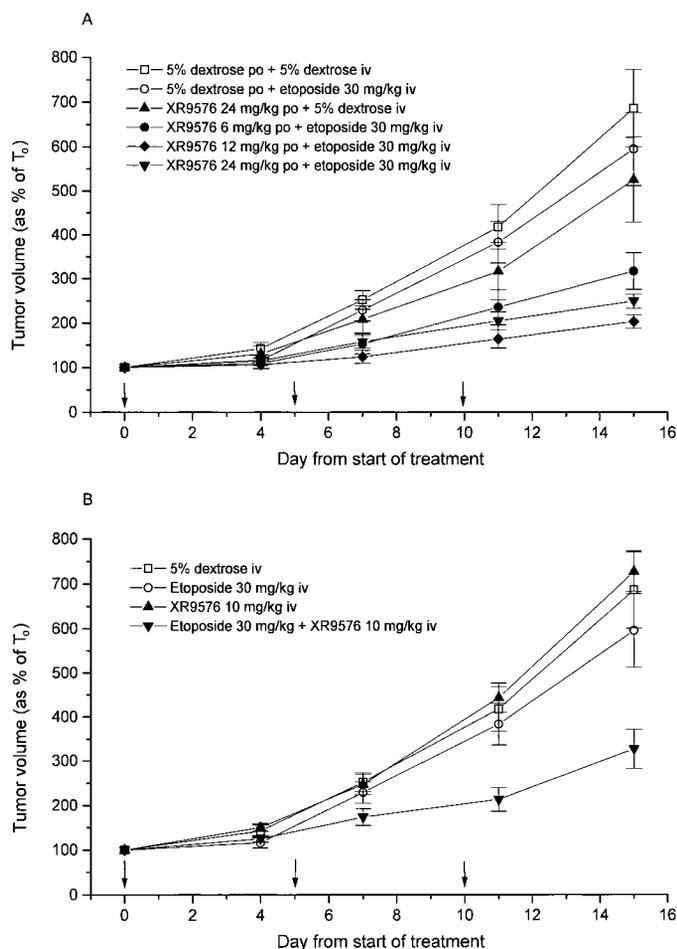


Fig. 10. Sensitization of etoposide antitumor effect on H69/LX4 SCLC xenografts by XR9576. Effects of p.o. (A) and i.v. (B) administration of XR9576 in combination with etoposide on the growth rate of tumors. Animals bearing s.c. tumors were treated three times at 5-day intervals. Arrows, the days of treatment. The modulator was administered p.o. 2 h before and i.v. at the same time as etoposide. Each group consisted of eight animals, and the values represent mean \pm SD. The growth rate of the tumors was significantly ($P < 0.01$, Mann-Whitney U test) reduced by coadministration of XR9576, either p.o. or i.v. with etoposide compared with drug-alone or vehicle-treated groups.

which any toxicity (IC_{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 ($50 \mu\text{M}$), 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, [^3H]azidopine. The direct interaction with P-gp was confirmed by equilibrium-binding studies with [^3H]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 ≥ 100 nM XR9576 and subsequent removal from the incubation medium. In contrast, PSC 833 ($1 \mu\text{M}$), CsA ($20 \mu\text{M}$) and Vpm ($50 \mu\text{M}$) 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 [^3H]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 *mdr1a* and *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

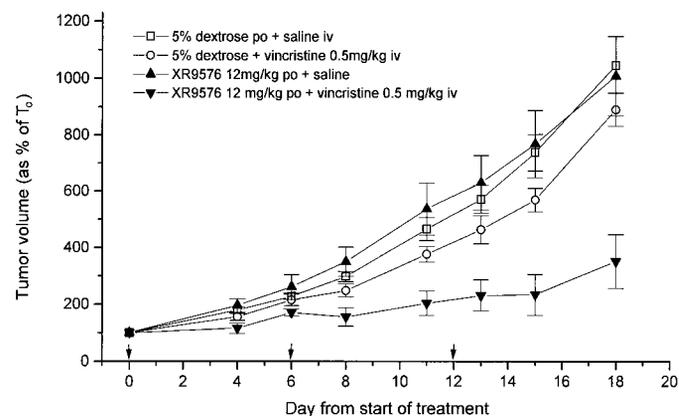


Fig. 11. Effect of XR9576 administered p.o. on the activity of vincristine in mice bearing H69/LX4 SCLC xenografts. Animals bearing s.c. tumors were treated 3 times at 6-day intervals. Arrows, the days of treatment. The modulator was administered 2 h before the cytotoxic agent. Each group consisted of five to seven animals. The growth rate of the tumors was significantly ($P < 0.01$, Mann-Whitney U test) reduced by administration of XR9576 with vincristine compared with cytotoxic-drug-alone or vehicle-treated groups.

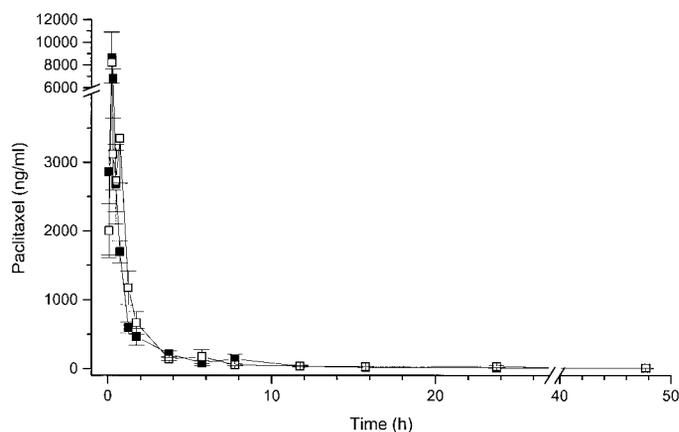


Fig. 12. Effect of XR9576 administration on plasma paclitaxel concentration. Male CD rats were dosed i.v. with paclitaxel alone (■; 10 mg/kg, 15-min infusion) or with XR9576 (□; 10 mg/kg, i.v.). The modulator was administered 15 min before paclitaxel, and plasma samples were collected and analyzed by LC-MS/MS as described in the "Materials and Methods" section. The values represent mean \pm SD of three animals per group.

nonspecific modulators such as VX-710 (24), CsA, and PSC 833 (25) have shown significant enhancement of pharmacokinetics and toxicity of cytotoxic drugs. If these results translate to humans, then XR9576 could be used in combination with a normal dose of cytotoxic agents in front-line therapy regimen. It has been proposed that such a regimen may improve the overall response to therapy by preventing the emergence of drug-resistant cells expressing either P-gp or other mechanisms of resistance in response to exposure to cytotoxic drugs (reviewed in Refs. 15 and 36).

The promising activity of XR9576 demonstrated *in vitro* was confirmed in *in vivo* efficacy studies. The modulator exhibited potent activity after i.v. and p.o. administration in mice bearing MDR murine and human tumor xenografts, restoring antitumor activity of several drugs without an apparent increase in toxicity. Against the intrinsically resistant MC26 murine colon tumor, which exhibits a low level of P-gp-mediated resistance (29), coadministration of XR9576 i.v. or p.o. significantly ($P < 0.001$) increased the antitumor activity of doxorubicin in a dose-related manner without an apparent increase in toxicity. Moreover, maximum modulatory activity was observed after a single administration of a low dose of XR9576 (between 2 and 4 mg/kg) by either route. Oral bioavailability of the modulator in BALB/c mice is ~80% (32), thus explaining the result that both routes of administration appear equipotent. Even in mice bearing human carcinoma xenografts exhibiting high levels of P-gp-mediated acquired MDR, coadministration of 6–12 mg/kg XR9576 p.o. was sufficient to restore the antitumor activity of paclitaxel, etoposide, and vincristine. All of the efficacious combination schedules with XR9576 appeared to be well tolerated, as indicated by the minimal changes in body weights of the various treatment groups. Furthermore, XR9576 did not enhance the activity of paclitaxel against the parental tumor. These observations along with the lack of effect on plasma pharmacokinetics of paclitaxel indicate that *in vivo* reversal of resistance is attributable to inhibition of P-gp function and is unlikely to be caused by an increase in the pharmacokinetics of the cytotoxic drug. The effect of XR9576 on pharmacokinetics of the other cytotoxic drugs is being investigated and will be reported separately.

In conclusion, XR9576 is one of the most potent *in vitro* and *in vivo* modulators of P-gp-mediated MDR described to date. In addition, it is selective, binds to P-gp with a high affinity, and has a long duration of action. XR9576 also exhibits good oral bioavailability and potent i.v. and p.o. activity, restoring sensitivity of MDR murine and human tumors to a range of chemotherapeutic drugs at well-tolerated doses. Moreover, XR9576 does not alter plasma pharmacokinetics of paclitaxel and hence the enhancement in its activity against MDR tumors by the modulator does not appear to be related to an increase in plasma concentrations of the drug. These data indicate that it may be possible to use XR9576 as part of a first-line therapy with paclitaxel in the clinic. This preclinical profile for XR9576 supported the progression of the modulator into Phase I clinical trials in healthy volunteers. These studies have included measurement of P-gp activity in CD56⁺ lymphocytes as a surrogate marker of efficacy. Complete inhibition of P-gp activity by XR9576 was observed in excess of 24 h after a single i.v. dose (2.0 mg/kg), which supports a long duration of action for this modulator (37). Thus, the properties of XR9576 and the Phase I studies indicate that it holds great promise for the treatment of P-gp-mediated MDR cancers.

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