The multi-drug resistance reversal agent SR33557 and modulation of vinca alkaloid binding to P-glycoprotein by an allosteric interaction

Catherine Martin, Georgina Berridge, Christopher F. Higgins & [1]Richard Callaghan

Imperial Cancer Research Fund Laboratories and Nuffield Department of Clinical Biochemistry, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DS

1   The interaction of the indolizin sulfone SR33557 with the multidrug resistance P-glycoprotein (P-gp), was used to explore the nature of drug binding site(s) on this transporter. The steady-state accumulation of [^{3}H]-vinblastine in P-gp expressing CH^{r}B30 cells was increased by SR33557 with greater potency than verapamil. Furthermore, SR33557 potentiated the affinity of verapamil to modulate vinblastine transport when added simultaneously.

2   Verapamil elicited a 1.5 to 2.5 fold stimulation of basal ATPase activity in CH^{r}B30 membranes, whereas SR33557 and vinblastine inhibited activity, but only at relatively high concentrations. However, SR33557 and vinblastine decreased the V_{max} but not the K_m for verapamil stimulation of ATPase activity. This is indicative of a non-competitive interaction, most likely at distinct sites.

3   The specific [^{3}H]-vinblastine binding to P-gp in CH^{r}B30 cell membranes was displaced by SR33557 with an IC$_{50}$ of 8.3 ± 4.5 nM. Moreover, SR33557 caused a 3 fold increase in the dissociation rate of vinblastine binding to P-gp indicating a negative allosteric effect on the vinca alkaloid acceptor site.

4   These results demonstrate that SR33557 interacts with a site on P-gp which is distinct from, but allosterically linked to the vinca alkaloid site. The apparent broad substrate specificity displayed by P-gp may be explained by a multiple drug binding site model.

Keywords: Multidrug resistance; P-glycoprotein; allosteric; drug binding

Introduction

The 180 kDa P-glycoprotein (P-gp) is a major factor responsible for the development of drug resistance in tumour cells (Kartner *et al.*, 1983). P-gp is an adenosine 5′-triphosphate (ATP) dependent drug transporter which reduces the intracellular concentration of cytotoxic compounds, thereby conferring drug resistance (Gottesman & Pastan, 1993). The basic organization of P-gp consists of 12 putative transmembrane α-helices and two cytoplasmic domains which bind and hydrolyze ATP (Gros *et al.*, 1986; Yoshinura *et al.*, 1989; Doige *et al.*, 1992; Al-Shawi & Senior, 1993; Loo & Clarke, 1995). However, comparatively little is known about the site(s) or nature of the molecular interactions between drugs and P-gp or how drug binding is coupled to ATP hydrolysis to elicit transport.

A large number of chemically unrelated compounds have been demonstrated to interact with P-gp (Endicott & Ling, 1989; Safa, 1992) and it has therefore been proposed that P-gp possesses a low specificity binding site. Indeed, the only apparent requirements for substrate interaction with P-gp are physical properties such as planarity, lipophilicity and a cationic nitrogen group (Zamora *et al.*, 1988). Early attempts to map the drug interaction site on P-gp used photoaffinity labelling with azidopine (Bruggeman *et al.*, 1989; Greenberger *et al.*, 1991). Data from these investigations suggested that sequences on each homologous half of P-gp approach each other to form a single substrate binding site. However, the highly flexible nature of azidopine allows labelling of many reactive sites not necessarily involved in drug binding (Glossmann *et al.*, 1987). Furthermore, the reactive group of photoaffinity ligands may not lie in the pharmacophoric region of the drug and, hence, may label the protein distant from the binding site (Borchers *et al.*, 1995). Investigations, in which mutational analyses were used in an attempt to locate drug binding sites

on P-gp, have demonstrated that alteration of several residues throughout the protein modify profiles of resistance to cytotoxic agents (Dhir *et al.*, 1993; Kajiji *et al.*, 1993). However it is not possible from such studies to distinguish whether the mutated residues are involved in substrate binding or another step in the translocation pathway. There is also little known about the number of drug binding sites or the possible communication between them. Equilibrium binding studies with P-gp containing membranes indicate a non-competitive interaction between vinblastine and azidopine, possibly due to interaction at multiple or distinct sites on the protein (Tamai & Safa, 1991). In addition, the novel calcium channel blocker SR33557 has been demonstrated to reverse multidrug resistance but does not prevent azidopine labelling (Jaffrezou *et al.*, 1991).

We have therefore used a pharmacological approach with the indolizin sulphone SR33557 to examine substrate-substrate interactions with P-gp at the level of drug binding, ATPase activity and transport. SR33557 behaved as a high affinity substrate for P-gp by altering vinblastine accumulation and displacing drug binding to P-gp. However, SR33557 modified the ability of verapamil to stimulate the ATPase activity of P-gp in a non-competitive fashion and also increased the dissociation rate of vinblastine from P-gp. These results are discussed in terms of multiple allosterically linked drug acceptor sites on P-gp.

Methods

Cell culture and membrane preparations

The drug resistant Chinese hamster ovary cell line CH^{r}B30 expressing P-gp was grown in α-minimum essential medium (α-MEM) supplemented with nucleosides and 30 μg ml^{-1} colchicine to maintain resistance as described previously (Kartner *et al.*, 1983). The parental drug sensitive AuxB1 cell line was

[1] Author for correspondence:

also grown in α-MEM supplemented with nucleosides but in the absence of colchicine.

Plasma membranes were isolated from CH^{r}B30 cells according to previously published methods (Lever, 1977). Cells were disrupted by nitrogen cavitation (1500 psi) and membranes purified by sucrose density centrifugation. Purified membranes were stored in 10 mM Tris. HCl pH 7.4, 0.25 M sucrose at $-70°$C for up to 6 months without loss of activity.

Steady-state [^{3}H]-vinblastine accumulation in Aux B1 and CH^{r}B30 cells

AuxB1 and CH^{r}B30 cells were grown to confluent monolayers in 6-well (60 mm) tissue culture plates and then washed with phosphate buffered saline (PBS). Competing drugs were added in the concentration range 10^{-9} to 10^{-5} M. Accumulation was started by the addition of [^{3}H]-vinblastine (0.1 μCi) and unlabelled vinblastine to a final concentration of 100 nM. The cells were incubated in a reaction volume of 1 ml for 60 min at 37°C under 5% CO$_2$. Following incubation, the monolayers were washed with cold PBS and extracted with 0.4 ml 0.4 M NaOH. The cell extract was neutralized with 0.8 ml 0.25 M ammonium acetate (pH6.6) and incorporated radioactivity measured by liquid scintillation counting.

ATPase activity in CH^{r}B30 membranes

The ATPase ativity of CH^{r}B30 membranes was determined by measuring inorganic phosphate liberation by a previously described colourimetric assay (Chifflet *et al*., 1988). Briefly, membranes (1–2 μg protein) were incubated with 2 mM Na$_2$ATP in a total assay volume of 100 μl, in buffer containing 50 mM Tris pH 7.4, 5 mM MgSO$_4$, 0.02% NaN$_3$, 150 mM NH$_4$Cl, for 20 min at 37°C. The ATPase activity of CH^{r}B30 membranes assigned to P-gp, was the vanadate sensitive fraction as previously described (Al-Shawi & Senior, 1993), and was linear to 40 min. All assays were performed in the absence of Na$^+$ and K$^+$ and the presence of sodium azide to minimize contributions from the Na$^+$/K$^+$-ATPase and V-type ATPases (Pedersen & Carafoli, 1987). Where required, SR33557, vinblastine and verapamil were added from stock solutions made up in dimethylsulphoxide (DMSO). The final concentration of DMSO was less than 1% (v/v).

Equilibrium binding of [^{3}H]-vinblastine

A rapid filtration assay was used to determine the equilibrium binding of [^{3}H]-vinblastine to CH^{r}B30 membranes as described previously (Ferry *et al*., 1992). Briefly, membranes (20 μg protein) were incubated with [^{3}H]-vinblastine (25–30 nM) at room temperature in the dark for 120 min. Following an appropriate incubation time, 3 ml of wash buffer was added and the samples vacuum-filtered through a combination of GF/F, and 0.2 μm nitrocellulose membranes in a Millipore Filtration Manifold. Filter-retained radioactivity was measured by liquid scintillation counting. Non-specific binding (usually about 20% total) was defined as the amount of [^{3}H]-vinblastine bound in the presence of 3 μM unlabelled vinblastine and was subtracted from all values.

Displacement of vinblastine binding by SR33557 (10^{-10} to 10^{-6} M) was performed in a total assay volume of 1 ml. SR33557 stock solutions were made up in DMSO and added to the samples as a 2 μl aliquot giving a final DMSO concentration of 0.2% (v/v); this solvent can displace [^{3}H]-vinblastine binding with an IC$_{50}$ value of 2.5% (v/v).

The kinetics of [^{3}H]-vinblastine dissociation were based on published procedures (Malkhandi *et al*., 1994). Briefly, following a 120 min incubation of membranes and ligand, samples were cooled to 12°C at which time vinblastine was added to a final concentration of 1.5 μM to block the association reaction in the presence or absence of 3 μM SR33557, 10 μM verapamil or 3 μM nicardipine. Samples were then equilibrated

for times indicated in figure legends and filtered as described above.

Data analysis

The potencies of SR33557 and verapamil as modifiers of [^{3}H]-vinblastine accumulation, in displacing [^{3}H]-vinblastine binding to P-gp and modulating the basal ATPase activity were assessed by non-linear regression by use of the general dose-response equation (De Lean *et al*., 1978):

$$Y = \left\{ (a - b)/(1 + (x/c)^d) \right\} + b$$

where Y = response; a = initial response; b = final response; c = IC$_{50}$ concentration; d = slope factor and x = antagonist concentration.

The response was defined as either the amount of [^{3}H]-vinblastine accumulated (pmol mg^{-1}), the fraction of drug bound or the basal ATPase activity (nmol min^{-1} mg^{-1}). The value of final response in the case of ATPase assays was equivalent to the maximal rate of hydrolysis (V$_{max}$).

Dissociation of [^{3}H]-vinblastine binding was plotted as the natural logarithm of the ratio of the amount bound at time t (B$_t$) over the amount bound at equilibrium (B$_e$), as a function of incubation time. The slope of the relationship obtained was defined as the dissociation rate constant k_{-1}.

Statistical analysis

All statistical analyses were made on comparisons 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.

Materials

SR33557 ((2-isopropyl-1-((4-(3-N-methyl-N-(3,4-dimethoxy-b-phenethyl) amino) propyloxy) benzenesulphonyl)) indolizine) was a generous gift from Sanofi Recherché. Verapamil hydrochloride and vinblastine sulphate were purchased from Sigma Chemicals (Dorset, U.K.). [^{3}H]-vinblastine (13–18 Ci mmol^{-1}) was purchased from Amersham Chemicals (Amersham, U.K.).

Results

The effects of verapamil and SR33557 on the accumulation of [^{3}H]-vinblastine in CH^{r}B30 cells

Drug transport by P-gp was assessed by measuring the steady-state accumulation of [^{3}H]-vinblastine in the P-gp expressing cell line CH^{r}B30 and the drug sensitive parental line AuxB1. The steady-state accumulation of [^{3}H]-vinblastine in the CH^{r}B30 cells (3.12 ± 0.59 pmol mg^{-1}, n = 5) was 22 fold lower than that observed in AuxB1 cells (69 ± 2 pmol mg^{-1}, n = 4, P < 0.001). The time course and ATP-dependence of the [^{3}H]-vinblastine accumulation for both these cell lines has been described previously (Cano-Gauci & , 1987).

Sigmoidal curves were obtained when the ability of verapamil and SR33557 to alter the accumulation of [^{3}H]-vinblastine in CH^{r}B30 cells was assessed, and the results are summarized in Table 1. Verapamil increased the accumulation of [^{3}H]-vinblastine in a dose-dependent fashion to a maximal value of 37.5 ± 4.3 pmol mg^{-1}. The verapamil concentration required to cause half-maximal effect (EC$_{50}$), which is in effect a measure of affinity, was 15.3 ± 2.9 μM (n = 5). SR33557 also increased the steady-state accumulation of [^{3}H]-vinblastine to a similar maximal value as verapamil (36.5 ± 3.9 pmol mg^{-1}). However the potency of SR33557 (EC$_{50}$ = 2.5 ± 0.4 μM, n = 4) was roughly 7 fold greater than that observed for verapamil (P < 0.05). No effect on the steady-state accumulation of [^{3}H]-vinblastine by AuxB1 cells was observed for either drug (data not shown).

Table 1 The effects of verapamil and SR33557 added alone or simultaneously on the accumulation of [^{3}H]-vinblastine in CH^{r}B30 cells

Variable modulator	Fixed modulator	Initial accumulation (pmol mg^{-1})	Final accumulation (pmol mg^{-1})	EC_{50} (μM)
SR33557		3.7±0.4	36.5±3.9	2.5±0.4*
Verapamil		4.2±1.5	37.5±4.3	15.3±2.9
Verapamil	SR33557 (10^{-8} M)	3.0±0.6	45.3±1.0*	17.4±3.6
Verapamil	SR33557 (10^{-7} M)	2.4±0.5	39.3±4.8	13.6±2.9
Verapamil	SR33557 (10^{-6} M)	12.6±2.3*	49.8±3.2*	13.5±2.3
Verapamil	SR33557 (10^{-5} M)	21.8±2.8*	34.6±4.3	8.3±2.0*

Incubation of [^{3}H]-vinblastine (100 nM), CH^{r}B30 cells and a variable modulator in the presence or absence of a fixed modulator was carried out at 37°C for 60 min. The fixed modulator was added at the concentration indicated and the variable modulator in the range 30 nM to 30 μM. Initial accumulation refers to the amount of [^{3}H]-vinblastine accumulated in the absence of a variable modulator. The EC_{50} value refers to the concentration of variable modulator required to increase [^{3}H]-vinblastine to half the maximal value obtainable. *Statistically significant difference ($P < 0.05$) from the parameter obtained with verapamil alone as the modulating agent.
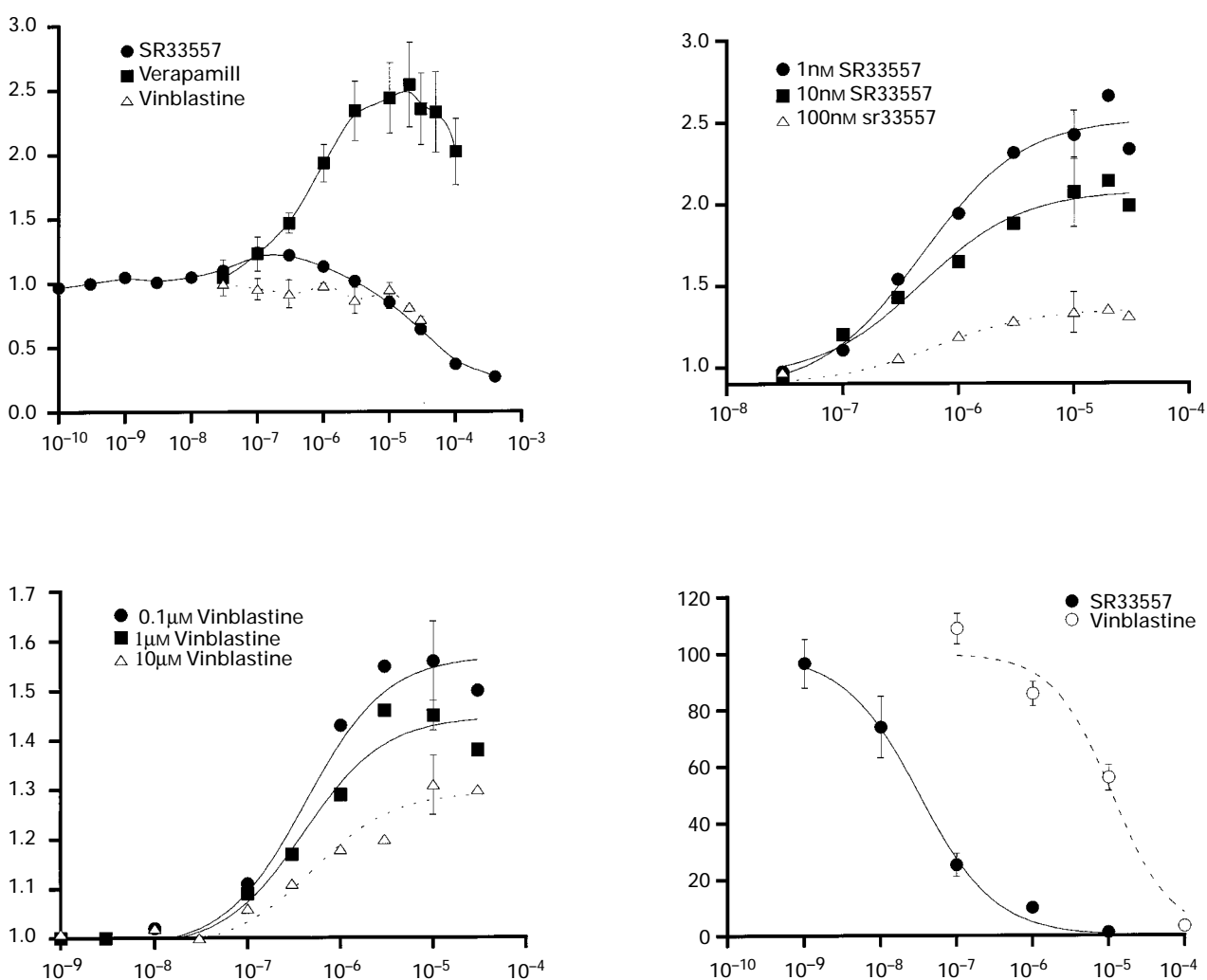

Figure 1 The effects of verapamil, vinblastine and SR33557 on the ATPase activity of P-glycoprotein (P-gp). (a) Dose-response analysis of the effects of verapamil, SR33557 and vinblastine on the basal ATPase activity in CH^{r}B30 membranes (1 μg protein), measured at 37°C as described in Methods. Values were obtained from 3–6 independent membrane preparations; vertical lines show s.e.mean. (b) The effects of 1, 10 or 100 nM SR33557 on the ability of verapamil to stimulate basal ATPase activity in CH^{r}B30 membranes. Values represent means obtained from 3–5 independent membrane preparations. S.e.means (vertical lines) are shown at a single verapamil concentration for clarity of presentation. (c) The effects of 0.1, 1 or 10 μM vinblastine on the ability of verapamil to stimulate basal ATPase activity in CH^{r}B30 membranes. Values represent means obtained from 3–5 independent membrane preparations. S.e.means (vertical lines) are shown at a single verapamil concentration for clarity of presentation. (d) Secondary plot showing the effects of SR33557 and vinblastine on the verapamil activation factor. The V_{max} for verapamil stimulation of ATPase activity in the absence of any drug was defined by an activation factor of 100. Values of the V_{max} for verapamil stimulation of ATPase activity at fixed concentrations of either SR33557 or vinblastine were expressed as a fraction of the value obtained in the presence of only verapamil. Data are the means obtained from 3–5 independent preparations; vertical lines show s.e.mean.

The interaction between the two reversing agents, verapamil and SR33557, and their ability to modulate [³H]-vinblastine accumulation was assessed by co-incubation. Verapamil was chosen as the variable modulator of [³H]-vinblastine accumulation in the presence of a fixed modulator, SR33557, at 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M (Table 1). As the amount of SR33557 was increased, the starting value of [³H]-vinblastine accumulation (i.e. in the absence of verapamil) was also increased, due to the inhibitory effects of SR33557 on transport by P-gp. However, the presence of SR33557 did not significantly increase the final extent of [³H]-vinblastine accumulation brought about by the variable modulator verapamil (Table 1). However, the presence of fixed SR33557 concentrations caused an apparent increase in the ability of verapamil to modulate [³H]-vinblastine accumulation, as reflected by a lower EC_{50} value (Table 1). This may indicate a synergistic interaction between the two reversing agents.

Substrate interaction between SR33557, vinblastine and verapamil; effects on the ATPase activity of P-gp in CHᵣB30 membranes

To assess more directly pharmacological interactions between P-gp modulators, their effects on ATP hydrolysis by the protein were examined (Figure 1a). Verapamil caused a dose-dependent 1.5 to 2.5 fold increase in the basal ATPase activity of P-gp (264 ± 26 nmol min^{-1} mg^{-1}) with an EC_{50} concentration of 0.61 ± 0.13 μM. The variation in the extent of maximal ATPase stimulation is attributed to the different membrane preparations used, and similar differences have been described elsewhere (Doige *et al.*, 1992; Al-Shawi & Senior 1993). The dose-response relationship for verapamil stimulation of ATPase activity was in fact a bell-shaped curve, with maximal stimulation occurring at 30 μM. SR33557, on the other hand, elicited a modest 1.24 ± 0.01 fold increase in the ATPase activity of P-gp at concentrations up to 10^{-5} M, with an EC_{50} value of 33 ± 12 nM ($n = 5$). At concentrations above 10^{-5} M, SR33557 inhibited basal activity reaching a maximum inhibition of 80% at a concentration of 300 μM ($P < 0.02$). The EC_{50} value for this inhibitory phase was 20 ± 4 μM ($n = 5$), which is significantly less than its potency to alter transport (Table 1) or displace drug binding to P-gp (Figure 2a). Vinblastine did not stimulate basal ATPase activity, but did cause inhibition at concentrations above 10^{-5} M with a maximum inhibition of 40% observed at 30 μM vinblastine ($P < 0.02$). However, a full dose-response curve for vinblastine was not possible due to the low solubility of this drug.

Subsequently, we examined the effect of adding combinations of P-gp substrates on ATPase activity. Verapamil was used as the variable modulator in the presence and absence of fixed concentrations of SR33557 or vinblastine. The concentrations of SR33557 ($10^{-9}–10^{-5}$ M) and vinblastine ($10^{-7}–10^{-4}$ M) used had previously been shown to have little effect on basal ATPase activity. In Figures 1b and c for clarity, we have shown the effect of three of the five concentrations of SR33557 or vinblastine used on the ability of verapamil to stimulate ATPase activity. Both SR33557 and vinblastine modulated the extent to which verapamil was able to stimulate ATPase activity.

In order to examine quantitatively the precise nature of the substrate interaction resulting in modulation of the ability of verapamil to alter basal ATPase activity, a secondary plot was drawn (Figure 1d). The V_{max} for verapamil stimulation in the absence of fixed modulator was assigned a value of 100 (verapamil activation factor). In the presence of each fixed modulator concentration, the V_{max} for verapamil stimulation was expressed as a fraction of this value, and was plotted as a function of fixed modulator concentration. Both SR33557 and vinblastine attenuated the verapamil activation factor in a dose-dependent manner. However, there was a marked difference in the potency of these drugs to alter verapamil stimulation of ATPase activity (EC_{50} of 32.5 ± 4.4 nM for SR33557 versus EC_{50} value of 11.2 ± 3.6 μM for vinblastine,
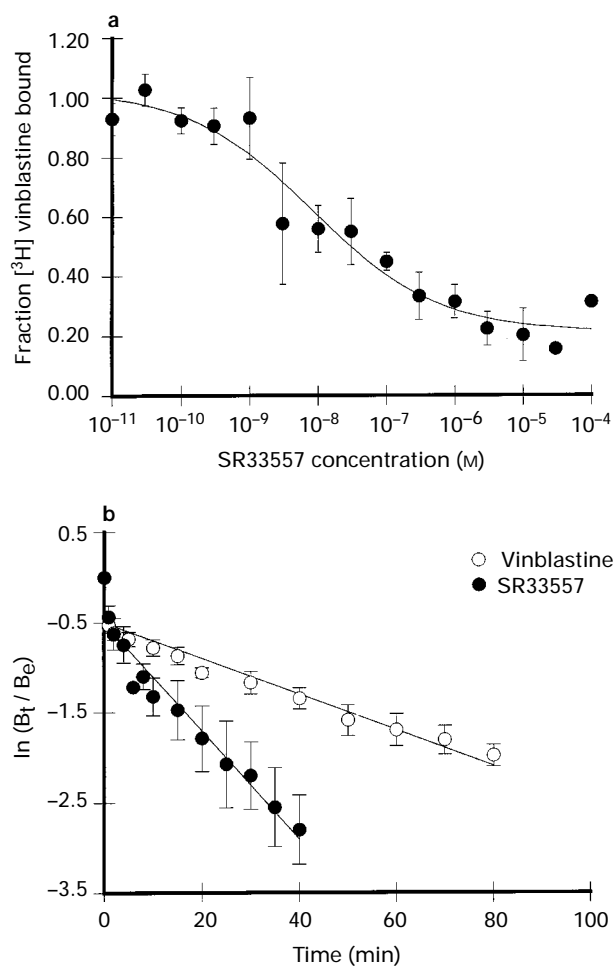

Figure 2 The effect of SR33557 on binding of [³H]-vinblastine to membranes from CHᵣB30 cells. (a) Displacement of the equilibrium binding of 25 nM [³H]-vinblastine to CHᵣB30 membranes (20 μg) by increasing SR33557 concentrations as described in Methods. All values obtained represent the mean from 3–5 independent membrane preparations; vertical lines show s.e.mean. (b) The dissociation of [³H]-vinblastine from CHᵣB30 membranes by 1.5 μM vinblastine or a combination of 1.5 μM vinblastine with 3 μM SR33557. The natural logarithm of the ratio of [³H]-vinblastine bound at each time (B_t) to that at equilibrium (B_e) was plotted against time and the slopes of the relationship used to determine the dissociation rates. All values obtained represent the mean from 3–5 independent membrane preparations; vertical lines show s.e.mean.

$P < 0.01$). In contrast, neither drug caused a change in K_m as evidenced in Figure 1b–c (data not shown). A fall in the verapamil activation factor, which is a measure of V_{max}, with no concomitant change in K_m for the SR33557 concentrations used, suggests that the attenuation of verapamil stimulated basal ATPase by SR33557 and vinblastine is non-competitive in nature.

Substrate interaction between SR33557, vinblastine and verapamil; effects on equilibrium and kinetic binding properties of [³H]-vinblastine to CHᵣB30 membranes

Binding assays were used to elucidate the molecular mechanism underlying the non-competitive interaction of SR33557 and vinblastine with verapamil, on ATP hydrolysis by P-gp. [³H]-vinblastine has previously been demonstrated to bind with high affinity ($K_d = 37$ nM) to P-gp in CHᵣB30 membranes (Callaghan *et al.*, 1997). In the present study SR33557 displaced approximately 80% of [³H]-vinblastine bound to P-gp with an IC_{50} 8.3 ± 4.5 nM ($n = 5$) and a slope value of 0.47 ± 0.12, implying a cooperative interaction between the substrates. Verapamil also displaced [³H]-vinblastine binding

to CH^{r}B30 membranes but with a 150 fold lower affinity (IC$_{50}$ = 1.27 ± 0.74 μM, slope = 0.91 ± 0.35, n = 4) than SR33557 (P < 0.02). There was no specific binding of [^{3}H]-vinblastine to membranes from the non-P-gp expressing AuxB1 cell line (data not shown). The non-specific binding component in CH^{r}B30 membranes (4.2 ± 0.2 pmol mg^{-1}) was reduced by less than 8% of total [^{3}H]-vinblastine binding by SR33557 (3.3 ± 0.2 pmol mg^{-1}, NS) and verapamil (3.3 ± 0.2 pmol mg^{-1}, NS).

The effects of a compound on the rate of dissociation from a target of a second substrate has frequently been used to examine binding site interactions (Kostenis *et al.*, 1996). In the presence of 1.5 μM vinblastine, to stop the association reaction, the dissociation of the [^{3}H]-vinblastine-P-gp complex was described by a rate constant of 0.0211 ± 0.0018 min^{-1} (n = 12) (Figure 2b and Table 2). In the presence of nicardipine (3 μM), which has previously been shown to increase the rate of [^{3}H]-vinblastine dissociation from human P-gp, there was significant elevation of the rate constant in CH^{r}B30 membranes (0.4259 ± 0.042 min^{-1}, P < 0.02, n = 3). The dissociation of the [^{3}H]-vinblastine-P-gp complex was accelerated 3 fold (P < 0.05) by 3 μM SR33557 to 0.0751 ± 0.0129 min^{-1} and 2–3 fold by 10 μM verapamil to 0.0902 ± 0.0212 min^{-1} (Figure 2b, Table 2). Such an increase in the vinblastine dissociation rate constant by SR33557 and verapamil is evidence of an allosteric interaction between these ligands and the vinca alkaloid binding site. This provides further evidence for the existence of multiple drug acceptor sites on P-gp.

Discussion

Due to the development of multiple drug resistance (MDR) in tumours, intensive efforts have been undertaken to develop chemotherapeutic strategies to overcome the actions of the drug efflux pump P-gp. However, two major problems exist, (1) the reversal agents developed thus far suffer from a lack of potency (Dalton *et al.*, 1989; Miller *et al.*, 1991) and (2) there is little known about the interaction site(s) of drugs with P-gp. The indolizin sulphone SR33557 has previously been shown to reverse more potently the MDR phenotype than the well characterized chemosensitizer verapamil (Jaffrezou *et al.*, 1991). However, in that study SR33557 was able to increase the accumulation of doxorubicin, but not displace azidopine labelling of P-gp in MDR cells. Since both of these compounds interact with P-gp, the presence of multiple drug interaction sites was proposed. In the present study, we have demonstrated multiple, allosterically linked binding sites on P-gp through the interaction of SR33557 with verapamil and vinblastine.

We have shown that SR33557 interacts with P-gp as a high affinity ligand, displacing vinblastine binding with a potency 150 fold greater than that of verapamil, and similar to that

Table 2 The effects of SR33557 and verapamil on the dissociation rate (k_{-1}) of [^{3}H]-vinblastine from CH^{r}B30 membranes

Dissociating agent	k_{-1} (min)	n	P
Vinblastine	0.0211 ± 0.0018	12	
Vinblastine + SR33557	0.0751 ± 0.0129	5	< 0.02
Vinblastine + verapamil	0.0902 ± 0.0212	5	< 0.05

The binding of [^{3}H]-vinblastine (25 nM) was allowed to reach equilibrium at 25°C for 120 min. The association reaction was then blocked by 1.5 μM unlabelled vinblastine alone or in the presence of 3 μM SR33557 or 10 μM verapamil at 12°C. Dissociation rate constants were obtained from the slope on plots of ln(B$_t$/B$_e$) versus time as described in Methods. Results are expressed as mean ± s.e.mean and the number of observations from independent experiments indicated by *n*.

shown for cyclosporin A (Ferry *et al.*, 1995). In addition to displacing the binding of vinblastine to P-gp, SR33557 was able to inhibit vinblastine accumulation in drug resistant cells with a greater potency than verapamil. A similar high potency for modulating daunorubicin transport has been previously observed in murine leukaemia cells (Jaffrezou *et al.*, 1991). Previous kinetic studies on transport in P-gp expressing cells suggest the presence of distinct sites for vinca alkaloids and anthracyclines (Spoelstra *et al.*, 1992; Ayesh *et al.*, 1996). Consequently, the observation that SR33557 affects the transport of both vinblastine and daunomycin may be explained by an interaction of SR33557 at multiple sites on P-gp. It has also been shown that verapamil interacts non-competitively on daunomycin and vinblastine transport by P-gp (Ferry *et al.*, 1992; Pereira *et al.*, 1994; Spoelstra *et al.*, 1994; Ayesh *et al.*, 1996). Additionally, the inability of SR33557 to displace azidopine labelling is suggestive of non-equivalent or multiple distinct binding sites on P-gp. Alternatively, it is also possible that SR33557, rather than interacting at multiple sites, may modulate transport of vinblastine or daunomycin transport through an allosteric mechanism.

By using combinations of P-gp substrates to assess ATPase activity, we were able to gain further insight into the nature of ligand interactions with this protein. Interestingly, not all drug binding events modulate ATPase activity in the same fashion, which suggests heterogeneity of the drug binding sites on P-gp. Furthermore, we have shown that SR33557 and vinblastine significantly modulated the stimulant effect of verapamil on basal ATPase activity, at concentrations where they elicited only minor effects when added alone. This modulation was non-competitive, due to the observed decrease in V$_{max}$ in the absence of an altered K_m for verapamil. Similar non-competitive interactions on the ATPase activity of P-gp have been observed for verapamil with several peptides and steroid hormones known to bind to P-gp (Sarkadi *et al.*, 1994; Orlowski *et al.*, 1996; Garrigos *et al.*, 1997). The non-competitive effects of the drugs on ATPase activity found in this study, also suggest the existence of multiple distinct but interacting binding sites on P-gp.

To determine whether there was cooperativity between the binding sites on P-gp, dissociation kinetic experiments were conducted. It has previously been shown that the 1,4,dihydropyridines interact at a distinct site to vinblastine by virtue of their ability to dissociate vinblastine from P-gp (Ferry *et al.*, 1992). An increase in the dissociation rate constant of a ligand from its receptor by another compound, according to the law of mass-action, can only be explained by allosteric modulation (Weiland & Molinoff, 1981; Motulsky & Mahan, 1983). The finding that SR33557 and verapamil accelerate the dissociation of vinblastine from P-gp, is evidence that these ligands bind to sites which are distinct, but linked by negative heterotropic cooperativity.

Thus, the data presented in this manuscript describing the interactions between SR33557, vinblastine and verapamil at the level of ATPase activity and drug dissociation kinetics, argue strongly for a multiple binding site model of P-gp. Furthermore, we have shown these distinct sites appear to be allosterically linked. There are several other lines of supporting evidence for this from studies investigating (a) transport of daunomycin and its interaction with several multidrug resistance modulators (Spoelstra *et al.*, 1992; Ayesh *et al.*, 1996), (b) the dissociation of vinca alkaloids by the 1,4-dihydropyridines (Ferry *et al.*, 1992), (c) interaction between steroid hormones and verapamil (Orlowski *et al.*, 1996), and (d) the non-competitive binding interaction between azidopine and vinblastine (Tamai & Safa, 1991). A multiple binding site model for P-gp, incorporating two possible mechanisms of non-competitive interaction, can be used to explain the effects seen with SR33557, vinblastine and verapamil in the present study. Firstly, it is possible that SR33557 and vinblastine do not disrupt verapamil binding to P-gp, but rather uncouple this binding from ATP hydrolysis. Alternatively, given the negative cooperativity shown between binding sites, it seems more likely

that the binding of SR33557 and vinblastine alters verapamil association with the protein at a distinct site.

In summary, by using the high affinity ligand SR33557, we have demonstrated multiple binding sites on P-gp which are allosterically linked, and have differential effects on the ATPase activity of the protein. Future work will be directed towards understanding the mechanisms underlying drug transport and how it may be coupled to ATP hydrolysis.

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