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The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein

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1 The kinetics and nature of equilibrium binding were used to characterize the molecular interaction of the anthranilic acid derivative [^{3}H]-XR9576 with the multidrug resistance P-glycoprotein (P-gp). XR9576 displayed specific high-affinity binding to P-gp ($B_{max} = 275$ pmol mg^{-1}, $K_d = 5.1$ nM). The transport substrates [^{3}H]-vinblastine and [^{3}H]-paclitaxel displayed 4 fold and 20 fold lower affinity respectively for P-gp. The duration of action of XR9576 with P-gp was increased in comparison to that of vinblastine which displayed a slower rate of association and a faster dissociation rate.

2 The relative affinities of several modulators and transport substrates to interact with P-gp were determined from displacement drug equilibrium binding assays. Vinblastine and paclitaxel could only fractionally displace [^{3}H]-XR9576 binding, displaying K_i values significantly different from their measured K_d values. This suggests a non-competitive interaction between XR9576 and the P-gp substrates vinblastine and paclitaxel.

3 XR9576 was shown to be a potent modulator of P-gp mediated [^{3}H]-vinblastine and [^{3}H]-paclitaxel transport as it increased the steady-state accumulation of these cytotoxics in CH^{r}B30 cells to levels observed in non-P-gp-expressing AuxB1 cells ($EC_{50} = 487 \pm 50$ nM). This inhibition of drug transport is not mediated through competition for transport since [^{3}H]-XR9576 accumulation was not influenced by P-gp expression or function.

4 These results demonstrate that the P-gp modulator XR9576 exhibits greater selectivity, duration of inhibition and potency of interaction with this transporter than any other reported modulators. Several lines of evidence suggest that XR9576 inhibits P-gp function by binding at a site which is distinct from the site of interaction of transport substrates. The two sites may be classified as serving modulatory or transport functions.

Keywords: P-glycoprotein; multidrug resistance; drug binding sites; high-affinity reversal agent

Abbreviations: ABC, ATP binding cassette; Aux B1, Chinese hamster ovary parental drug-sensitive cell-line; CH^{r}B30, Chinese hamster ovary drug resistant cell-line; MDR, multidrug resistance; MRP, multidrug resistance protein

Introduction

The successful chemotherapy of many solid and haematological tumours has been severely compromised by intrinsic or acquired resistance to a wide range of chemically and functionally distinct cytotoxic drugs, a phenomenon known as multidrug resistance (MDR). A key factor mediating MDR in cancer cells is the overexpression of the 180 kDa P-glycoprotein (P-gp) on the surface of resistant cells. P-gp acts as an energy-dependent, and apparently non-specific, drug efflux pump which reduces the intracellular concentration of cytotoxics, thereby conferring the MDR phenotype.

Due to the apparent lack of substrate specificity, a major focus of research in the field has been the screening of established pharmacological agents for their ability to reverse the MDR phenotype by blocking P-gp mediated transport. A plethora of compounds has been proposed as potential MDR modulators or chemosensitizers and include calcium channel antagonists (verapamil and nicardipine), calmodulin inhibitors (trifluoperazine), immunosuppressants (cyclosporin A) and steroids (progesterone). Verapamil has been one of the most extensively tested in the clinic (Dalton *et al*., 1989; Miller *et al*., 1991) being used in conjunction with combination chemotherapy strategies. However, it has been of limited success due to the cardiac toxicity associated with the high plasma levels required to effectively reverse the MDR phenotype. The need to focus on agents specifically targeting P-gp was recognized. A second generation of reversal agent emerged (e.g. dexniguldipine and dexverapamil), comprising of chemically modified versions of earlier compounds, which elicited fewer side effects. However, the efficacy of some of these agents in blocking P-gp was compromised by the failure to reach high enough plasma levels of drug.

A third generation of P-gp-related MDR inhibitor has been borne from a recognition of the need to design compounds lacking other pharmacological effects and, thereby confer greater selectivity and specificity for P-glycoprotein. The first such compounds described were the acridonecarboxamide derivative GF120918 (GG918) (Hyafil *et al*., 1993) and PSC833 (Boesch *et al*., 1991) which were developed from a chemical programme designed specifically to make progressive modifications of prototype MDR inhibitors. These modulators displayed efficacy which was 100–200 times more potent than modulators such as verapamil (Tsuro *et al*., 1981), tamoxifen (Ramu *et al*., 1984) and cyclosporin A. More recently, the diketopiperazine derivative XR9051 (Dale *et al*., 1998) and the anthranilic acid derivative XR9576 (Mistry *et al*., 1998;

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Stewart *et al*., 1998) have also shown an ability to reverse MDR. XR9051 and XR9576 have been demonstrated to completely reverse the resistance of P-gp expressing cell lines to a wide range of cytotoxic agents including colchicine, doxorubicin and vinblastine (Mistry *et al*., 1998; Stewart *et al*., 1998). The emergence of compounds displaying greater selectivity and higher affinity for P-gp (see Figure 1) offer hope in combating MDR, without the adverse side effects associated with less potent inhibitors.

Despite the obvious clinical benefits of using agents displaying greater selectivity and potency for P-gp, they can also be used as tools to probe the nature of drug interaction sites on this protein. There exists a dearth of information concerning the mechanism underlying the transport function of P-gp and the seemingly promiscuous nature of its drug-handling ability. How is binding of substrate coupled to ATP hydrolysis to generate transport? How do MDR reversers interact with the protein to block its drug efflux function? Are there distinct sites on P-gp for transported substrates and modulators? In fact there is evidence emerging suggesting a dichotomy of interaction sites in terms of function (i.e. distinct sites mediating drug efflux and modulatory effects respectively), and that these sites can communicate with each other (Ferry *et al*., 1992; Martin *et al*., 1997; Pascaud *et al*., 1998). It is hoped that the newly available high-affinity inhibitors can be used to address these outstanding but fundamental issues concerning the mechanism of action of P-gp.

The purpose of the present work is to characterize the molecular interaction of the anthranilic acid derivative XR9576 with P-gp in cultured cells. The interaction of this compound with P-gp has also been compared with that of the known transported substrates vinblastine and paclitaxel and with other modulators. The results of this study provide important information suggesting an interaction with P-gp at a site that is distinct from that of vinblastine and paclitaxel. Based on the findings of this study, a multi-site model for P-gp drug interaction is proposed.

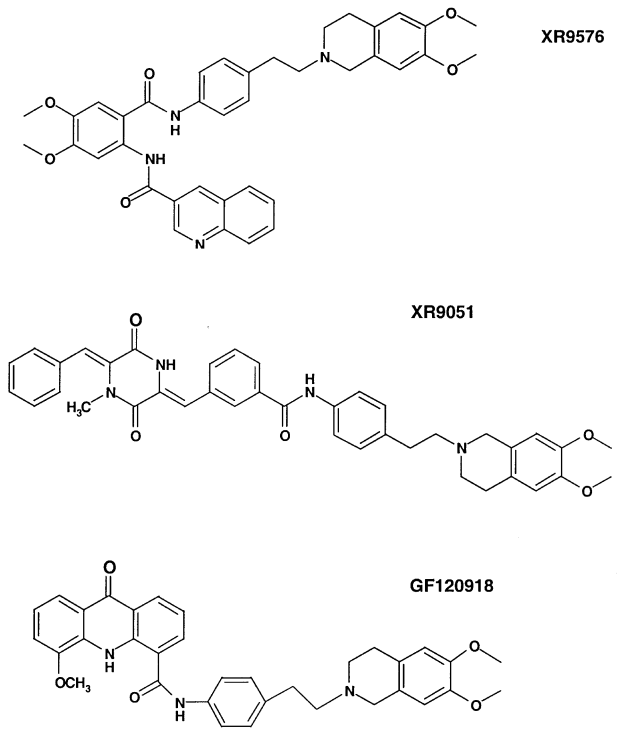

Figure 1 Chemical structures of the anthranilic acid derivative XR9576, the diketopiperazine XR9051 and the acridonecarboximide derivative GF120918. All are potent MDR reversal agents used in this investigation.

Methods

Materials

[^{3}H]-vinblastine (13–18 Ci mmol^{-1}) was purchased from Amersham Chemicals (Amersham, U.K.), and [^{3}H]-paclitaxel (2.4–6.1 Ci mmol^{-1}) from Campro Scientific (Veenendaal, Netherlands). Vinblastine, nicardipine, paclitaxel and sodium orthovanadate were purchased from Sigma (Poole, U.K.) and the detergent compatible protein assay kit from Bio-Rad (Hemel Hempstead, U.K.). XR9576, XR9051 and GF120918 were synthesized and provided by Xenova Ltd. (Slough, U.K.). XR9576 provided by Xenova was tritiated to a specific activity of 32 Ci mmol^{-1}.

Cell culture

The Chinese hamster ovary parental (sensitive) AuxB1 and the resistant CH^{r}B30 cells were grown as previously described in α-minimum essential medium (α-MEM) containing 10% foetal calf serum (Kartner *et al*., 1983). The CH^{r}B30 cells, derived from AuxB1 cells by step wise selection in colchicine (Kartner *et al*., 1983), express P-gp and selection pressure was maintained by supplementing media with 30 μg ml^{-1} colchicine.

Plasma membrane preparation

Plasma membranes were prepared following disruption of CH^{r}B30 cells using nitrogen cavitation and collection with sucrose density centrifugation as previously described (Lever, 1977). The final membrane preparation was stored at −70°C, at protein concentrations of 5–10 mg ml^{-1} in buffer containing 0.25 M sucrose, 10 mM Tris HCl (pH 7.5) and including the protease inhibitors leupeptin (0.1 mg ml^{-1}), pepstatin A (0.1 mg ml^{-1}) and benzamidine (1 mM).

Steady-state drug accumulation assay

AuxB1 and CH^{r}B30 cells were grown to confluency in 12-well (24 mm) tissue culture dishes and the steady-state accumulation of [^{3}H]-vinblastine was measured as previously described (Martin *et al*., 1997). Accumulation was initiated by the addition of 0.1 μCi [^{3}H]-vinblastine and unlabelled vinblastine to a final concentration of 100 nM. The accumulation of [^{3}H]-paclitaxel was measured using 0.1 μCi [^{3}H]-paclitaxel and unlabelled drug to a final concentration of 1 μM. Cells were incubated in a reaction volume of 1 ml for 60 min at 37°C under 5% CO_2 in order to reach steady-state. The effect of the modulators XR9576 and GF120918 on [^{3}H]-ligand accumulation was investigated in the concentration range 10^{-9}–10^{-6} M. Modulators were added from a DMSO stock giving a final solvent concentration of 0.2% (v v^{-1}). Following cell harvesting, accumulated drug was measured by liquid scintillation counting and normalized for cell protein content. Plots of amount accumulated as a function of modulator concentration were fitted with the general dose-response equation (De Lean *et al*., 1978)

$$Y = \{(a - b)/(1 + (X/c)^d)\} + b \quad (1)$$

where; Y = response
a = initial response
b = final response
c = EC_{50} concentration
d = slope value
X = drug concentration

The accumulation of [^{3}H]-XR9576 was also measured in AuxB1 and CH^{r}B30 cells using several concentrations of radiolabelled drug (1–300 nM) in the presence and absence of 1 μM GF120918 and followed over a 60 min period, as described above.

ATP hydrolytic activity of P-gp in CH^{r}B30 membranes

A previously described colorimetric assay was used to measure inorganic phosphate liberation following ATP hydrolysis (Chifflet *et al.*, 1988). Membranes (1 μg protein) were incubated with Na_2ATP (2 mM) in a total assay volume of 50 μl in buffer containing (mM): Tris pH 7.4 50, $MgSO_4$ 5, 0.02% NaN_3, NH_4Cl 150 for 20 min at 37°C. The ATPase activity was linear to 40 min at 37°C. Modulators (from DMSO stocks) and the ATPase inhibitor vanadate, were added in the concentration range 10^{-9}–10^{-5} M. The final DMSO concentration was always <1%, a level known not to alter ATPase activity. The effect of drugs on the ATPase activity was fitted by the general dose-response relationship (see above).

Specific drug binding to P-glycoprotein

A rapid filtration assay was used to measure the binding of [^{3}H]-vinblastine, [^{3}H]-paclitaxel and [^{3}H]-XR9576 to P-gp in CH^{r}B30 membranes as previously described (Ferry *et al.*, 1992). Membranes were incubated with appropriate radioligand in a total buffer volume of 200 μl (50 mM Tris pH 7.4) for a period of 2–3 h to reach equilibrium. Washing buffer (3 ml) containing 20 mM $MgSO_4$, 20 mM Tris (pH 7.4) was then added and the samples filtered under vacuum through a single GF/F filter in a filtration manifold to separate bound and free ligand. After further washing (2 × 3 ml) the amount of bound ligand was determined by liquid scintillation counting. Non-specific binding was defined as the amount of [^{3}H]-ligand bound in the presence of at least a 100 fold excess of competing ligand (indicated in Results) and was subtracted from all values.

Determination of the capacity and affinity of [^{3}H]-ligand binding was achieved by saturation isotherm analysis. Membranes were incubated with increasing concentration of labelled drug and the amount bound (pmol mg^{-1}) plotted as a

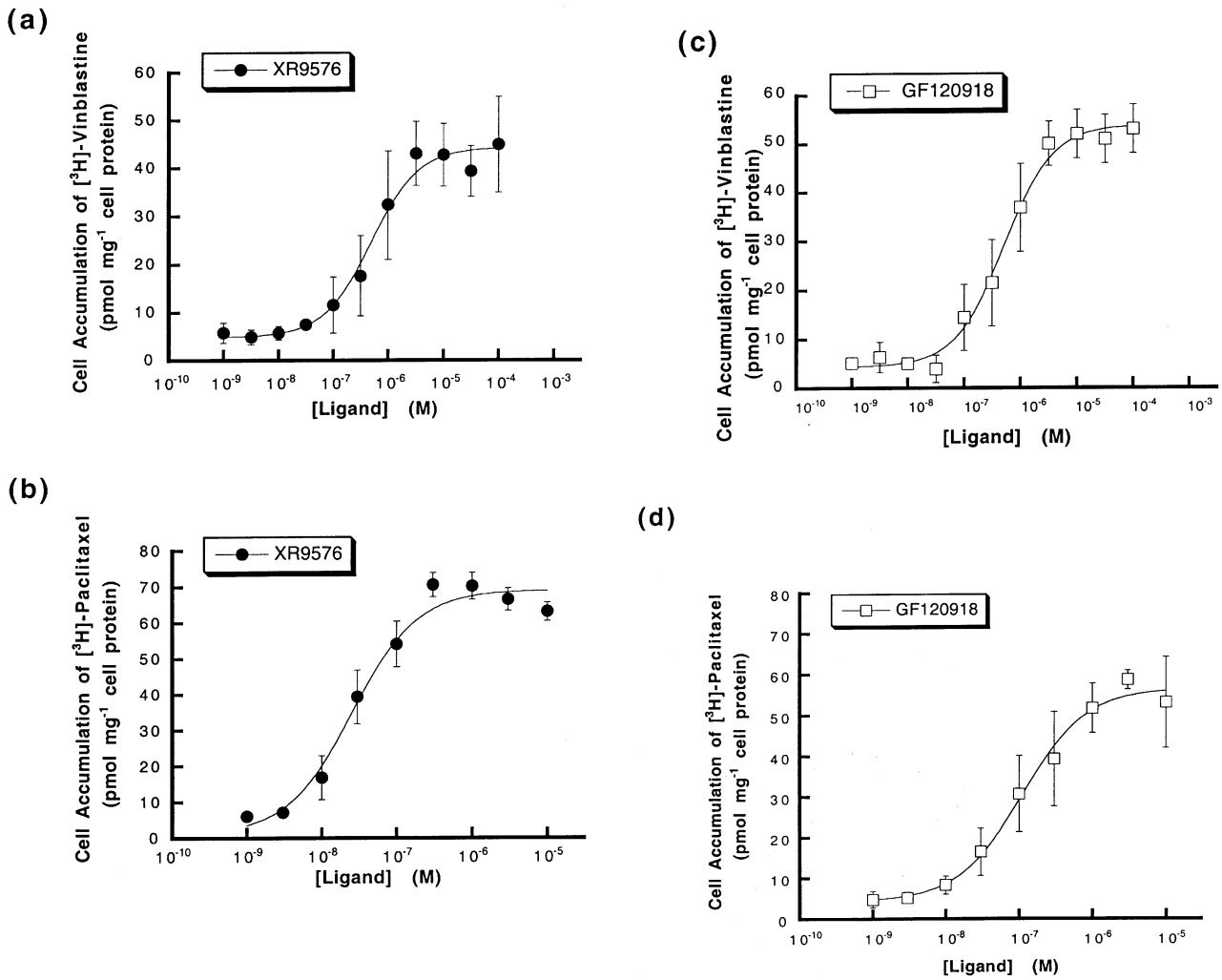

Figure 2 The effect of modulator on the steady-state accumulation of [^{3}H]-vinblastine (100 nM) and [^{3}H]-paclitaxel (1 μM) was measured in CH^{r}B30 cells at 37°C as described in Methods. (a) and (b) show accumulation of vinblastine and paclitaxel in the presence of XR9576. (c) and (d) show the effect of GF120918 on the steady-state accumulation of these drugs. Values represent the means ± s.e.mean of three independent experiments.

function of free ligand concentration. The amount bound (B_d) was fitted by the following equation;

$$B_d = (B_{max} \bullet [L])/(K_d + [L]) \quad (2)$$

where; K_d = dissociation constant (nM)
B_{max} = maximal binding capacity (pmol mg^{-1})
[L] = ligand concentration (nM)

Displacement of drug equilibrium binding by various substrates and modulators (dissolved in DMSO) of P-gp was also determined for [^{3}H]-vinblastine, [^{3}H]-paclitaxel and [^{3}H]-XR9576. Total volume was 200 μl and DMSO concentration was 0.1% (v v^{-1}). Data were plotted as the fraction of radiolabel bound as a function of displacing agent concentration (range 10^{-9}–10^{-6} M). A potency value (IC_{50}) was determined using the general dose-response equation (see above). Values of IC_{50} were converted to inhibition constants (K_i) using the Cheng-Prusoff equation (Cheng & Prusoff, 1973) where;

$$K_i = IC_{50}/(1 + [L]/K_d) \quad (3)$$

where; K_i = inhibition constant (nM)
IC_{50} = concentration antagonist to reduce binding to 50% of the maximal amount (nM)
L = concentration of ligand (nM)
K_d = dissociation constant (nM)

The dissociation kinetics of [^{3}H]-ligand receptor complex were followed using previously published procedures (Ferry *et al.*, 1995). Membranes were allowed to equilibrate with either [^{3}H]-vinblastine (30 nM) or [^{3}H]-XR9576 (12 nM) in the dark at room temperature. Association reactions were blocked by the addition of a 100 fold molar excess of unlabelled ligand (1–3 μM) and the dissociation of the ligand-P-gp complex followed over a period 40 min. The data was linearized by plotting the natural logarithm of the amount bound at time t (B_t) over the amount bound at equilibrium (B_e) as a function of time. The slope of this relationship was defined as the dissociation rate constant k_{-1} where;

$$Ln(B_t/B_e) = k_{-1}.t \quad (4)$$

The rate of [^{3}H]-ligand association with P-gp was also followed by a rapid filtration binding assay. Membranes containing P-gp were added to varying concentrations of [^{3}H]-vinblastine and [^{3}H]-XR9576 in the dark at room temperature for varying time periods. Binding to membranes (nM) was determined following filtration and plotted as a function of association time. From this plot the amount bound at equilibrium (B_e) for each experiment was determined. The rate of association of [^{3}H]-ligand (K_{obs}) with P-gp for each experiment was determined by modelling the following equation (Bowman & Rand, 1984; Kenakin, 1997);

$$B_t = \{B_e * (1 - e^{-(Kobs.t)})\} \quad (5)$$

where; B_t = amount bound at time t
B_e = amount bound at equilibrium
K_{obs} = observed association rate (nM^{-1} min^{-1})
t = time (min)

The value for K_{obs} is not a measure of the association rate constant as it is dependent on the concentration of ligand used

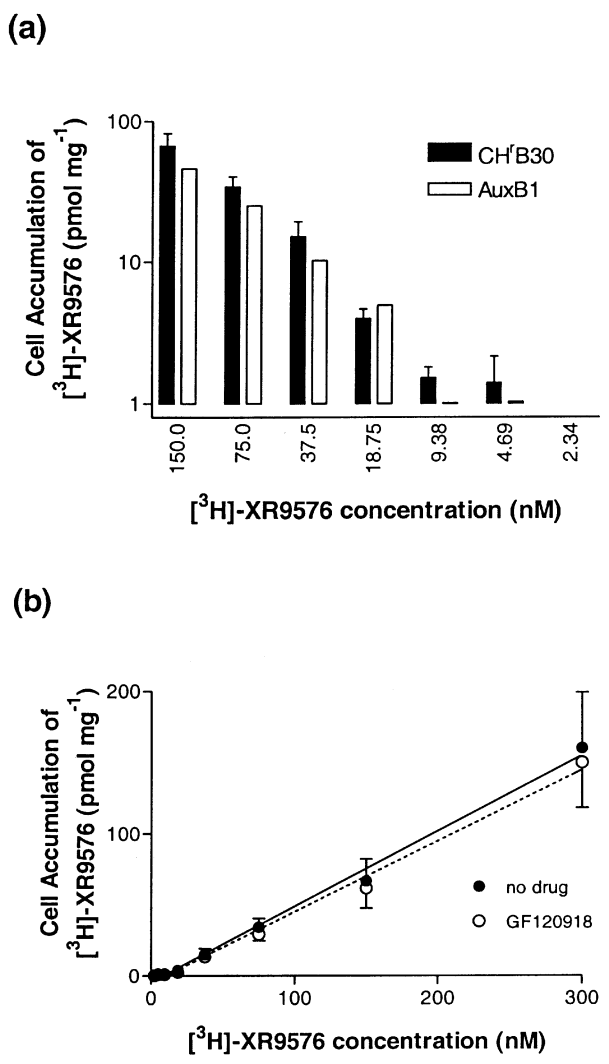

Figure 3 The cellular accumulation profiles for [^{3}H]-XR9576. (a) The relative accumulation of various concentrations of [^{3}H]-XR9576 in the drug sensitive AuxB1 and drug resistant CH^{r}B30 cells at 37°C. (b) Accumulation of [^{3}H]-XR9576 by CH^{r}B30 cells in the presence or absence of 1 μM GF120918. Values represent the means ± s.e.mean of three independent experiments.

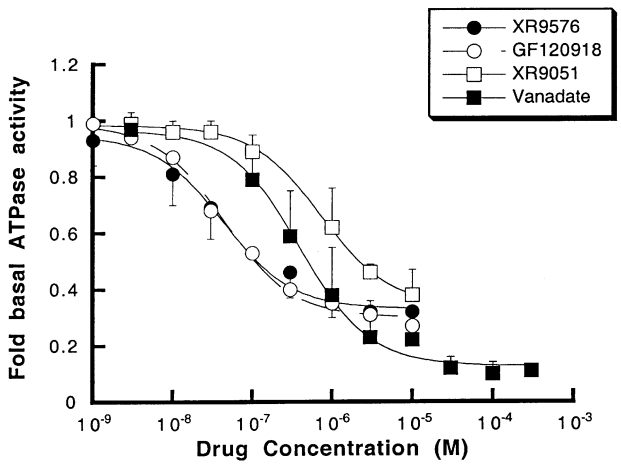

Figure 4 The effect of XR9576, GF120918, XR9051 and vanadate on the ATPase activity of P-gp-containing CH^{r}B30 membranes (1 μg). Activity was measured over 20 min as described in Methods. Values represent means ± s.e.mean of three independent experiments.

and on the amount of receptor used. It can be described by the following relationship (Kenakin, 1997);

$$K_{obs} = k_1 \bullet [L] + k_{-1} \quad (6)$$

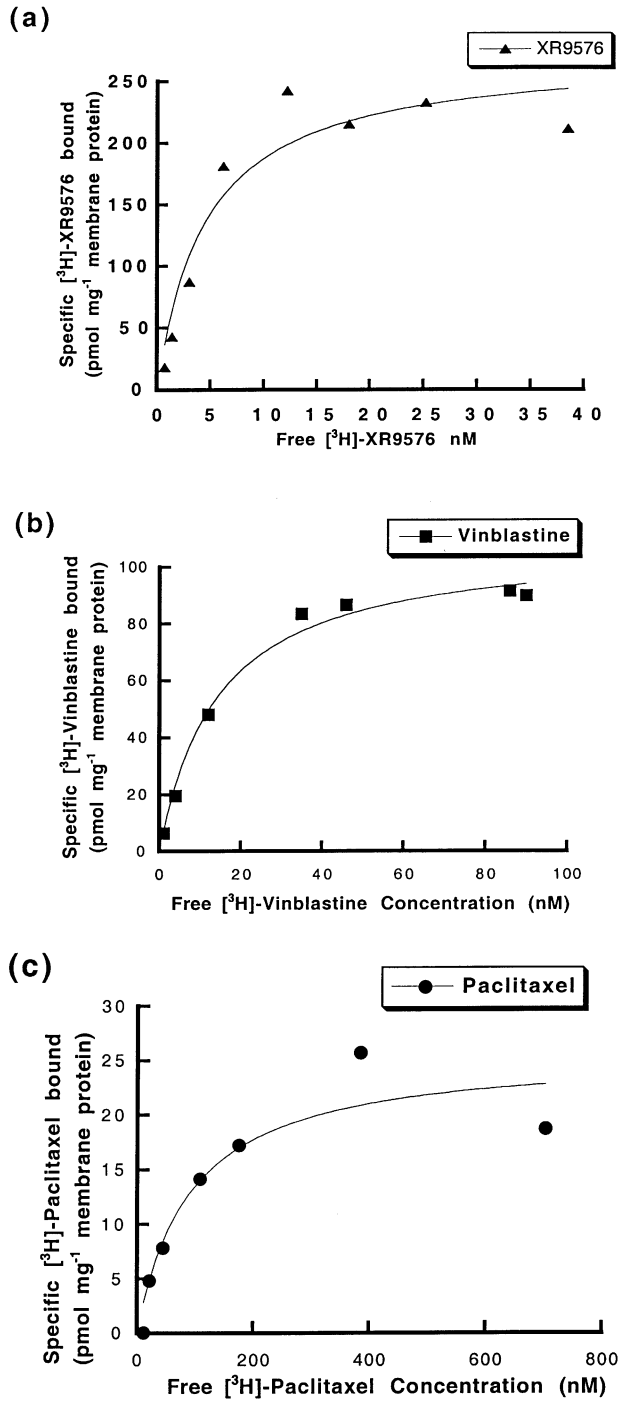

Figure 5 Saturation isotherms of [^{3}H]-XR9576, [^{3}H]-vinblastine and [^{3}H]-paclitaxel binding to CH^{r}B30 membranes. (a) Membranes (2 μg) were incubated with [^{3}H]-XR9576 (0–40 nM) for 3 h at room temperature. Non-specific binding was determined in the presence of 1 μM unlabelled GF120918. (b) CH^{r}B30 membranes (5 μg) were incubated with [^{3}H]-vinblastine (0–100 nM) for 2 h at room temperature. Non-specific binding was measured in the presence of 10 μM unlabelled verapamil. (c) Membranes (20 μg) were incubated with [^{3}H]-paclitaxel (0–800 nM) for 2 h at room temperature. Non-specific binding was determined in the presence of 5 μM cold vinblastine. Representative saturation isotherm plots are shown. Values for B_{max} and K_d were determined as described in Methods.

where; k_{-1} = dissociation rate constant (min^{-1})
k_1 = association rate constant (min^{-1} nM^{-1})
[L] = ligand concentration (nM)

The association rate constant for [^{3}H]-ligand with P-gp was determined by using a secondary plot where K_{obs} (obtained from 5) was plotted versus ligand concentration (using equation 6). The values for k_1 and k_{-1} were determined from the slope and y-intercept of this relationship respectively. Alternatively, the experimentally derived value of the dissociation reaction (from equation 4) was used in equation 6 to calculate the value of k_1.

Statistical analysis

All statistical analyses were made on comparison of sample means by use of two-tailed, unpaired Student's *t*-test. In all cases, a *P* value of 0.05 was considered statistically significant.

Results

Effect of XR9576 on cellular accumulation of [^{3}H]-vinblastine and [^{3}H]-paclitaxel

The non-P-gp-expressing parental cell line, AuxB1, reached a steady-state accumulation of 40 ± 5 pmol mg^{-1} for 0.1 μM [^{3}H]-vinblastine and 43 ± 3 pmol mg^{-1} for 1 μM [^{3}H]-paclitaxel after 60 min, and these levels of accumulation were not altered by adding the P-gp modulating agents XR9576 or GF120918 at any concentration tested (data not shown). However, the P-gp over-expressing CH^{r}B30 cells accumulated 8 fold less [^{3}H]-vinblastine (5 ± 2 pmol mg^{-1}, $P<0.05$) and 6 fold less [^{3}H]-paclitaxel (7.4 ± 1.1 pmol mg^{-1}, $P<0.05$). In contrast to the parental cell line, the accumulation of [^{3}H]-vinblastine was increased in a dose-dependent fashion by the modulators XR9576 ($EC_{50} = 487 \pm 50$ nM) and GF120918 ($EC_{50} = 512 \pm 25$ nM) (see Figure 2). The maximal accumulation levels achieved in the presence of XR9576 and GF120918 were 44 ± 2 pmol mg^{-1} and 53 ± 2 pmol mg^{-1} respectively and were not significantly different from those achieved in the non-P-gp-expressing AuxB1 cells. Similarly, the accumulation of [^{3}H]-paclitaxel was increased to 69 ± 2.4 pmol mg^{-1} ($EC_{50} = 25.4 \pm 6.9$ nM) and 56.4 ± 1.7 pmol mg^{-1} ($EC_{50} = 109 \pm 21$ nM) for XR9576 and GF120918 respectively. Therefore, both XR9576 and GF120918 act as potent modulators of P-gp-mediated transport. The extent of the increases in steady-state levels of both [^{3}H]-vinblastine and [^{3}H]-paclitaxel accumulation, indicates complete inhibition of P-gp in CH^{r}B30 cells. Both modulators reversed the accumulation deficit of [^{3}H]-paclitaxel with greater potency than was displayed to inhibit [^{3}H]-vinblastine transport, which may be a reflection of the relative affinities of these transport substrates for P-gp.

Is [^{3}H]-XR9576 transported by P-gp?

In order to determine how XR9576 reversed the accumulation deficit for [^{3}H]-vinblastine and [^{3}H]-paclitaxel, investigations were undertaken to examine whether this modulator affects P-gp mediated transport by acting as a competitive substrate. Steady-state accumulation of [^{3}H]-XR9576 was measured over a series of ligand concentrations, in order to examine any dose-dependent effects and to look for saturability of the transport process (Figure 3). There was no difference in the steady-state levels of [^{3}H]-XR9576 between the P-gp expressing CH^{r}B30 cell line and the parental AuxB1 cells at any ligand concentration

examined (Figure 3a). Furthermore, the accumulation displayed a linear relationship (Figure 3b) with the amount of added ligand over a wide concentration range (1–300 nM). In addition, the P-gp modulator GF120918 had no effect on the steady-state accumulation of [^{3}H]-XR9576 in either cell line. These two lines of evidence suggest that the accumulation of [^{3}H]-XR9576 is not only independent of P-gp expression, but follows a passive diffusion process. Therefore, it does not appear that XR9576 mediates its effect on vinblastine transport by acting as a competing substrate, but perhaps by altering substrate binding or the transport process in an indirect or allosteric fashion.

Effects of MDR modulators on the ATPase activity of P-gp

The next series of experiments were done to examine whether XR9576 alters P-gp-mediated transport by affecting its rate of ATP hydrolysis. The ATPase activity of CH^{r}B30 membranes assigned to P-gp is the vanadate-sensitive fraction ($IC_{50}=0.4\pm0.06$ μM; $n=3$), and this activity (200 nmol min^{-1} mg^{-1}) occurs in the absence of added substrate (Figure 4). Numerous reports have described the sensitivity of this ATPase activity to drugs which interact with P-gp (Sharom *et al*., 1995; Urbatsch *et al*., 1994). The MDR modulators XR9576, GF120918 and XR9051 were able to inhibit 60–70% of the vanadate-sensitive ATPase activity, with potent IC_{50} values of 43 ± 9 nM, 44 ± 5 nM and 0.7 ± 0.09 μM ($n=3$) respectively. This inhibition of the ATPase activity also supports their interaction with P-gp as modulatory in nature, rather than as competitors for transport since substrates would be expected to stimulate ATPase activity.

Potency and capacity of drug binding to P-gp

In order to directly determine the nature and affinity of the interaction of XR9576 with P-gp, saturation binding was measured and compared with the substrates vinblastine and paclitaxel. As shown in Figure 5, all three radioligands bound specifically to CH^{r}B30 membranes. No specific binding was observed in the non-P-gp-expressing AuxB1 cell membranes (data not shown). Non-specific binding was determined in the presence of excess cold ligand and was linear with increasing concentrations of [^{3}H]-ligand. [^{3}H]-XR9576 bound to CH^{r}B30 membranes with the highest affinity ($K_d=5.1\pm0.9$ nM, $n=7$) and a binding capacity (B_{max}) of 275 ± 15 pmol mg^{-1} membrane protein. The specific binding of [^{3}H]-vinblastine ($K_d=21\pm3.2$ nM, $n=4$) and [^{3}H]-paclitaxel ($K_d=102\pm17$ nM, $n=3$) displayed 4 fold and 20 fold lower affinity for P-gp than that observed for [^{3}H]-XR9576 respectively. In addition, the maximal binding capacity for [^{3}H]-XR9576 to CH^{r}B30 membranes was approximately 3 fold greater than that observed for [^{3}H]-vinblastine (93 ± 10 pmol mg^{-1}, $n=4$) and 10 fold greater than the B_{max} for [^{3}H]-paclitaxel binding (26 ± 4 pmol mg^{-1}, $n=3$).

Kinetics of drug binding to P-gp

Measurement of the association and dissociation rate constants of drug-receptor complexes provides information on the onset and duration of drug action. Dissociation of drug from P-gp following attainment of equilibrium was initiated by the addition of a large molar excess of unlabelled ligand. The resultant dissociation profiles for [^{3}H]-XR9576 and [^{3}H]-vinblastine were both monophasic. The dissociation rate constant for the P-gp-vinblastine complex was 0.027 ± 0.001 min^{-1} ($n=4$) (Table 1) which corresponds to a half-life of 26 min. The rate of dissociation was significantly slower for the P-gp-XR9576 complex which was described by a rate constant of 0.0166 ± 0.0004 min^{-1} ($P<0.05$) and a half-life of 42 min.

The association of [^{3}H]-XR9576 and [^{3}H]-vinblastine with P-gp in CH^{r}B30 membranes was allowed to proceed until equilibrium was reached. A rate constant for the association (K_{obs}) was attained for each concentration of labelled ligand studied. The absolute association rate constants for vinblastine and XR9576 were estimated from the relationship between ligand concentration and K_{obs} ($K_{obs}=k_1\cdot[L]+k_{-1}$) using the experimentally derived value for k_{-1} (Table 1). The rate constant for [^{3}H]-XR9576 association with P-gp (0.00421 ± 0.00043 min^{-1} nM^{-1}) was roughly 10 fold greater than that describing [^{3}H]-vinblastine-P-gp association (0.00048 ± 0.00013 min^{-1} nM^{-1}, $P<0.05$).

From the kinetic data above it is clear that the ligand-P-gp complex is formed more rapidly and is more stable with XR9576 than vinblastine. The values for these two processes ($K_d=k_{-1}/k_1$) may be used to estimate a value for the dissociation constant. The resultant derived K_d values were similar to the values experimentally obtained from saturation binding analysis (Table 1).

Relative potencies of drug interaction with P-gp

A final characterization of the interaction between P-gp and XR9576 was done by comparing its potency of binding to that

Table 1 Kinetics of [^{3}H]-XR9576 and [^{3}H]-vinblastine binding to P-gp in CH^{r}B30 membranes

Ligand	*Association rate constant* k_1 (min^{-1} nM^{-1})	*Dissociation rate constant* k_{-1} (min^{-1})	*Kinetic Kd* k_{-1}/k_1 (nM)	*Equilibrium Kd* (nM)
Vinblastine	0.00048 ± 0.00013 ($n=8$)	0.027 ± 0.001 ($n=3$)	56.3	21 ± 3.2 ($n=4$)
XR9576	0.00421 ± 0.00043 ($n=7$)	0.0166 ± 0.0004 ($n=4$)	3.9	5.1 ± 0.9 ($n=7$)
P	<0.05	<0.05		

The association (k_1) and dissociation (k_{-1}) rate constants for binding of [^{3}H]-XR9576 and [^{3}H]-vinblastine to P-gp were measured at room temperature as described in Methods. Dissociation rate constants were measured, following attainment of equilibrium between [^{3}H]-ligand and P-gp, by the addition of excess unlabelled compound. The dissociation profiles were monophasic and plotted as the natural logarithm of the fraction of maximal binding remaining (B_t/B_{eq}) as a function of time. The dissociation rate constant was obtained from the slope of this relationship. Rate constants for the association process (K_{obs}) were measured at several different concentrations of ligand and used to estimate the value of k_1 using the experimentally derived value of k_{-1} from the equation $K_{obs}=k_1.[L]+k_{-1}$. All values represent means ± s.e.mean for the number of observations indicated. The kinetic constants k_1 and k_{-1} were used to obtain a kinetically derived dissociation constant (K_d) for comparison with the value obtained in equilibrium binding studies.

obtained for several other modulators and substrates. The potency estimations were obtained by investigating the displacement of the equilibrium binding of [^{3}H]-XR9576 (Figure 6), [^{3}H]-paclitaxel and [^{3}H]-vinblastine by various compounds, and the data are summarized in Table 2. From the displacement curves obtained, an IC_{50} value (concentration of antagonist reducing maximal binding by 50%) for antagonist potency was determined. However, IC_{50} values cannot be used as a true measure of antagonist affinity, as this value is dependent on the experimental conditions used, in particular the concentration of radioligand employed and receptor number. The IC_{50} value was therefore converted to an antagonist affinity constant (K_i) by use of the Cheng-Prusoff transformation as described in Methods.

The P-gp substrates [^{3}H]-paclitaxel and [^{3}H]-vinblastine were completely displaced by all compounds examined with less than 15% of the specific binding remaining. Interestingly, paclitaxel was only able to displace 20% of the [^{3}H]-XR9576 binding with a K_i value (1640 nM) 10 fold lower than its equilibrium dissociation constant (K_d = 102 nM). Vinblastine, a more potent (c.f. paclitaxel) transported substrate of P-gp was also only able to fractionally displace the binding of [^{3}H]-XR9576 (70%). Furthermore, the K_i value (1250 ± 510 nM) for this displacement also differed greatly from its dissociation constant (K_d = 21 nM). In contrast, the K_i value for displacement of [^{3}H]-paclitaxel by vinblastine (K_i = 19.9 nM) was almost identical to its dissociation constant concentration. The poor correlation between the dissociation constants for vinblastine and paclitaxel and their K_i values to displace XR9576 binding, suggests a non-competitive interaction between the transport substrates and the modulator XR9576.

The high potency modulators XR9051 and GF120918 did not display large variations in their K_i values for displacing either [^{3}H]-XR9576, [^{3}H]-vinblastine or [^{3}H]-paclitaxel binding. In each case, the amount of [^{3}H]-ligand remaining was less than 10% of the total bound. In contrast, the 1,4-dihydropyridine nicardipine displayed a large variation in the K_i values to displace the binding of [^{3}H]-XR9576 (885 ± 152 nM), [^{3}H]-paclitaxel (10.8 ± 1.4 nM) and [^{3}H]-vinblastine (182 ± 9 nM). This variation in the K_i values for nicardipine also indicates that several distinct modulator drug interaction sites may exist on P-gp.

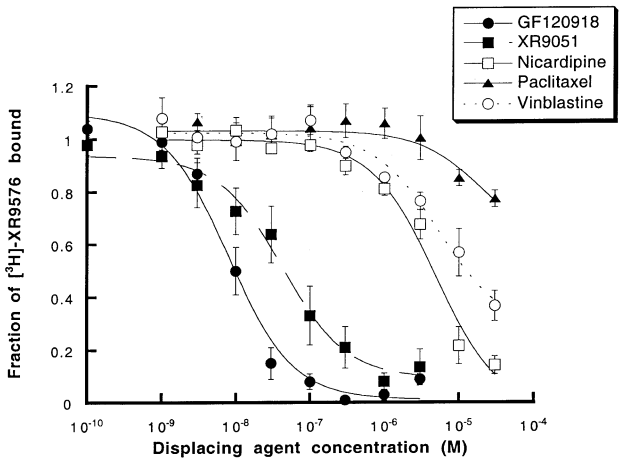

Figure 6 The effects of GF120918, XR9051, nicardipine, paclitaxel and vinblastine on the binding of [^{3}H]-XR9576 to CH^{r}B30 membranes. Binding was carried out at room temperature using 12 nM labelled drug, 2 μg membrane protein and displacing compounds at the concentrations shown. All values represent the means ± s.e.mean for 3–5 independent observations.

Discussion

The failure to render tumour cells sensitive to anticancer drugs has been due in part to the relative lack of selectivity and potency of currently available pharmacological reversal agents. This problem is compounded further by the lack of understanding of the mechanism of P-gp drug efflux function, a key target of reversal agents. In more recent years, steps have been taken to develop drugs which will be targeted more specifically to P-gp with a view to creating modulators displaying greater selectivity and potency of action. In this study, the interaction of the anthranilic acid derivative XR9576 with P-gp has been characterized, and has been used to gain insight into its mode of inhibition of P-gp function.

The molecular interaction of XR9576 with P-gp was examined using drug equilibrium binding, as this method provides a direct and quantitative means to determine the affinity of drug interaction. XR9576 was shown to interact specifically with P-gp, and displayed several fold higher affinity than either of the transported substrates vinblastine or paclitaxel in doing so. This high potency of interaction would indicate that this agent is a good candidate with which to inhibit P-gp activity in the clinic. The specificity of XR9576 for P-gp was shown by the absence of binding to the parental cell line.

To further examine the interaction of XR9576 with P-gp and compare it to that for transported substrates, the relative affinities of several modulators and transport substrates for P-gp were obtained by measuring K_i values for displacement of equilibrium drug binding. K_i values estimated from the Cheng-Prusoff transformation assume that the agonist and antagonist are interacting in a reversible competitive fashion at a single

Table 2 The relative affinities of drug interaction with P-gp

	[^{3}H]-XR9576		*[^{3}H]-paclitaxel*		*[^{3}H]-vinblastine*	
Displacing agent	*Fraction displaced*	K_i (nM)	*Fraction displaced*	K_i (nM)	*Fraction displaced*	K_i (nM)
Vinblastine	0.7 ± 0.1	1250 ± 510	0.77 ± 0.13	19.9 ± 7.5		
Paclitaxel	0.18 ± 0.02	1640 ± 270			0.91 ± 0.05	335 ± 6
Nicardipine	0.88 ± 0.05	885 ± 152	0.92 ± 0.04	10.8 ± 1.4	0.99 ± 0.01	182 ± 9
XR9576			0.86 ± 0.07	4.2 ± 1.4	1.0	2.6 ± 0.8
GF120918	0.93 ± 0.02	1.5 ± 0.2	0.94 ± 0.03	2.54 ± 0.15	0.99 ± 0.07	1.4 ± 0.4
XR9051	0.91 ± 0.03	11 ± 4.2	0.86 ± 0.04	10.6 ± 1.4	1.0	1.8 ± 0.3

Displacement drug equilibrium binding studies were used to look at the ability of various substrates and modulators to displace the binding of [^{3}H]-XR9576 (12 nM), [^{3}H]-paclitaxel (100 nM) and [^{3}H]-vinblastine (30 nM) from CH^{r}B30 membranes. IC_{50} values for antagonist activity were obtained from displacement binding curves (see Figure 6) and converted to inhibition affinity constants (K_i) as described in Methods. The fraction of bound ligand displaced by antagonist has also been shown. The data represent the means ± s.e.mean of 3–5 independent experiments.

site (Barlow *et al*., 1997; Larazeno & Birdsall, 1993). Therefore, comparison of measured K_d values with the K_i value may provide some clues as to the type of drug interaction occurring. The fact that (i) vinblastine and paclitaxel could only fractionally displace XR9576 binding and (ii) the K_i values differed significantly from their measured K_d values, suggests that interaction of these agents does not comply with the assumptions on which the Cheng-Prusoff transformation is based. This suggests that XR9576 interacts non-competitively with vinblastine and paclitaxel on P-gp, most likely at a distinct site.

There is evidence emerging from the literature supporting a model for P-gp having distinct sites for transported substrate and modulator on P-gp. For instance, it has been shown through studies of the ATPase activity of P-gp that the binding sites for vinblastine and modulators such as verapamil and steroid hormones are distinct (Orlowski *et al*., 1996). Furthermore, it was also shown that verapamil and nicardipine (a 1,4-dihydropyridine) interacted at distinct but communicating sites (Pascaud *et al*., 1998). Kinetic binding studies have demonstrated that vinblastine interacts at a site distinct from, but allosterically linked to the 1,4-dihydropyridine site (Ferry *et al*., 1995) and the site for the indolizin sulphone SR33557 (Martin *et al*., 1997). We would like to propose that, on the basis of the observations above and evidence from the literature (Ayesh *et al*., 1996; Shao *et al*., 1997), there exist multiple drug interaction sites on P-gp. Moreover, our data suggests that the multiple sites may actually be functionally classified as transport or modulatory in nature.

This hypothesis was probed further by looking at the effect of XR9576 interaction on P-gp's transport function. In this study, it was shown that XR9576 could effectively block efflux of vinblastine and paclitaxel, reported transported substrates of P-gp (Aziz *et al*., 1998; Doige & Sharom, 1992; Horio *et al*., 1988). Moreover, it displayed significantly greater potency than the widely used reversal agent verapamil in doing so (Spoelstra *et al*., 1994). The specificity of XR9576's reversal activity for P-gp has previously been shown by its lack of effect on the transport activity of the related ABC transporter, Multidrug Resistance Protein (MRP) (Stewart *et al*., 1998). We have shown that the molecular mode of XR9576's inhibition of transport was not through competition for this process on P-gp, as expression of P-gp did not have any effect on the accumulation levels of XR9576, even over a wide concentration range of XR9576. It has been reported that the drug-efflux function of P-gp is a saturable process (Miyamoto *et al*., 1996) and the fact that there was no observed saturation of XR9576 accumulation is indicative of a passive rather than either a facilitated diffusion or indeed an active process. Therefore, unlike vinblastine and paclitaxel, XR9576 does not appear to be a transport substrate for P-gp. A possible interpretation is that this provides further evidence that XR9576 is interacting at a site distinct from the vinblastine and paclitaxel site(s), and one that is modulatory in nature. Indeed, previous studies have shown that reversal agents can inhibit transport through non-competitive interaction, for instance verapamil was shown to modulate transport of daunomycin and vinblastine non-competitively (Ayesh *et al*., 1996; Spoelstra *et al*., 1994). Additionally, these sites of interaction not only appear to be distinct but are allosterically linked (Ferry *et al*., 1992). These findings provide further evidence supporting a multi-site model of P-gp containing sites that either function to elicit transport of cytotoxics or mediate inhibition of this process.

The results of this paper explain the molecular mode of XR9576's inhibition of the transport of vinblastine and paclitaxel by P-gp in terms of a multiple-binding site model where binding of XR9576 can (a) prevent substrate binding; (b) alter ATP hydrolytic ability of P-gp; or (c) a combination of these two possibilities. It is well documented that ATP hydrolysis is required to fuel the drug efflux activity of P-gp (Horio *et al*., 1988; Schlemmer *et al*., 1995; Sharom *et al*., 1993). We have shown that XR9576 binding inhibited the P-gp vanadate-sensitive fraction of membrane ATPase activity with very high potency. However, we know that not all modulators affect transport through inhibition of ATP hydrolysis. For example, verapamil and nicardipine both block P-gp transport function even though they stimulate ATPase activity, implying a mechanism of inhibition that uncouples ATP hydrolysis from transport. This also indicates that interaction of modulators with the protein can elicit opposing effects on ATP hydrolytic activity and could be interpreted in terms of either distinct binding sites for modulators on the protein or opposing efficacies following binding to a single site.

In this paper an initial characterization of the molecular interaction of [^{3}H]-XR9576 with P-gp has been conducted. The nature of the interaction of XR9576 with P-gp was assessed both directly and quantitatively, in terms of its transport, ATP hydrolysis and binding functions. The anthranilic acid derivative, XR9576, interacts with this transporter with very high affinity and can potently inhibit P-gp function as a result. Additionally, the duration of action, as demonstrated by the kinetic binding data, appears longer than that for vinblastine. This augurs well for the use of this agent as a P-gp-related MDR reverser in the clinic and indeed XR9576 is currently in clinical trials. The potency and selectivity of XR9576 has also provided a means by which the molecular mode of modulator mediated inhibition of transport might be elucidated. Future studies with this compound will involve further investigation of the number and nature of drug interaction sites on P-gp and directly determine whether (a) they are competitive or non-competitive; (b) if there is interaction between binding sites; and (c) the possible nature of binding site interactions. In addition to determining information concerning the binding sites on P-gp, future efforts should also focus on the interaction of transport substrates and modulators with the transporter during its catalytic cycle to help elucidate its underlying mechanism of action.

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