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Communication between Multiple Drug Binding Sites on P-glycoprotein

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ABSTRACT

P-glycoprotein, a member of the ATP-binding cassette transporter family, is able to confer resistance on tumors against a large number of functionally and chemically distinct cytotoxic compounds. Several recent investigations suggest that P-glycoprotein contains multiple drug binding sites rather than a single site of broad substrate specificity. In the present study, radioligand-binding techniques were used to directly characterize drug interaction sites on P-glycoprotein and how these multiple sites interact. The drugs used were classified as either 1) substrates, which are known to be transported by P-glycoprotein (e.g., vinblastine) or 2) modulators, which alter P-glycoprotein function but are not themselves transported by the protein (e.g., XR9576). Drug interactions with P-glycoprotein were either competitive, at a common site, or noncompetitive, and therefore at distinct sites. Based on these data, we can assign a minimum of four drug binding sites on P-glycoprotein. These sites fall into two categories: transport, at which translocation of drug across the membrane can occur, and regulatory sites, which modify P-glycoprotein function. Intriguingly, however, some modulators interact with P-glycoprotein at a transport site rather than a regulatory site. The pharmacological data also demonstrate that both transport and regulatory sites are able to switch between high- and low-affinity conformations. The multiple sites on P-glycoprotein display complex allosteric interactions through which interaction of drug at one site switches other sites between high- or low-affinity conformations. The data are discussed in terms of a model for the mechanism of transport by P-glycoprotein.

ATP-binding cassette (ABC) protein family members mediate the transmembrane transport of molecules varying from ions through large polypeptides, yet despite this diversity of substrate, most individual ABC transporters display tightly restricted substrate specificity. An exception is mammalian P-glycoprotein (P-gp), which is able to interact with a large number of drugs that are structurally or functionally unrelated (Zamora et al., 1988; Ferry and Kerr, 1994). The over-expression of P-gp in cancer cells causes multidrug resistance (MDR), which results in the failure of cancer chemotherapy for a wide range of cytotoxic agents (Gottesman and Pastan, 1993). To circumvent this clinical problem, much effort has focused on finding pharmacological inhibitors of P-gp. Several generations of inhibitors, comprising both established pharmacologic agents and novel compounds, have been developed to overcome P-gp–mediated MDR (Woodhouse and Ferry, 1995; Ferry, 1998). How, then, does P-gp interact with such a large number of diverse compounds in an apparent contravention of the "lock-key" hypothesis for protein-substrate binding?

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The presence of multiple drug binding sites on P-gp could potentially account for the wide range of compounds known to interact with this protein. Indeed, several lines of evidence argue for multiple drug interaction sites. First, several pharmacological studies measuring ATP hydrolysis and cellular drug accumulation indicate a multiplicity of drug interactions with P-gp (Malkhandi et al., 1994; Ayesh et al., 1996; Martin et al., 1997; Pascaud et al., 1998). These distinct sites may interact as demonstrated by the stimulation of Hoechst 33342 transport by rhodamine 123 (Shapiro and Ling, 1997). Photoaffinity labeling using azidopine has identified interaction sites on both the N- and C-terminal halves of P-gp and it has been proposed that these two sites may then form a single region in the overall structure (Greenberger et al., 1991; Morris et al., 1994). In contrast, functional differences exist between the two distinct sites of iodoarylazidoprazosin labeling on P-gp (Dey et al., 1997). Additionally, there is evidence for allosteric regulatory sites distinct from transport sites. Thus, compounds such as the indolizin sulfone SR33557 (Martin et al., 1997) and the 1,4-dihydropyridines (Ferry et al., 1992) are able to confer allosteric control to the binding site on P-gp for the transported substrate vinblas-

ABBREVIATIONS: ABC, ATP binding cassette; P-gp, P-glycoprotein; MDR, multidrug resistance.

tine. Several issues, however, remain unresolved, such as the number of sites that exist, their proximity and location, whether the sites for transported substrates and those for modulators are entirely separate, and the manner in which these sites might influence each other.

Most mechanistic descriptions of transporter proteins require that substrate binding site(s) alternate between high- and low-affinity conformations exposed at alternate faces of the membrane during the transport process (Van Winkle, 1999). This may be caused by stimuli such as drug binding and/or ATP hydrolysis. Conformational changes in P-gp have been demonstrated using ^{2}H/H-exchange kinetics (Sonveaux et al., 1996, 1999), proteolytic accessibility (Wang et al., 1998), and changes in antibody epitope recognition (Mechetner et al., 1997).

In the present investigation, drug interactions on P-gp were characterized using radioligand binding studies with several substrates and modulators of P-gp. Noncompetitive interactions indicate distinct binding sites and may be detected in radioligand binding assays as an altered binding capacity, an increase or decrease in the rate of drug dissociation, or a slope from Schild analysis that is not equal to unity (Kenakin, 1997). On this basis, we are able to assign a minimum of four distinct drug binding sites on P-gp. It is conceivable, however, that more sites may exist. There seems to be a network of interactions between these sites and these allosteric communications may shift any given site between high- and low-affinity conformations.

Experimental Procedures

Materials. Vinblastine, paclitaxel, nicardipine, verapamil, rhodamine 123, and benzamidine were purchased from Sigma (Poole, UK). Hoechst 33342 was purchased as a 10 mg/ml solution from Molecular Probes (Leiden, Netherlands) and the detergent-compatible protein assay kit from BioRad (Hemel Hempstead, UK). [^{3}H]Vinblastine sulfate (13–18 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Amersham, UK) and [^{3}H]XR9576 (32 Ci/mmol) was supplied by Xenova Ltd (Slough, UK). XR9576 (an anthranilic acid derivative), XR9051 (a diketopiperazine derivative), and GF120918 (an acridonecarboxamide) were all synthesized and provided by Xenova Ltd. Ready Protein scintillation fluid was purchased from Beckman Instruments (High Wycombe, UK) and dimethyl sulfoxide from BDH (Poole, UK).

Cell Culture and Membrane Preparation. Drug-resistant (CH^{r}B30) and sensitive (AuxB1) Chinese hamster ovary cell lines were grown as described previously (Kartner et al., 1983) in α-minimum essential medium supplemented with 10% (v/v) fetal calf serum. The drug resistant CH^{r}B30 cells were grown in the presence of 30 μg/ml colchicine to maintain selection pressure on P-gp.

Plasma membrane preparations were prepared from both CH^{r}B30 and AuxB1 cell lines as described previously (Lever, 1977). Cells were disrupted using nitrogen cavitation and plasma membranes were harvested after centrifugation on a sucrose cushion. Membranes were stored for up to 6 months at −70°C in 0.25 M sucrose/10 mM Tris·HCl buffer, pH 7.4, containing the protease inhibitors (0.1 mg/ml leupeptin, 0.1 mg/ml pepstatin A, and 1 mM benzamidine).

Equilibrium Binding of [^{3}H]Vinblastine and [^{3}H]XR9576. The binding of [^{3}H]vinblastine to P-gp was followed by allowing CH^{r}B30 plasma membrane (5 μg of protein) and radioligand (0 to 150 nM) added in a total volume of 100 μl of binding buffer (50 mM Tris·HCl, pH 7.4) to come to equilibrium (120 min) at room temperature in the dark. After this incubation, 3 ml of ice-cold wash buffer (50 mM $MgSO_4$, 20 mM Tris·HCl, pH 7.4) was added and the samples rapidly filtered through Whatman GF/F filters briefly presoaked in wash buffer containing 0.1% bovine serum albumin. The amount of radioactivity trapped on the filter was determined by liquid scintillation counting. The amount of nonspecific binding was determined in the presence of 10 μM verapamil and subtracted from the total amount bound at each concentration of [^{3}H]vinblastine. It is important to avoid using an unlabeled analog of the radioligand to ensure that a true measure of nonspecific binding is determined (Kenakin, 1997). Modulators were added in the concentration range (10^{-9} to 10^{-5} M) from 100× stock solutions in dimethyl sulfoxide to maintain low solvent concentration.

The binding of [^{3}H]XR9576 and [^{3}H]paclitaxel was measured as described above with minor modifications. Assays using [^{3}H]XR9576 and [^{3}H]paclitaxel required 2 and 20 μg of membrane protein, respectively, and the equilibration time for [^{3}H]XR9576 was increased to 180 min. Nonspecific binding was determined for [^{3}H]XR9576 assays with 3 μM vinblastine and 1 μM GF120918 for [^{3}H]paclitaxel.

The data were plotted as the amount of radioligand bound (picomoles per milligram of membrane protein) as a function of radioligand concentration, and similar curves were plotted at each concentration of modulator used. The following curve describing saturation binding was fitted to the data by nonlinear regression and estimates made of maximal binding capacity (B_{max}) and affinity of binding (K_d):

$$B_d = \frac{B_{max} \cdot [L]}{(K_d + [L])} \quad (1)$$

The Effects of Competitive/Noncompetitive Agents on Radioligand Binding. For modulators that decreased the binding of radioligand to P-gp, the estimates of B_{max} were plotted as a function of modulator concentration. From such a plot, the potency of a modulator to alter binding capacity was determined from the EC_{50} value by nonlinear regression of the general dose-response equation;

$$Y = \frac{(Y_{max} - Y_{min})}{(1 + ([B]/EC_{50})^n)} + Y_{min} \quad (2)$$

where Y_{max} = maximal binding capacity (pmol/mg), Y_{min} = minimum binding capacity obtained (pmol/mg), [B] = modulator concentration (M), EC_{50} = concentration of modulator that causes a 50% reduction in B_{max} (M), and n = slope factor.

Modulators may, however, act by altering only the affinity of radioligand for binding to P-gp, in which case Schild analysis is required (Kenakin, 1997). From a series of saturation curves in the presence of increasing modulator concentrations, the apparent dissociation constant was determined as described above. These values for apparent K_d were divided by the dissociation constant obtained in the absence of modulator to give a dose-ratio (DR). Therefore, the DR is a measure of the parallel shift to the right in a saturation binding curve by a modulator. The DRs [log(DR-1)] were then expressed as a function of modulator concentration and fitted using linear regression with the following equation:

$$\log_{10}(DR - 1) = n \cdot \log_{10}[B] - \log_{10}(K_b) \quad (3)$$

where n = slope, [B] = concentration of modulator (M), and K_b = modulator affinity constant.

Linear Schild plots with slopes of 1 provide accurate measures of the modulator affinity of binding to a protein and also proof of competitive interactions between drugs (Kenakin, 1997). Anomalies may occur in this relationship if there is significant drug disposition/metabolism or heterogenous tissue responses. However, these types of scenarios did not occur in a plasma membrane system.

Kinetics of Radioligand Binding to P-gp. The kinetics of drug binding to P-gp in CH^{r}B30 membranes was also followed using the rapid filtration assay described above. The membranes and a single concentration of either [^{3}H]vinblastine (30 nM) or [^{3}H]XR9576 (3 nM) were allowed to reach equilibrium. After reaching binding equilibrium, unlabeled ligand (3 μM vinblastine or 1 μM XR9576), in the presence or absence of a range of modulator concentrations, was

added for various times before filtration. Samples containing [^{3}H]vinblastine were incubated with modulators for 1, 2, 4, 6, 8, or 10 min, although samples containing [^{3}H]XR9576 were incubated with modulators for 10, 30, 45, 55, or 60 min. Profiles of the amount of radioligand bound (B_d) as a function of time were linearized by plotting the natural logarithm of the amount bound at time t (B_t), over the amount bound at equilibrium (B_e). The dissociation rate constant is the slope of this relationship and was determined by linear regression. The dissociation rate constant for each ligand was subsequently plotted as a function of modulator compound and the general dose-response relationship (eq. 2) was fitted by nonlinear regression. The value for Y_{max} corresponds to the maximal dissociation rate, and the EC_{50} value reflects the potency of a modulator to increase the dissociation of radioligand.

Data Analysis. All curve fitting and regression analyses were done using Prism 2.0 (GraphPad Software, San Diego, CA). Statistical comparisons of mean values were done using the Student's *t* test and a *P* value of .05 was considered significant.

Results

Binding of [^{3}H]Vinblastine and [^{3}H]XR9576 to CH^{r}B30 Membranes. The P-gp substrate [^{3}H]vinblastine and the high-affinity modulator [^{3}H]XR9576 were used as primary ligands to measure drug binding to P-gp. There was no measurable specific binding of [^{3}H]vinblastine to membranes from the non-Pgp expressing cell line (AuxB1). Total binding of [^{3}H]vinblastine to CH^{r}B30 membranes was <5% of the total radioactivity added and nonspecific binding accounted for 10 to 20% of total binding. [^{3}H]vinblastine bound to CH^{r}B30 membranes with an affinity of K_d = 10 ± 2 nM and a density of binding sites of 85.4 ± 10 pmol/mg. Saturation binding curves for the [^{3}H]vinblastine concentrations used were best described by a single class of binding site (*F* test, $P < .05$). The kinetics of [^{3}H]vinblastine binding to CH^{r}B30 membranes was also investigated. In the presence of excess ligand (3 μM vinblastine), the dissociation of Pgp-[^{3}H]vinblastine complexes was characterized by a rate constant of 0.093 ± 0.002 min^{-1} (n = 5). Using a dilution method, the dissociation rate constant was not different from that obtained with excess unlabeled ligand indicating a simple rather than cooperative binding of [^{3}H]vinblastine binding to P-gp (data not shown).

The high-affinity modulator [^{3}H]XR9576 also displayed specific binding to membranes from the P-gp expressing CH^{r}B30 cells but not from the parental AuxB1 cells. Total binding did not exceed 15% of the total radioactivity added and nonspecific binding accounted for <5% of the total binding. [^{3}H]XR9576 bound with high affinity (K_d = 2.9 + 0.1 nM) and a binding capacity of B_{max} = 140 ± 20 pmol/mg. [^{3}H]XR9576, like [^{3}H]vinblastine, bound at a single class of site (*F* test, $P < .05$) (Martin et al., 1999). The dissociation kinetics of the P-gp–[^{3}H]XR9576 was also investigated using an excess of unlabeled ligand (1 μM XR9576). The dissociation rate constant for this species was 0.0230 ± 0.0011 min^{-1} (n = 5).

Possible reasons for the 2-fold difference in binding capacity of P-gp for [^{3}H]vinblastine and [^{3}H]XR9576 may be that either XR9576 binds at a single class of site, but there is twice the number of this class of site on P-gp compared with the vinblastine binding site class, or drug binding sites exist in an equilibrium between high- and low-affinity states and that the distributions are not the same for vinblastine and XR9576 binding sites. The present study was not able to detect the low-affinity binding because of an inability to reach appropriately high concentrations of radioligand.

Effects of XR9576, XR9051, GF120918, and Nicardipine on the Equilibrium and Kinetic Binding of [^{3}H]Vinblastine. The compounds XR9051, XR9576, GF120918, and nicardipine have been previously demonstrated to reverse MDR by modulating P-gp transport rather than acting as competing substrates (Hyafil et al., 1993; Dale et al., 1998; Martin et al., 1999). A common property of these modulators is the ability to reduce the maximal binding capacity of P-gp for [^{3}H]vinblastine (Fig. 1). All four modulators were able to reduce the B_{max} to below 5% of the value obtained in the absence of modulator, but their respective potencies varied. Figure 1a shows the relationship between the maximal binding capacity of [^{3}H]vinblastine as a function of XR9576 concentration. XR9576, an anthranilic acid derivative, was able to abrogate the binding of [^{3}H]vinblastine to P-gp in CH^{r}B30 membranes. XR9576 reduced the B_{max} for [^{3}H]vinblastine with an EC_{50} value of 11.8 ± 0.3 nM (n = 3). Similar reductions in the binding capacity of P-gp for [^{3}H]vinblastine were observed for the modulators XR9051, GF120918, and nicardipine (Fig. 1, b–d). XR9051 (EC_{50} = 19.2 ± 0.4 nM; n = 3), a diketopiperazine, and the acridonecarboxamide GF120918 (EC_{50} = 11.2 ± 0.2; n = 3) displayed high potencies for altering [^{3}H]vinblastine binding, whereas nicardipine, a 1,4-dihydropyridine, displayed the lowest potency (EC_{50} = 459 ± 12 nM, n = 3). There was no measurable alteration in the affinity of [^{3}H]vinblastine binding to P-gp by any of the modulators.

In summary, the modulators XR9576, XR9051, GF120918, and nicardipine alter [^{3}H]vinblastine binding in a noncompetitive fashion because the B_{max} for vinblastine binding, but not the K_d, was reduced in each case. This demonstrates that the modulators interact on P-gp at a site distinct from that for vinblastine. Moreover, it suggests an allosteric communication between sites for vinblastine binding and those for the modulators.

To prove this allosteric interaction, the influence of these compounds on the dissociation rate of [^{3}H]vinblastine was investigated (Table 1). All four modulators caused statistically significant 3- to 4-fold increases in the rate of dissociation for [^{3}H]vinblastine. The modulators with the greatest potency were XR9051 (EC_{50} = 290 ± 11 nM) and GF120918 (EC_{50} = 168 ± 11 nM). The potency of nicardipine (EC_{50} = 1.2 ± 0.2 μM) was roughly 10-fold less than that of these two compounds, consistent with the less potent effect of nicardipine in reducing the B_{max} for [^{3}H]vinblastine. However, the relatively poor potency of XR9576 (EC_{50} = 456 ± 40 nM) is surprising compared with its high potency in inhibiting [^{3}H]vinblastine binding, and this purely quantitative difference may be related to the nature of the experiment. Displacement assays involved coequilibration of all drugs with membranes, whereas dissociation assays allowed radioligand and membranes to reach equilibrium before the addition of unlabeled ligand and modulator.

These data show that not only do the modulators XR9576, XR9051, GF120918, and nicardipine bind at a site(s?) distinct from [^{3}H]vinblastine, but also that they are able to reduce the number of binding sites for the radioligand through a negative allosteric interaction.

Influence of Paclitaxel, Rhodamine 123, and Hoechst 33342 on [^{3}H]Vinblastine Binding Parameters. None of the modulators examined in the preceding section has been

demonstrated to be transported by P-gp. Do substrates of P-gp alter [^{3}H]vinblastine binding in a fashion similar to that of modulators, or do they display a competitive interaction for a common transport site? To address this point, the effect of known transported substrates such as paclitaxel, rhodamine 123, and Hoechst 33342 on [^{3}H]vinblastine binding was investigated. As shown in Fig. 2, a to c, all three transported substrates were able to reduce the binding capacity of P-gp for [^{3}H]vinblastine in a manner similar to the modulators described in the previous section. Paclitaxel and rhodamine 123 completely abolished the binding of [^{3}H]vinblastine with potencies of EC_{50} = 4.8 ± 0.1 μM (n = 3) and EC_{50} = 9.0 ± 0.4 μM (n = 3), respectively. Hoechst 33342 also reduced the B_{max} to less than 5% of the value in its absence (EC_{50} = 1.26 ± 0.01 μM; n = 3), although in this case the slope factor for the relationship was >3. In comparison, the relationships

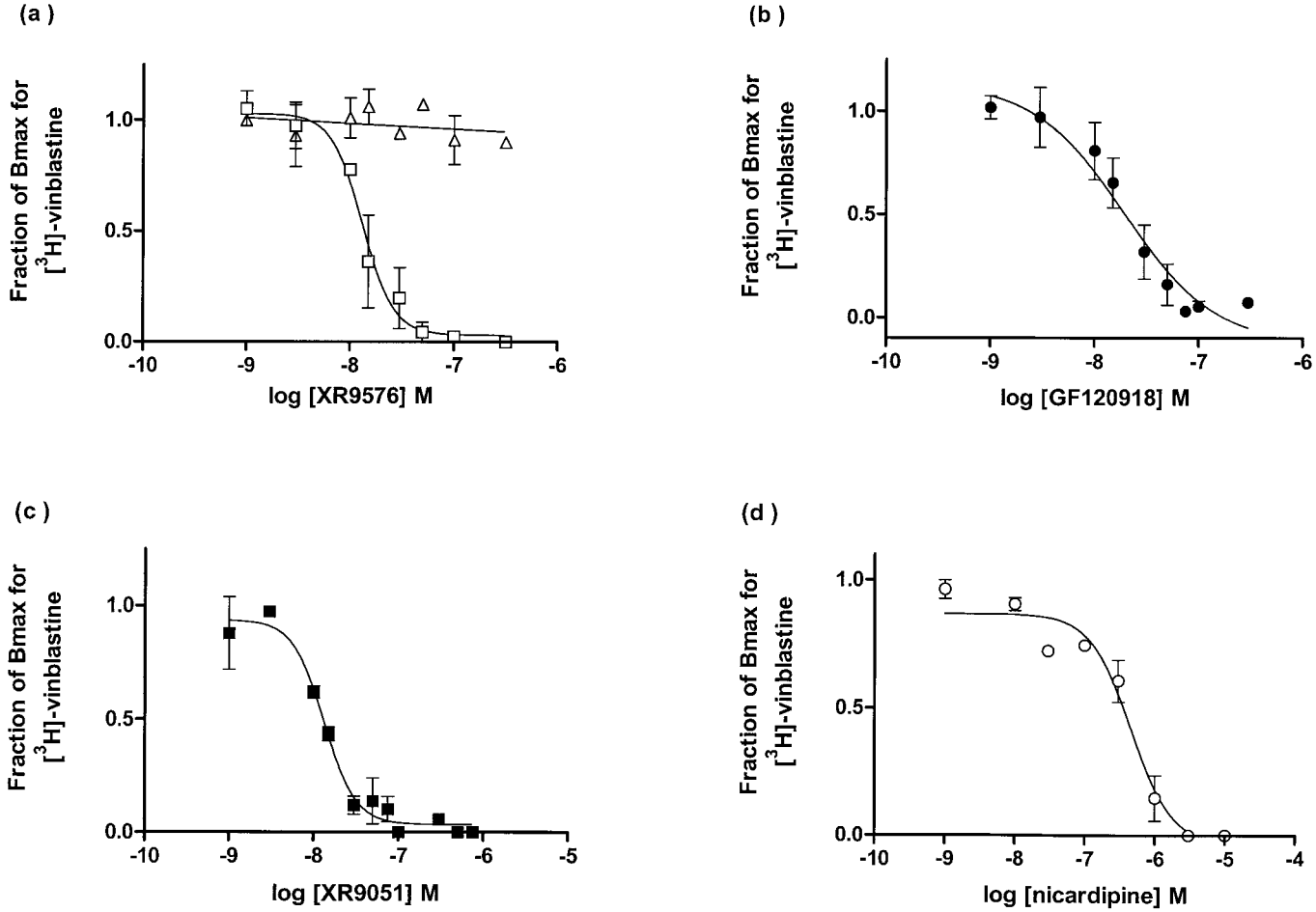

Fig. 1. Effect of modulators on [^{3}H]vinblastine binding. The maximal binding capacity of [^{3}H]vinblastine (1–150 nM) to P-gp in CH^{r}B30 membranes was determined over a range of concentrations of XR9576 (a), GF120918 (b), XR9051 (c), and nicardipine (d). The dose-dependent effect of each drug on the binding of [^{3}H]vinblastine was used to derive a potency for each compound. Data are also shown (△) for XR9576 to illustrate the lack of effects on affinity (K_d) of [^{3}H]vinblastine binding by any modulator. Values of B_{max} in the presence of modulator were expressed as a fraction of the value in the absence of modulator. The values represent mean ± S.E. of at least three independent determinations.

TABLE 1

The kinetics of drug interactions between modulators and substrates on P-gp

The dissociation rate constants for the outlined compounds on the binding of either [^{3}H]vinblastine or [^{3}H]XR9576 were determined as described under *Experimental Procedures*. The dissociation rates were determined at a number of drug concentrations and the maximal rate constant (k_{-1}) was estimated from nonlinear regression of the general dose-response equation. The potency of each compound to increase the dissociation rate constant for radioligand is shown as an EC_{50} value.

Drug (n > 3)	[^{3}H]Vinblastine			[^{3}H]XR9576		
	Maximal k_{-1}	Fold Change	EC_{50}	Maximal k_{-1}	Fold Change	EC_{50}
	min^{-1}		μM	min^{-1}		nM
Vinblastine	0.093					
XR9576	0.493	5.3	0.456	0.0230		
XR9000	0.293	3.2	0.170	0.0315	1.4	78
Nicardipine	0.452	4.9	1.2	0.0494	1.5	1700
XR9051	0.370	4	0.29			
Paclitaxel	0.114	1.2	0.024			
Hoechst 33342	0.317	3.4	18			

for paclitaxel and rhodamine 123 were best fitted with slope factors of 1 (F test, P values). This implies a degree of cooperativity in the relationship between Hoechst 33342 and [^{3}H]vinblastine. There was no effect of paclitaxel, rhodamine 123, or Hoechst 33342 on the affinity of [^{3}H]vinblastine binding to P-gp. Thus, in a manner similar to the modulators, albeit with lower potency, the transported compounds paclitaxel, rhodamine 123, and Hoechst 33342 displayed a noncompetitive interaction with [^{3}H]vinblastine binding.

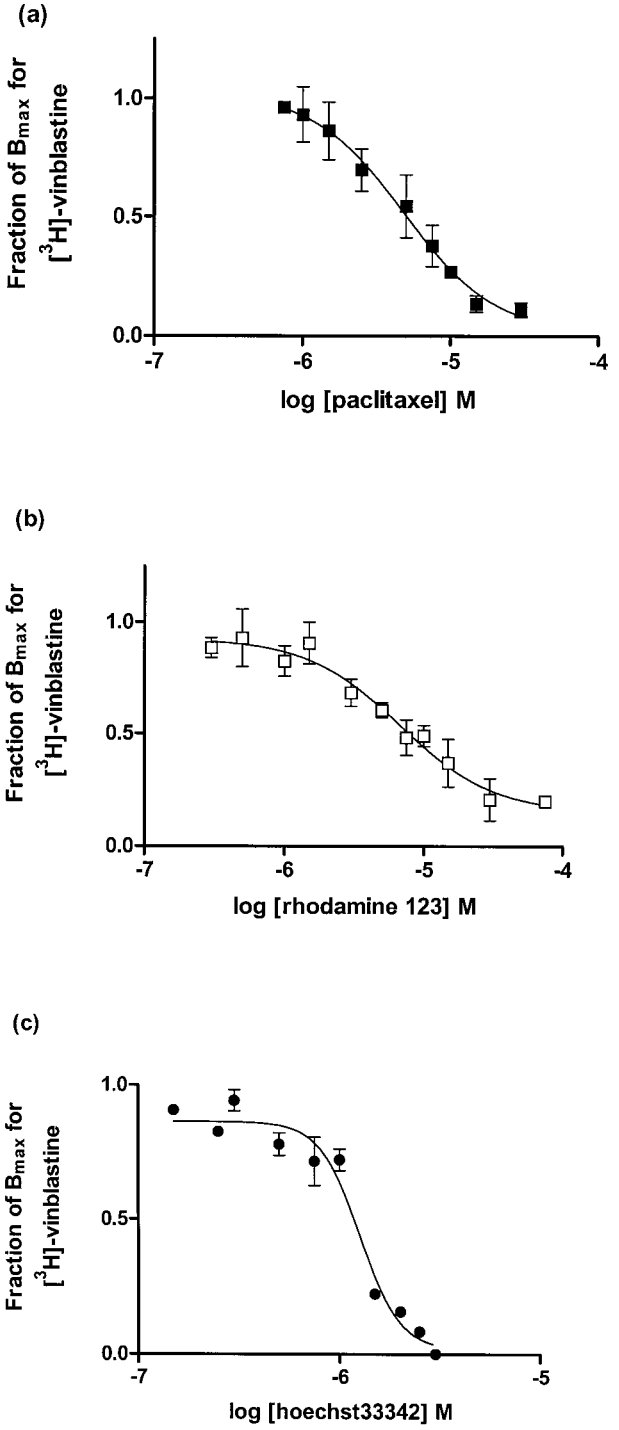

Fig. 2. Effect of transported substrates on [^{3}H]vinblastine binding. The effects of paclitaxel (a), rhodamine 123 (b), and Hoechst 33342 (c) on the binding of [^{3}H]vinblastine were determined from saturation isotherms of the radioligand. The results displayed describe the effects of these compounds on the maximal binding capacity of [^{3}H]vinblastine on CH^{r}B30 membranes. The values represent mean ± S.E. of at least three independent determinations.

Hoechst 33342 caused a 3.4-fold increase in the dissociation rate of [^{3}H]vinblastine (Table 1) with a potency of EC_{50} = 18 ± 2 μM (n = 3). Consequently, Hoechst 33342 is able to reduce the number of [^{3}H]vinblastine binding sites on P-gp through an allosteric action that promotes breakdown of the Pgp-[^{3}H]vinblastine complex. There was a small but statistically nonsignificant increase in the dissociation rate of [^{3}H]vinblastine by paclitaxel. In addition, there was no measurable effect of rhodamine 123 on the dissociation rate for [^{3}H]vinblastine. This may reflect merely a poor allosteric efficacy of these two compounds after their binding to P-gp, in contrast to the modulators outlined in the previous section.

Alteration of [^{3}H]XR9576 Binding to CH^{r}B30 Membranes by Vinblastine and Hoechst 33342. The above data demonstrate that P-gp modulators and substrates are able to noncompetitively affect the [^{3}H]vinblastine binding site. To assess whether allosteric communication occurs in the opposite direction, the effects of vinblastine and Hoechst 33342 on [^{3}H]XR9576 were investigated. Vinblastine did not cause a depression in the binding capacity of [^{3}H]XR9576 in the concentration range 10^{-6} to 3.10^{-4} M. However, there was a dose-dependent shift to the right in the saturation binding curves of [^{3}H]XR9576 to CH^{r}B30 membranes. The resultant Schild plot (Fig. 3a) had a slope of 0.52 ± 0.05 and showed a potency for vinblastine of K_b = 6.56 ± 0.56 μM (n = 4). The slope was significantly different ($P < .01$) from 1.0 and is thus indicative of a noncompetitive interaction between [^{3}H]XR9576 and vinblastine, confirming the same conclusion reached above. The affinity constant (K_b) obtained for vinblastine was more than 500-fold higher than the measured value for the dissociation constant, a finding also indicative of noncompetitive binding. Vinblastine did not cause an increase in the dissociation rate for [^{3}H]XR9576 from P-gp, suggesting that either the communication between these two distinct sites is not equivalent or that vinblastine has a much weaker allosteric coefficient than XR9576.

Hoechst 33342 also caused a reduction in the affinity of [^{3}H]XR9576 for binding to P-gp in CH^{r}B30 membranes. There was no effect of Hoechst 33342 on the shape of the binding isotherm, which may indicate a lack of allosteric modulation. The Schild plot of the interaction between Hoechst 33342 and [^{3}H]XR9576 binding is shown in Fig. 3b. The slope of this relationship was 1.14 ± 0.20, which is not significantly different from 1.0 (F test, $P < .05$), suggesting a competitive interaction between [^{3}H]XR9576 and Hoechst 33342. The affinity constant obtained for Hoechst 33342 from the Schild plot was 726 ± 76 nM (n = 3), which is similar to its reported potency (Shapiro and Ling, 1997). Hoechst 33342 did not alter the dissociation rate of [^{3}H]XR9576 (Table 1), providing further evidence that the two compounds interact competitively at the same site. In conclusion, based on these three lines of evidence, Hoechst 33342 and XR9576 interact at the same site and this site is distinct from the vinblastine-binding site.

Effects of XR9051, GF120918 and Nicardipine on the equilibrium and kinetic binding of [^{3}H]XR9576. As shown above, the modulators XR9576, XR9051, GF120918, and nicardipine all modulated the binding of [3H]vinblastine to P-gp by reducing the binding capacity. To determine

whether this effect occurs from a common binding site for all of these modulators, or from separate sites, the effects of XR9051, GF120918, and nicardipine on the binding of [^{3}H]XR9576 were examined. XR9051 and nicardipine both increased the K_d value for [^{3}H]XR9576 binding without any effect on the binding capacity (Fig. 4, a–c). The Schild plot describing the interaction between [^{3}H]XR9576 and XR9051 (Fig. 4a) had a slope of 0.981 ± 0.075, which is not significantly different from 1 (F test, $P < .05$) and an affinity of K_b = 89.3 ± 9.3 nM. Combined with the absence of any increase in the dissociation rate of [^{3}H]XR9576 in the presence of XR9051, their interaction with P-gp seems to be purely competitive. This is not unexpected, because the structure of XR9576 evolved from that of XR9051 (Martin et al., 1999). The Schild plot for the effects of nicardipine on [^{3}H]XR9576 binding (Fig. 4b) also generated a slope that was not significantly different from 1.0 (0.945 ± 0.066, n = 4). The value of K_b for XR9051 agrees with the reported potency for its binding to P-gp (Dale et al., 1998). However, the affinity constant for nicardipine (K_b = 4.89 ± 0.34 μM) is roughly 5- to 10-fold higher than that expected from its ability to modulate [^{3}H]vinblastine binding. Furthermore, nicardipine caused an increase in the dissociation rate of [^{3}H]XR9576 from P-gp (Table 1), which can only occur through a noncompetitive allosteric interaction. Thus XR9576, XR9051, and Hoechst 33342 (see above) seem to bind to the same site, whereas nicardipine must bind to a third site distinct from both the vinblastine and XR9576 sites.

The acridonecarboxamide GF120918 differed from XR9051

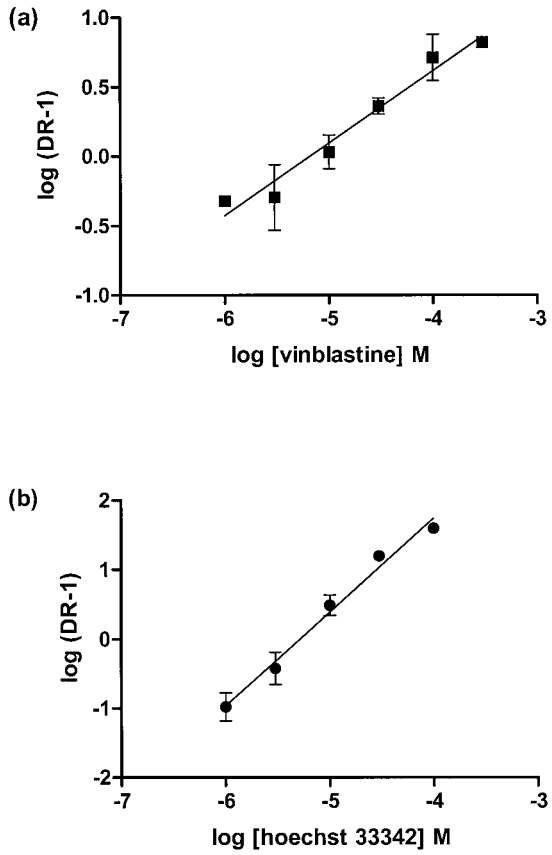

Fig. 3. Effects of vinblastine and Hoechst 33342 on [^{3}H]XR9576 binding. Schild plots describing the interaction between vinblastine (a) or Hoechst 33342 (b) on [^{3}H]XR9576 binding to P-gp. The shift in potency of the equilibrium binding of [^{3}H]XR9576 to P-gp in the presence of vinblastine and Hoechst 33342 was used to determine a DR. The DR was plotted as a function of unlabeled ligand. The slope of the relationship and the x-axis intercept are used to characterize the interaction between compounds on P-gp. Each value represents the mean ± S.E. from four independent experiments.

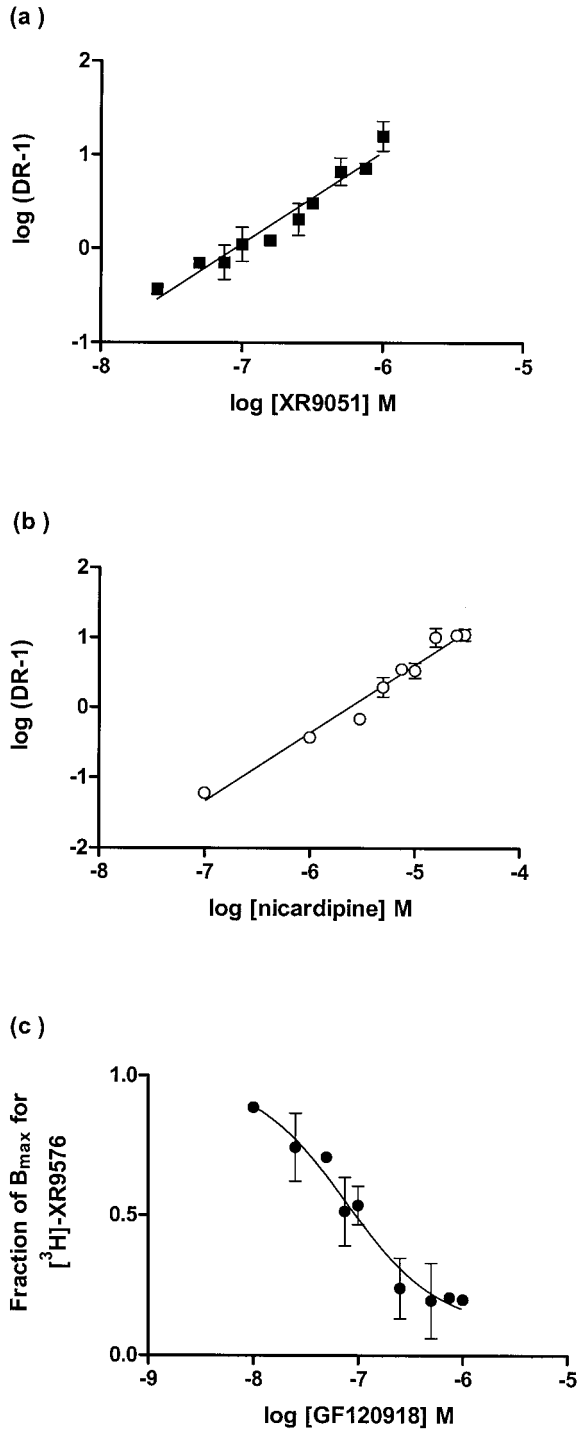

Fig. 4. Effects of XR9051, GF120918, and nicardipine on [^{3}H]XR9576 binding. a, interaction between XR9051 and [^{3}H]XR9576 described by plotting DR as a function of XR9051 concentration as described under *Experimental Procedures*. b, interaction between nicardipine and [^{3}H]XR9576 described by plotting DR as a function of nicardipine concentration. c, the effect of GF120918 on the maximal binding capacity of [^{3}H]XR9576 to P-gp. All values represent mean ± S.E. and were obtained from at least three independent experiments.

and nicardipine in that it caused a dose-dependent decrease in the B_{max} value obtained for [^{3}H]XR9576 binding without any effect on the affinity of binding (Fig. 4c). GF120918 caused a 75% reduction in the binding capacity of P-gp for [^{3}H]XR9576 with a potency of 85.9 ± 0.9 nM (n = 4). In addition, GF120918 was able to increase the dissociation of the Pgp-[^{3}H]XR9576 complex (Table 1), which also indicates a noncompetitive interaction. Therefore, GF120918 also does not bind to the vinblastine or XR9576 sites (see above) and it has been shown previously, with the use equilibrium binding assays, that GF120918 and paclitaxel interact in a noncompetitive fashion (Woodhouse, 1998). Therefore, it seems that the GF120918 (and perhaps nicardipine, because it was not possible to discriminate their respective binding sites) binds to a fourth site on P-gp.

Rhodamine 123 has previously been shown (Shapiro et al., 1999) to interact at a site distinct from Hoechst 33342; the latter, in our investigation, is common to XR9576/9051. Consequently, rhodamine 123 may bind to the GF120918, paclitaxel, or perhaps at an independent site. Our investigations could not discriminate between these options.

Discussion

The large number of substrates and modulators known to interact with P-gp suggests the presence of greater than one binding site. The presence of at least four distinct drug interaction sites on P-gp was detected using equilibrium and kinetic radioligand binding assays in this study and the conclusions are summarized in Fig. 5. However, it is quite possible that more sites may exist for drug interaction on P-gp. Sites I, II, and III may be classified as sites for transport because they interact with vinblastine, paclitaxel, rhodamine 123, and Hoechst 33342, all of which have previously been demonstrated to be substrates of P-gp (Ambudkar et al., 1999). In contrast, site IV seems to act as a regulatory site because modulators but not substrates could be assigned to interact with it. The four binding sites were able to allosterically communicate in a negative heterotropic manner. The data presented are in agreement with the recent studies by Shapiro et al. (1999) that demonstrated distinct sites for transport of rhodamine 123 and Hoechst 33342 in addition to a modulatory site for prazosin/progesterone. In fact, a number of studies using various techniques, such as photoaffinity labeling, ATP hydrolysis, and cellular drug efflux assays, have suggested multiple binding sites on P-gp (Bruggeman et al., 1989; Greenberger, 1993; Ayesh et al., 1996; Orlowski et al., 1996; Pascaud et al., 1998).

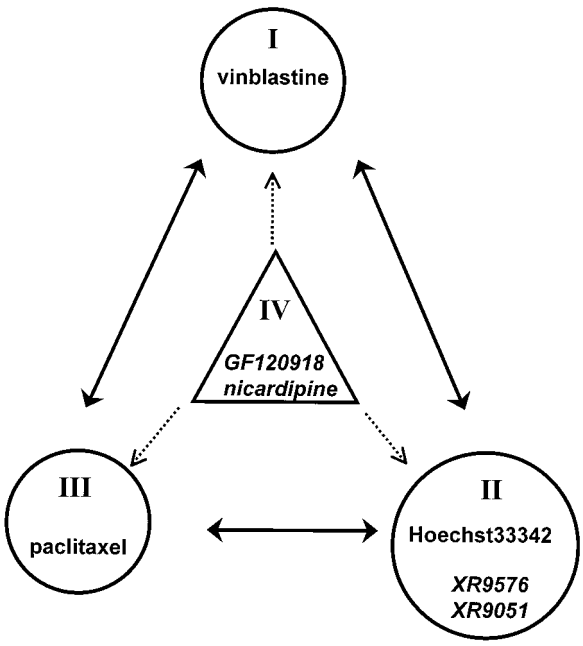

Fig. 5. Classification of four drug binding sites on P-gp. Based on the interactions described, the ligands used in this investigation may be classified into four binding sites on P-gp. However more drug binding sites may exist on the protein, and it was not possible to conclusively assign a site for rhodamine 123. The italicized compounds are modulators rather than transported substrates of P-gp. Arrows indicate communication between the four sites.

Site II, which binds both Hoechst 33342 and XR9576, is not distinct, but it is the only one for which evidence exists of both transport and regulatory functions. More precisely, the substrate Hoechst 33342 and the modulator XR9576, which is not a P-gp substrate and is able to inhibit ATP hydrolysis by the protein (Martin et al., 1999), cause distinct effects on P-gp from a common binding site. However, the allosteric communication of this site II with the other sites, for example the vinblastine site, is identical for both Hoechst 33342 and XR9576 binding. Does this information shed light on the mechanism of transport by P-gp? One of two events will most likely occur on occupation of a transport site by a modulator. First, occupation by a modulator may simply prevent the binding site from gaining access to the opposite side of the membrane and thereby prevent transport. Alternatively, normal reorientation of the binding site may still occur, but there is little or no dissociation of the modulator when exposed to the opposite side of the membrane, again preventing transport. Indeed, it has been demonstrated previously that the dissociation rate of [^{3}H]XR9576 is significantly slower than that of [^{3}H]vinblastine (Martin et al., 1999). Investigating the communication between binding sites may assist in demonstrating which possible scenario is the most likely. It is possible that any of the drug binding sites may undertake transport or modulatory roles and that the factor dictating the precise role is the compound bound and how it interacts with the site.

The model in Fig. 6 has been formulated to describe the nature of communication between the multiple drug binding sites on P-gp. The central tenet of this model is that each site has a high- and low-affinity state for its ligand and an equilibrium exists between the two. Moreover, the allosteric interactions between the sites are proposed to alter the equilibrium between the two states of a site. What is the consequence of negative heterotropic allosteric communication on a drug-binding site? In terms of binding characteristics, the major outcome is that, in the absence of an alteration in affinity, a reduction in the number of radioligand binding sites occurs in the presence of allosteric modulators. Such a marked allosteric effect on a binding site will occur through conformational alterations after inhibitor binding. Biophysical studies using ^{2}H/H-exchange kinetics and fluorescence quenching techniques have shown that nucleotides, substrates, and modulators are all able to elicit conformational changes in P-gp (Liu and Sharom, 1996; Sonveaux et al., 1996, 1999; Romsicki and Sharom, 1999). It has also been demonstrated by following antibody epitope and proteolytic

accessibility changes that these conformational changes display sensitivity to the various stages of the ATP hydrolytic cycle of P-gp (Mechetner et al., 1997; Wang et al., 1998). Furthermore, direct communication between substrate binding sites and the ATP binding site have been demonstrated using P-gp labeled with the fluorescent probe MIANS [2-(4-maleimidoanilino)naphthalene-6-sulfonic acid] (Liu and Sharom, 1996).

Several proteins mediating solute translocation across membranes also contain multiple sites for drug interaction; therefore, the presence of four substrate/modulator sites on P-gp is not unprecedented. For example, the L-type calcium channel contains three allosterically linked binding sites for different classes of drug (Glossmann et al., 1984). Unfortunately, in the case of P-gp, the location of these sites on the protein is yet to be elucidated. It is possible that the sites classified in this study may be found on distinct regions of P-gp. Alternatively, the sites may exist as part of a large binding pocket with discrete domains of specificity, as has been suggested previously (Shapiro et al., 1999). Regulatory sites such as those shown in Fig. 5 may reside outside this binding pocket. Data from the many mutagenesis investigations on P-gp do not clarify the locations of drug binding sites (for review, see Ambudkar et al., 1999). Most of the studies demonstrate that modification of specific residues leads to altered substrate specificity; however, it is not clear whether the sites are actual drug-recognition sites or affect some part of the translocation pathway. These approaches describe a clustering of residues that alter substrate specificity of P-gp to two pockets around membrane spanning segments 5/6 and 11/12. In the absence of structural details, it is not possible to ascertain whether these two regions form a single large binding pocket in the overall protein or whether they contain multiple small binding sites. Photoaffinity labeling approaches to localize the binding site region suffer because of the inherent mobility of the probes, which may therefore label residues distinct from the true binding site (Glossmann et al., 1987). The drug binding sites have been suggested to be accessible from the lipid bilayer (Raviv et al., 1990; Homolya et al., 1993), which is consistent with the hydrophobic nature of substrates and modulators. Biophysical parameters such as hydrophobicity indices, lipid diffusibility, and hydrogen bond acceptor strengths have been used to characterize structural features on drugs that mediate their interaction with P-gp (Chiba et al., 1996; Ecker et al., 1999). This type of analysis may indeed eventually provide biophysical characterization of drug binding sites on P-gp.

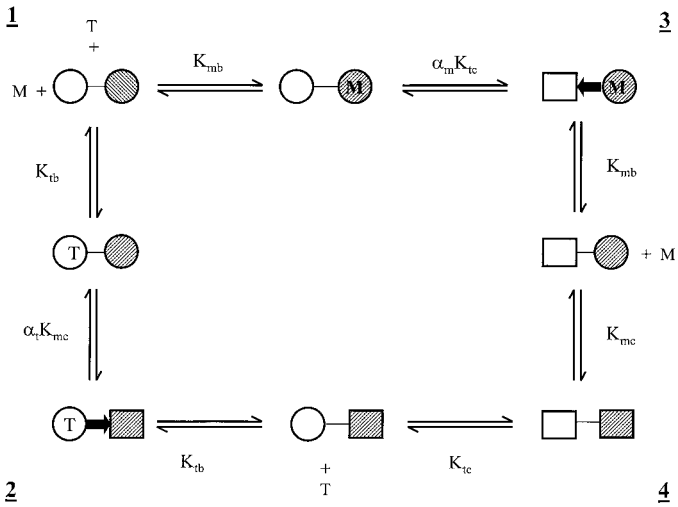

Fig. 6. Multiple site transition model of distinct drug binding sites on P-gp. The model presented depicts P-gp with two independent, spatially distinct but interacting ligand-binding sites for drugs T (○) and M (◎). Only two of the four sites designated in Fig. 5 are shown, for clarity. These two sites may exist in either high- (○) or low-affinity (□) configurations for their respective ligands. Straight lines between the sites indicate a communication route and a solid arrow depicts an allosteric effect. P-gp may exist in one of four distinct configurations with respect to the ligand binding sites shown. Measurable binding of a drug is only possible to the high-affinity conformation of its binding site. For example, both drugs could bind to state 1 of P-gp, but no measurable binding would be detected in state 4. The binding of drugs M and V to the binding sites is described by the equilibrium constants K_{mb} and K_{tb}, respectively. Transitions between high- and low-affinity states for the M and T binding sites are governed by the equilibria K_{mc} and K_{tc}, respectively. The symbols α_t and α_m correspond to the allosteric potencies of each drug to modify the other binding site.

What is the orientation or conformation of drug binding sites in their low-affinity states? Modulators may allosterically cause a decrease in the number of high-affinity sites by simply altering or occluding radioligand binding. Alternatively, the allosteric agent may lead to a reorientation and, importantly, a stabilization of the substrate binding site that mimics the "external" facing low-affinity stage of a transport cycle. There is good evidence for substrate-, modulator-, and nucleotide-induced conformational changes, but there is scant data on how these alterations are mediated. Perhaps the allosteric agent is able to alter the radioligand-binding site via interactions through the nucleotide binding domains of the protein. It has been demonstrated that trapping ADP and vanadate in the nucleotide binding domains was able to alter the photoaffinity labeling of P-gp by the modulator prazosin (Dey et al., 1997), thus indicating communication between the domains. Nucleotide binding has also been demonstrated to alter the binding of glibenclamide and its analogs to the sulfonylurea receptor, a related member of the ABC family of transporters (Hambrook et al., 1999). Future work will focus on the way that allosteric communication is transmitted throughout the P-gp molecule between the drug and nucleotide binding domains of P-gp.

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