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Competitive and Allosteric Interactions in Ligand Binding to P-glycoprotein as Observed on an Immobilized P-glycoprotein Liquid Chromatographic Stationary Phase

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ABSTRACT

A liquid chromatographic stationary phase containing immobilized P-glycoprotein (Pgp) was synthesized using cell membranes obtained from Pgp-expressing cells. The resulting Pgp-stationary phase was used in frontal and zonal chromatographic studies to investigate the binding of vinblastine (VBL), doxorubicin (DOX), verapamil (VER), and cyclosporin A (CsA) to the immobilized Pgp. The compounds were added individually to the chromatographic system with or without ATP in the running buffer. Using this approach, dissociation constants were calculated for VBL (23.5 ± 7.8 nM), DOX (15.0 ± 3.2 μM), VER (54.2 ± 4.7 μM), and CsA [97.9 ± 19.4 nM (without ATP) and 62.5 ± 4.6 nM (with ATP)]. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that competitive interactions occurred between VBL and DOX, cooperative allosteric interactions occurred between VBL and CsA and ATP and CsA, and anticooperative allosteric interactions occurred between ATP and VBL and VER. The chromatographic studies indicate that the immobilized Pgp was modified by ligand and cofactor binding and that the stationary phase can be used to study drug-drug binding interactions on the Pgp molecule.

P-glycoprotein (Pgp) is a member of the ATP binding cassette (ABC) superfamily of transport proteins (Loe et al., 1996; Doyle et al., 1998). It is a 170-kDa cell membrane protein with two ATP binding sites and ATPase activity (Rosenberg et al., 1997). Pgp acts as an efflux drug transporter whose substrates include anthracycline antibiotics and Vinca alkaloids (Cordon-Cardo et al., 1989; Clarke et al., 1993; Clarke and Leonessa, 1994), steroids (Barnes et al., 1996), verapamil (VER) (Yusa and Tsuro, 1989), peptides (Foxwell et al., 1989), and quinolines (Kusuhara et al., 1997). Pgp is expressed in normal tissues and appears to be a major contributor to the blood-brain barrier (Cordon-Cardo et al., 1989; Tsuji et al., 1992). Expression also has been detected in breast cancer where it is associated with a poor clinical response (Trock et al., 1997).

Pgp's broad substrate specificity has not been definitively explained. Several indirect and direct models for Pgp activity have been proposed (Shapiro and Ling, 1994). The most popular model is the "membrane vacuum cleaner" mechanism in which Pgp binds its substrate from the inner leaflet of the plasma membrane and releases it into the extracellular fluid (Gottesman and Pastan, 1993). In a related mechanism, Pgp activity has been described as a "flippase" that transports its substrates from the inner to the outer leaflet of the plasma membrane (Raviv et al., 1990; Higgins and Gottesman, 1992).

The number of binding sites on the Pgp molecule has not been determined. There is evidence for the existence of multiple binding sites as some substrates bind to Pgp in a mutually noncompetitive manner (Raviv et al., 1990; Ferry et al., 1992, 1995). Other data suggesting multiple binding sites include synergistic activity on ATPase activation (Garrigos et al., 1997), substrate discriminating effect of specific Pgp mutations (Devine et al., 1992), and differential effect of chemosensitizers on the photoaffinity labeling at two different locations on the Pgp molecule (Dey et al., 1997).

One experimental approach to determine Pgp selectivity and transport mechanism has been the isolation of the transporter followed by purification using a combination of anion exchange and affinity chromatography (Shapiro and Ling, 1994; Sharom, 1995). The isolated protein was then reconstituted into proteoliposomes either by the detergent dilution method (Shapiro and Ling, 1994) or by detergent dialysis followed by Sephadex-G50 chromatography (Sharom, 1995). In the proteoliposomes prepared by either method, >90% of Pgp was reconstituted with an inside-out orientation, i.e., ATP-binding and cytoplasmic domains exposed to the ex-

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ABBREVIATIONS: Pgp, P-glycoprotein; VBL, vinblastine; DOX, doxorubicin; VER, verapamil; CsA, cyclosporin A; IAM, Immobilized Artificial Membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; BCA, bicinchoninic acid.

travesicular medium (Sharom, 1995). The reconstituted Pgp could be used to study and characterize both drug-stimulated ATPase activity and ATP-dependent transport. Using this approach, the effect of verapamil and daunorubicin on [^{3}H]vinblastine ([^{3}H]VBL) accumulation in the proteoliposomes, a measure of transport, could be measured (Sharom, 1995). The effect of verapamil on the ATPase kinetics (K_m and V_{max}) also could be determined (Shapiro and Ling, 1994).

Another approach to the determination of the effect of compounds on Pgp transport used the transepithelial flux of digoxin across Caco-2 cells (Wandel et al., 1999). This method was used to determine the IC_{50} for digoxin transport for 14 compounds. An in vivo method for Pgp transport in tumors and the blood-brain barrier also has been reported (Hendrikse et al., 1999). This approach used [^{11}C]verapamil and [^{11}C]daunorubicin as the transport substrates and positron emission tomography as the detection method.

The binding of compounds to Pgp has been investigated by measuring the displacement of [^{3}H]vinblastine and [^{3}H]verapamil from human intestinal Caco-2 cells overexpressed with Pgp (Doppenschmitt et al., 1999). The assays were performed in 96-well plates, and the method was designed to be adapted to high-throughput screens. Using this method, K_m and IC_{50} values for nine compounds were determined.

An alternative experimental approach to the determination of binding affinities is affinity chromatography. We have previously reported the synthesis of a liquid chromatographic stationary phase containing immobilized Pgp and its use in the determination of Pgp binding affinities (Zhang et al., 2000). The present work expands the characterization of the Pgp-stationary phase and uses frontal and zonal chromatographic techniques to investigate the binding of vinblastine, doxorubicin, verapamil, and cyclosporin A (CsA) to the immobilized Pgp. The compounds were added individually to the chromatographic system with or without ATP in the running buffer. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that both competitive and allosteric interactions occurred during the chromatographic studies and that the binding affinities of immobilized Pgp are altered by the presence or absence of ATP.

Experimental Procedures

Materials. Immobilized Artificial Membrane (IAM) particles were obtained from Regis Chemical Co. (Morton Grove, IL). A glass column (HR5/5) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). [^{3}H]Vinblastine and [^{3}H]cyclosporin A were purchased from Amersham Life Science Products (Boston, MA). [^{3}H]Verapamil was from NEN Life Science Products, Inc. (Boston, MA). Vinblastine, verapamil, doxorubicin, cyclosporin, CHAPS, glycerol, benzamidine, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). GF/C glass microfiber filters were from Whatman (Ann Arbor, MI). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT).

Preparation of Membranes. As previously described, the Pgp-positive MDA435/LCC6^{MDR1} cell line was obtained by transduction of Pgp-negative-expressing MDA435/LCC6 human breast cancer cells with a retroviral vector carrying MDR1 cDNA (Pgp) (Leonessa et al., 1996). Approximately 8×10^7 cells were harvested in 10 ml of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 μM leupeptin, 2 μM phenylmethanesulfonyl fluoride, and 4 μM pepstatin). The suspension of cells was homogenized twice for 30 s (with a cooling period in between) with a Brinkmann (Westbury, NY) Polytron homogenizer. The homogenized cells were centrifuged first at 1,000*g* for 10 min, the pellets were discarded, and the supernatant was collected and centrifuged at 150,000*g* for 30 min. The membrane pellets were collected.

Immobilization of Pgp on IAM Particles. The membrane pellets were resuspended in 6 ml of solubilization solution (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 15 mM CHAPS, 2 mM dithiothreitol, 10% glycerol) for 3 h at 4°C. This was mixed with 100 mg of dried IAM particles and stirred for 1 h at room temperature. The suspension of Pgp-IAM was then dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine) for 36 h at 4°C (1.5 liters for every 12 h).

Preparation of the Liquid Chromatographic Column. The IAM particles with immobilized Pgp were packed into a HR5/5 glass column (0.5 × 0.8 cm) after centrifugation three times at 350*g* for 3 min at 4°C. Then the column was equilibrated with buffer B (50 mM Tris-HCl, pH 7.4) at room temperature for 3 h.

Frontal Chromatographic Studies. The chromatographic system has been previously described (Zhang et al., 2000) and was primarily based upon the Pgp-IAM column connected on-line to a flow scintillation monitor (Radiometric FLO-ONE Beta 500 TR instrument; Packard Instruments). All chromatographic experiments were conducted at room temperature using a flow rate of 0.5 ml/min.

The marker ligand, either [^{3}H]VBL (1.0 nM), [^{3}H]VER (0.3 nM), or [^{3}H]CsA (2.0 nM) were applied to the Pgp-IAM column in sample volumes of 25 to 50 ml. The solutions containing the marker ligands were supplemented with a range of concentrations of either cold VBL, VER, doxorubicin, or CsA. Elution profiles were obtained showing front and plateau regions as illustrated for [^{3}H]VER (Fig. 1). The observed elution volume data were used for calculation of ligand dissociation constants. The K_d values of VER and CsA were calculated by nonlinear regression using Prism (GraphPad Software, San Diego, CA) and a one-site binding (hyperbola) equation (1) (Klotz, 1983)

$$Y = B_{max} \cdot X/(K_d + X) \quad (1)$$

in which X is the concentration of VER or CsA; Y is equal to [verapamil] $(V - V_{min})$ or [CsA]$(V - V_{min})$, where V_{min} is the elution volume of VER or CsA under conditions where specific interactions

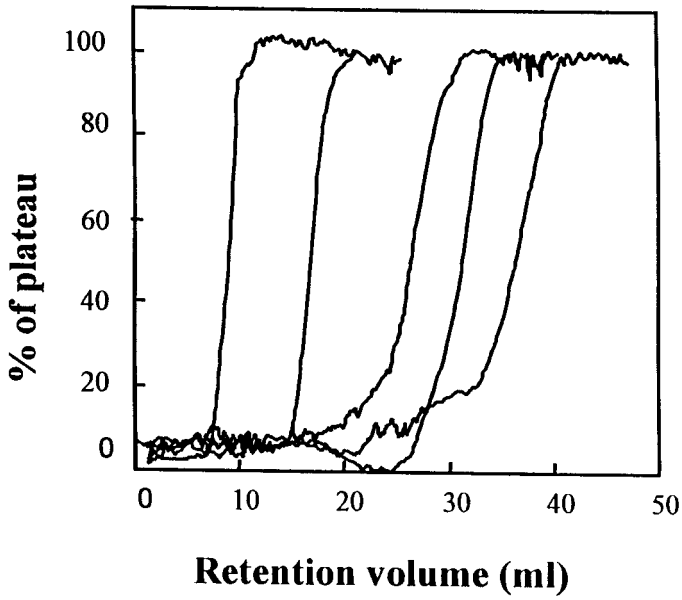

Fig. 1. Frontal analysis of interactions of Pgp with verapamil on an immobilized Pgp-IAM column (0.5 × 0.8 cm). The elution profiles of 1.0 nM [^{3}H]verapamil in solution with 10, 40, 60, 200, and 400 μM nonradioactive verapamil are shown (from right to left). Running buffer was 50 mM Tris-HCl, pH 7.4, at a flow rate of 0.5 ml/min.

are completely suppressed and V is the retention volume of VER or CsA at different concentrations (0.3–400 μM for VER and 2.5–100 nM for CsA).

Two series of runs were made to determine the K_d value for VBL and the K_d values for doxorubicin and CsA. One series was performed with cold VBL (3–100 nM) to displace [^{3}H]VBL, and the other was performed with cold doxorubicin (5–70 μM) or CsA (10–250 nM) with [^{3}H]VBL as the displaced ligand. The K_d value of VBL and the K_d values of doxorubicin and CsA were calculated using eqs. 2 and 3 (Winzor, 1985; Brekkan et al., 1996; Zhang et al., 1998).

$$(V_{max} - V)^{-1} = (1 + [VBL]K_{VBL}) \cdot (V_{min}[P]K_{VBL})^{-1} + (1 + [VBL]K_{VBL})^2 \cdot (V_{min}[P]K_{VBL}K_i)^{-1} \cdot [I]^{-1} \quad (2)$$

$$(V - V_{min}) - 1 = (V_{min}[P]K_{VBL})^{-1} + (V_{min}[P])^{-1}[VBL] \quad (3)$$

where I represents doxorubicin or CsA; [P] represents the concentration of active receptor in the volume; V_{min} represents the elution volume of VBL under conditions where the specific interaction is completely suppressed; V_{max} is the elution volume obtained with 1.0 nM [^{3}H]VBL.

Control Experiments. Membranes from the Pgp-negative parental cell line MDA435/LCC6 (Leonessa et al., 1996) were prepared and immobilized on an IAM support as described above. Using the procedure described above, the Pgp-negative-IAM support was packed into a glass column (0.5 × 0.8 cm), and a second glass column (0.5 × 0.8 cm) was packed with untreated IAM support. The three columns, IAM support (negative control), Pgp-negative-IAM (positive control), and Pgp-IAM (experimental), were separately connected on-line to a flow scintillation monitor and used in zonal chromatographic experiments. In these studies, a mobile phase composed of Tris-HCl (50 mM, pH 7.4) was constantly pumped through the column at a flow rate of 0.5 ml/min. A single 100-μl injection of the marker ligand [^{3}H]VER (23.5 nM) was injected onto the column, and the radioactive signal (cpm) was recorded every 6 s. The chromatographic data was summed up in 0.5-min intervals and smoothed using the Microsoft Excel program with a 5-point moving average.

Membrane Binding Assays. The binding assays were accomplished using a previously described method (Ferry et al., 1995). Briefly, 50 μl of [^{3}H]VBL [3–100 nM with 2% ethanol (v/v)] was incubated with Pgp-containing or Pgp-negative membranes (150 μg in 50 μl) or bare IAM particles and 50 μl of cold VBL (12 μM) for 2 h at room temperature. Bound and free drug were separated by rapid filtration through Whatman GF/C filters that had been presoaked with 0.1% bovine serum albumin in Tris-HCl (50 mM, pH 7.4). The filters were then washed with 2 portions of 5 ml of ice-cold 20 mM Tris-HCl, 20 mM $MgCl_2$ buffer. The filters were dried, and retained radioactivity was quantitated by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding.

Protein Assay. The amount of membrane and the immobilized membrane were determined by bicinchoninic acid (BCA) protein assay. The sample was diluted with NaOH (0.1 M). A protein standard (0.3–37.5 μg in 50 μl) was prepared with albumin standard (Pierce, Rockford, IL). The measurement procedure followed the instruction in the Pierce BCA protein assay kit in which 20 ml of reagent A was mixed with 0.4 ml of reagent B. Aliquots (50 μl) of standards and samples were added in triplicate to a 96-well plate and 200 μl of BCA reagent (A + B) were added to each well. The standards and samples were incubated at room temperature for 3 h, and the resulting absorbance at λ = 570 nm was determined using a spectrophotometer. The amount of protein was calculated by using the Microsoft Excel program.

Results

Chromatographic Studies with Vinblastine and Doxorubicin. The dissociation constants (K_d) of VBL and doxorubicin were determined on the Pgp-IAM stationary phase using displacement chromatography with [^{3}H]VBL as the marker ligand (Table 1). The calculated K_d of VBL was 23.5 ± 7.8 nM, consistent with the previously reported values of 37.0 ± 10 nM (Ferry et al., 1995) and 36 ± 5 nM (Korzekwa et al., 1998). The K_d value of 15.0 ± 3.2 μM determined for doxorubicin was also consistent with the reported value of 31.0 ± 7.3 μM (Ferry et al., 1995).

The chromatographic results also were consistent with the results obtained from binding assays using the same membranes used in the construction of the Pgp-IAM stationary phase. In these studies, membrane extracts were prepared from the Pgp-expressing cell line MDA435/LCC6^{MDR1} and the Pgp-negative cell line MDA435/LCC6 (Hendrikse et al., 1999). VBL binding to the two membrane extracts and the IAM support was determined using a previously described rapid filtration method (Ferry et al., 1995). No specific binding was observed with the Pgp-negative cell membranes or the IAM particles, while a K_d value of 54.5 ± 40.8 nM was determined using the membranes from the Pgp-expressing cell line. The calculated affinity was consistent with the previously published value, 37 ± 10 nM, obtained using the same experimental approach (Ferry et al., 1995).

Chromatographic Studies with Verapamil and Vinblastine. When VER was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for VER was 54.2 ± 4.6 μM. This value was significantly higher than the previously reported values of 0.45 ± 0.05 μM (Ferry et al., 1995) and 0.6 ± 0.18 μM (Ferry et al., 1992). When the experimental conditions were reversed and [^{3}H]VER was the marker ligand and VBL the displacer, no displacement of [^{3}H]VER was observed when 50 and 100 nM concentrations of VBL were added to the mobile phase (Table 2).

The specificity of the chromatographic interactions of VER with the immobilized Pgp were investigated through the independent immobilization of membrane extracts from the Pgp-expressing cell line and the Pgp-negative cell line on the IAM support. Zonal chromatographic studies were conducted with columns containing either the Pgp-IAM, Pgp-negative-IAM, or IAM support. When a 100-μl sample of [^{3}H]VER was injected onto the columns containing either the Pgp-negative-IAM support or the IAM support alone, the retention volumes on both columns were less than 4 ml (Fig. 2, curves

TABLE 1
The K_d values calculated using frontal affinity chromatography on the immobilized Pgp-IAM stationary phase

Drugs	K_d[a]	K_d
Vinblastine	23.5 ± 7.8 nM	37.0 ± 10 nM[b] 36.0 ± 5 nM[c]
Verapamil	54.2 ± 4.6 μM	0.45 ± 0.05 μM[b]
Doxorubicin	15.0 ± 3.2 μM[d]	31 ± 7.3 μM[b]
Cyclosporin A	62.5 ± 5.6 nM[e] 97.9 ± 19.4 nM[d]	18 ± 3.6 nM[b]

[a] These values were measured in the present work by frontal affinity chromatography with immobilized Pgp-IAM.
[b] These values are from Ferry et al. (1995).
[c] This value is from Callaghan et al. (1997).
[d] These values were obtained by displacing [^{3}H]vinblastine (see *Experimental Procedures*).
[e] This value was measured when 3 mM ATP was in the running buffer.

1 and 2). The volumes of these columns (as well as the Pgp-IAM column) are 0.5 ml, thus a retention of 4 ml indicates that it takes 8 column volumes to elute the [^{3}H]VER, indicating that an interaction occurred between the solute and both of the stationary phases. On the column containing the Pgp-IAM support, the retention volume of [^{3}H]VER was >20 ml (Fig. 2, curve 3).

Chromatographic retention on biopolymer containing stationary phases is a combination of nonspecific and specific interactions. The former interactions are due to the physicochemical properties of the solute and stationary phase, i.e., electrostatic and hydrophobic interactions, while the latter (specific) interactions are due to interactions between the solute and a specific binding site(s) on the biopolymer. The 5-fold increase in retention volume between the Pgp-IAM and both the Pgp-negative-IAM and IAM support alone indicates that specific binding interactions occur between [^{3}H]VER and the immobilized membrane extracts obtained from the Pgp-expressing cells.

Chromatographic Studies with Cyclosporin A and Vinblastine. When CsA was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for CsA was 97.9 ± 19.4 nM, compared with the previously reported value of 18.0 ± 3.6 nM (Ferry et al., 1995) (Table 1). When [^{3}H]CsA was used as the marker ligand and migrated alone through the Pgp-IAM, the retention volume was 7.8 ml (Table 2), and no specific retention was observed (Fig. 3A). The addition of 50 nM VBL to the running buffer increased the retention volume of [^{3}H]CsA to 15.7 ml (Table 2) and produced the expected frontal chromatogram (Fig. 3B). When the VBL concentration was increased to 100 nM, the observed retention of the frontal chromatogram increased to 18.8 ml (Fig. 3D; Table 2).

Effect of ATP on the Chromatographic Properties of the Pgp-IAM. The addition of 3 mM ATP to the running buffer resulted in changes in the retention volumes of CsA, VBL, and VER. The concentration of ATP was selected based upon the previously reported studies of the secondary and tertiary structures of reconstituted Pgp (Sonveaux et al., 1996).

In the case of CsA, the addition of ATP increased the retention volume from 7.8 to 17.5 ml (Table 2). In addition to the change in elution volume, the observed chromatogram changed from a frontal curve indicative of nonspecific retention (Fig. 3A) to a frontal chromatogram characteristic of specific retention due to binding interactions between the CsA and the immobilized Pgp-IAM (Fig. 3C). With 3 mM ATP in the running buffer, [^{3}H]CsA was displaced from Pgp by the addition of unlabeled CsA. The results from the CsA displacement studies were used to calculate a K_d value of 62.5 nM for CsA binding to the immobilized Pgp.

When VBL was the marker ligand, the addition of 3 mM ATP decreased the retention volume from 32.1 to 8.4 ml (Table 2). The presence of ATP in the running buffer also changed the observed chromatograms from a frontal curve demonstrating specific retention (Fig. 4A) to a nonspecific curve (Fig. 4B). A similar effect was observed for VER as the addition of 3 mM ATP to the running buffer reduced the elution volume from 34.2 to 5.9 ml (Table 2) with a resulting loss in specific retention, as demonstrated by the shape of the frontal curve (data not shown).

Discussion

Quantitative affinity chromatography is an extensively studied and documented approach for the measurement of ligand-protein interactions (cf. Jaulmes and Vidal-Madjar, 1989). This technique uses both frontal and zonal chromatography to perform equilibrium, thermodynamic, and kinetic studies. In addition, displacement chromatographic techniques can be used to observe binding interactions between two or more ligands binding at the same or separate sites. In this manner, competitive and allosteric (cooperative or anticooperative) interactions can be readily identified.

In this study, both zonal and frontal chromatography were used to evaluate Pgp-ligand and ligand-ligand binding interactions. Using zonal chromatography, a comparison of the chromatographic retention of verapamil, a known Pgp substrate, on the native chromatographic support and the Pgp-positive and Pgp-negative supports (Fig. 2) demonstrated that, for Pgp substrates, the observed chromatographic retentions were a function of specific interactions between the substrate and the immobilized Pgp.

The relationship between chromatographic retention on the Pgp-IAM stationary phase and Pgp binding affinity was also illustrated by comparison of substrate affinities calculated using frontal chromatography on the Pgp-IAM column and the results from classical filtration binding assays (Table 1). The initial studies in this series were conducted using [^{3}H]VBL as the marker ligand and Tris buffer (50 mM, pH 7.4) as the running buffer. Under these conditions, CsA displaced [^{3}H]VBL, producing a calculated K_d value of 97.9 nM (Table 1), which is consistent with results from filtration binding assays (Ferry et al., 1992, 1995).

The displacement of [^{3}H]VBL by CsA indicated that CsA specifically and competitively binds to immobilized Pgp, but frontal chromatography with [^{3}H]CsA alone in the running buffer produced a low retention volume, 7.8 ml (Table 2), and no detectable specific retention (Fig. 3A). This indicates that under the experimental conditions, [^{3}H]CsA did not specifically bind to immobilized Pgp. However, the addition of 50 nM VBL to the running buffer produced a classical frontal

TABLE 2

Retention volumes of [^{3}H]vinblastine and [^{3}H]cyclosporin A were obtained when 1) no ATP was present in the running buffer, 2) 3 mM ATP was added in the running buffer, 3) 50 nM cold vinblastine was supplemented in the sample (no ATP in the buffer), and 4) 100 nM cold vinblastine was in the sample (no ATP in the buffer)

Drugs	Retention Volume (ml) at			
	No ATP	3 mM ATP	50 nM Vinblastine (No ATP)	100 nM Vinblastine (No ATP)
[^{3}H]Vinblastine	32.1	8.4	11.0	9.5
[^{3}H]Verapamil	34.2	5.9	34.1	34.0
[^{3}H]Cyclosporin A	7.8	17.5	15.7 (15.4)[a]	18.8

[a] 15.7 ml was measured at the condition of no ATP present in the running buffer, and 15.4 ml was obtained when 3 mM ATP was in the running buffer.

chromatogram for [^{3}H]CsA (Fig. 3B) and increased the retention volume to 15.7 ml (Table 2). When the VBL concentration was increased to 100 nM, the retention volume also increased to 18.8 ml (Table 2; Fig. 3D).

The results from the studies with [^{3}H]VBL and [^{3}H]CsA as the marker ligands indicate that the addition of VBL to the running buffer produced a cooperative allosteric interaction in the binding process between [^{3}H]CsA and the immobilized Pgp. This suggests that the binding of VBL to the immobilized Pgp alters the protein in such a manner that the site at which CsA binds is formed or made accessible to the ligand.

The data also indicated that once the VBL-induced change had occurred CsA bound to Pgp and displaced VBL through competitive and/or anticooperative allosteric interactions. The addition of CsA to the running buffer did not change the shape of the [^{3}H]VBL frontal chromatograms, demonstrating that the displacement was competitive in nature. One explanation of these results is that the VBL-induced CsA binding site is contiguous with or part of the VBL site. Thus, CsA binding to the induced site does not directly compete with VBL for the same site but inhibits VBL binding through steric interactions. Korzekwa et al. (1998) have proposed a similar model for enzymatic inhibition and activation of cytochrome P450 isoforms. In this model, the simultaneous but independent binding of two different substrates in the active site of the enzyme results in steric interactions that produce the displacement (inhibition) or reorientation (activation) of one of the substrates.

In these studies, the addition of increasing concentrations of VER to the running buffer reduced the retention volumes of [^{3}H]VBL without changing the shapes of the frontal chromatograms. This indicates that VER competitively displaced VBL from its binding to Pgp, although the calculated K_d value was significantly higher than previously reported values (Table 1). However, VBL was unable to displace [^{3}H]VER from the immobilized Pgp. These results suggest that VER binds to two or more distinct sites on the Pgp molecule including the site at which VBL binds. Furthermore, the site common to VBL and VER is not the primary, high-affinity VER binding site. Thus, the K_d value calculated from the frontal chromatographic studies (Table 1) appears to be the sum of VER binding affinities. It could not be determined from the experimental conditions used in this study whether the VER and VBL sites are allosterically linked. Further studies will be required to select specific markers for the high- and low-affinity VER binding sites.

The existence of multiple binding sites on the Pgp molecule has been previously proposed. Using classical filtration binding assays, Ferry et al. (1992) obtained evidence of nonoverlapping binding sites for Vinca alkaloids and dihydropyridine substrates and for Vinca alkaloids and doxorubicin. Also, distinct sites for steroids and Vinca alkaloids (Garrigos et al., 1997), steroids and VER (Orlowski et al., 1996), VER and dihydropyridines (Pascaud et al., 1998), and between differ-

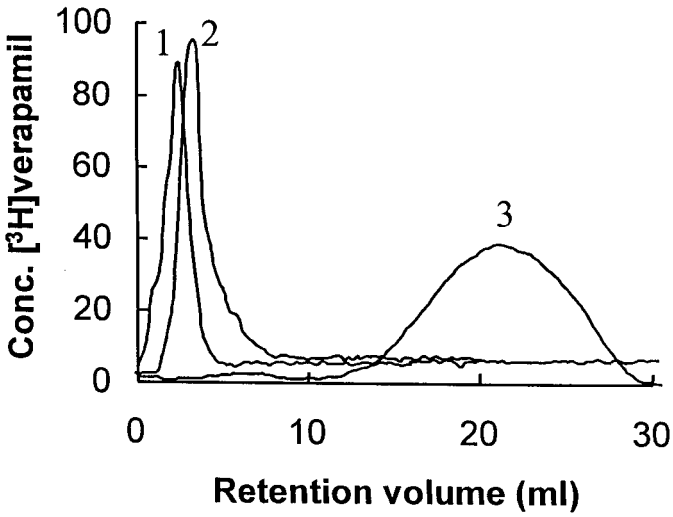

Fig. 2. Zonal affinity chromatographic profiles of 100 μl of 23.5 nM [^{3}H]verapamil at a flow rate of 0.5 ml/min with 50 mM Tris-HCl, pH 7.4, buffer. 1, from Pgp-negative-IAM column; 2, from IAM particles column; and 3, from Pgp-IAM column.

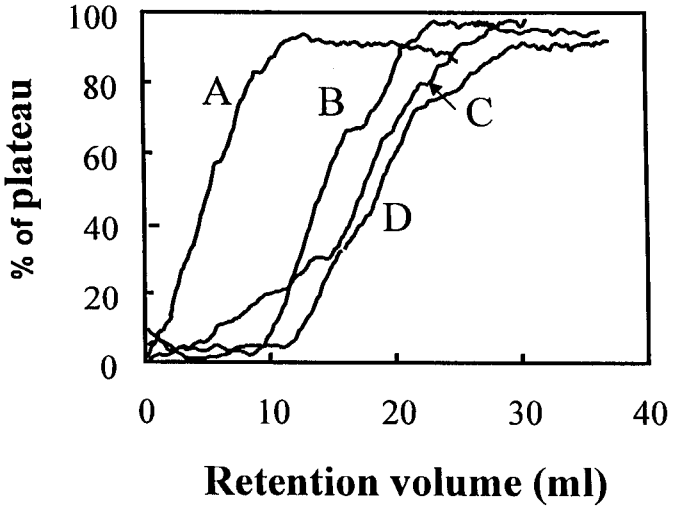

Fig. 3. Frontal affinity analysis of 1.0 nM [^{3}H]cyclosporin A. A, [^{3}H]cyclosporin A was in the sample alone; B, 50 nM cold vinblastine was supplemented in the sample; C, 3 mM ATP was in the sample and running buffer; D, 100 nM cold vinblastine was added in the sample. The running buffer was 50 mM Tris-HCl, pH 7.4.

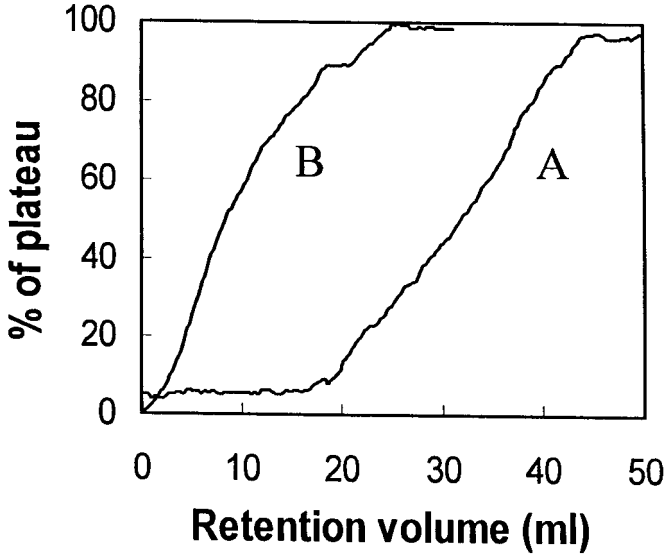

Fig. 4. Frontal affinity chromatographic analysis of 1 nM [^{3}H]vinblastine with Pgp-IAM on a column of 0.5 × 0.8 cm at a flow rate of 0.5 ml/min. A, 1.0 nM [^{3}H]vinblastine only; B, 1.0 nM [^{3}H]vinblastine supplemented with 3 mM ATP. The running buffer for both A and B was 50 mM Tris-HCl, pH 7.4, with 1.6% ethanol.

ent steroids (Orlowski et al., 1996) were supported by the results of studies using an ATPase activation endpoint. Moreover, separate binding sites have been suggested for VER and anthracyclines (Spoelstra et al., 1994; Litman et al., 1997), VER and colchicine (Korzekwa et al., 1998), and cyclosporins and dihydropyridines (Tamai and Safa, 1991).

Pgp contains two ATP binding sites (Rosenberg et al., 1997). A previous study has investigated the effect of ATP binding on the secondary and tertiary structures of Pgp using infrared attenuated total reflection spectroscopy (Sonveaux et al., 1996). In this work, purified Pgp was functionally reconstituted into liposomes, and the effect of ATP, ATP with VER, VER alone, and ADP on the structure of Pgp was investigated. No effects were observed with VER alone or with ADP. However, the addition of ATP induced a change in the tertiary structure of Pgp.

Sonveaux et al. (1996) used 3 mM ATP versus no ATP as the two experimental states for Pgp. In this study, we have used a running buffer without ATP and one to which we have added the same concentration of ATP (i.e., 3 mM). Thus, the chromatographic results with ATP in the running buffer should reflect the shift in Pgp tertiary structure indicated by Sonveaux et al. (1996). Indeed, the addition of 3 mM ATP to the running buffer increased the retention volume of [^{3}H]CsA from 7.8 to 17.5 ml (Table 2), produced a classical frontal chromatogram for [^{3}H]CsA (Fig. 3C), and permitted the calculation of a K_d value of 62.5 nM (Table 1). These results indicate that the addition of ATP to the running buffer produced a cooperative allosteric interaction that increased the binding affinity of Pgp for CsA. Similar results were obtained in the VBL-CsA binding interaction studies.

The presence of ATP in the running buffer produced the opposite effect on the retention volumes of [^{3}H]VBL and [^{3}H]VER. With [^{3}H]VBL, the addition of 3 mM ATP reduced the observed retention from 32.1 to 8.4 ml (Table 2; Fig. 3), and the retention volume for [^{3}H]VER was reduced from 34.2 to 5.9 ml, with the loss of specific retention in both cases. These results suggest an ATP-induced anticooperative allosteric interaction. Allosterically produced reductions in retention volume can be distinguished from competitive displacements as illustrated by the effect of the addition of VBL on the retention volume of [^{3}H]VBL (Table 2). In this case, the retention volume decreased, but the specific frontal chromatographic curves were retained (data not shown).

Thus, the addition of ATP to the running buffer produced changes in the chromatographic interactions between the ligands and the immobilized Pgp (i.e., specific to nonspecific and vice versa) that are consistent with the changes in the tertiary structure identified by Sonveaux et al. (1996). In this case, the consequence of the change in Pgp tertiary structure was the creation of a specific binding site for CsA. The same change that increased the binding affinity for CsA also altered the site at which VBL binds, decreasing the affinity of Pgp for VBL. The effect of VBL on CsA binding affinity and the effect of ATP on the binding affinities of both VBL and CsA indicate that separate, but closely linked, binding sites for CsA and VBL exist on the Pgp molecule.

The immobilized Pgp liquid chromatographic stationary phase described in this report appears to reproduce Pgp substrate binding as determined by classical filtration binding assays. The observed binding is Pgp-specific, is highly sensitive to changes in the protein's tertiary conformation caused by Pgp interactions with substrates and ATP, and reflects changes occurring in the functional cycle of Pgp. Thus, Pgp-affinity chromatography represents a promising tool for a quick and reproducible evaluation of potential Pgp substrates and/or inhibitors and a useful probe of the transport mechanism. The data obtained through this approach provide new information on Pgp's mechanism of action, including evidence of binding sites for verapamil and for cyclosporins distinct from the ones for Vinca alkaloids. The data directly support a model of Pgp's action where these substrates can bind to distinct, although often allosterically connected, regions.

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References

Barnes KM, Dickstein B, Cutler GB, Fojo T and Bates SE (1996) Steroid transport, accumulation and antagonism of P-glycoprotein in multidrug-resistant cells. *Biochemistry* **35:**4820–4827.

Brekkan E, Lundqvist A and Lundahl P (1996) Immobilized membrane vesicle or proteoliposome affinity chromatography. Frontal analysis of interactions of cytochalasin B and D-glucose with the human red cell glucose transporter. *Biochemistry* **35:**12141–12145.

Callaghan R, Berridge G, Ferry DR and Higgins CF (1997) The functional purification of P-glycoprotein is dependent on maintenance of a lipid-protein interface. *Biochim Biophys Acta* **1328:**109–124.

Clarke R and Leonessa F (1994) Cytotoxic drugs and hormones in breast cancer: Interactions at the cellular level, in *Drug and Hormonal Resistance in Breast Cancer: Cellular and Molecular Mechanisms* (Dickson RB and Lippman ME eds) pp 407–432, Ellis Horwood, Chichester, UK.

Clarke R, Thompson EW, Leonessa F, Lippman J, McGarvey M and Brunner N (1993) Hormone resistance, invasiveness and metastatic potential in human breast cancer. *Breast Cancer Res Treat* **24:**227–239.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* **86:**695–698

Devine SE, Ling V and Melera PW (1992) Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance. *Proc Natl Acad Sci USA* **89:**4564–4568.

Dey S, Ramachandra M, Pastan I, Gottesman MM and Ambudkar S (1997) Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proc Natl Acad Sci USA* **94:**10594–10599.

Doppenschmitt S, Langguth P, Regardh CG, Andersson TB, Hilgendorf C and Spahn-Langguth H (1999) Characterization of binding properties to human P-glycoprotein: Development of a [^{3}H]verapamil radioligand-binding assay. *J Pharmacol Exp Ther* **288:**348–357.

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK and Ross DD (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* **95:**15665–15670

Ferry DR, Malkandi PJ, Russell MA and Kerr DJ (1995) Allosteric regulation of [^{3}H] vinblastine binding to P-glycoprotein of MCF-7 ADR cells by dexniguldipine. *Biochem Pharmacol* **49:**1851–1861.

Ferry DR, Russel MA and Cullen MH (1992) P-glycoprotein possesses a 1,4-dihydropyridine-selective acceptor site which is allosterically coupled to a vinca-alkaloid-selective binding site. *Biochem Biophys Res Commun* **188:**440–445.

Foxwell BM, Mackie A, Ling V and Ryffel B (1989) Identification of the multidrug resistance-related P-glycoprotein as a cyclosporin binding protein. *Mol Pharmacol* **36:**543–546.

Garrigos M, Mir LM and Orlowski S (1997) Competitive and non-competitive inhibition of the multidrug-resistance-associated P-glycoprotein ATPase. Further experimental evidence for a multisite model. *Eur J Biochem* **224:**664–673.

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann Rev Biochem* **62:**385–427.

Hendrikse NH, de Vries EGE, Eriks-Fluks L, van der Graaf TA, Hospers GAP, Willemsen ATM, Vaalburg W and Franssen EJF (1999) A new *in vivo* method to study P-glycoprotein transport in tumors and the blood-brain barrier. *Cancer Res* **59:**2411–2416.

Higgins CF and Gottesman MM (1992) Is the multidrug transporter a flippase? *Trends Biochem Sci* **17:**18–21.

Jaulmes A and Vidal-Madjar C (1989) Theoretical aspects of quantitative affinity chromatography: An overview, in *Advances in Chromatography, Volume 28* (Giddings JC, Grushka E and Brown PR eds) pp 1–64, Marcel Dekker, New York.

Klotz IM (1983) Ligand-receptor interactions: What we can and cannot learn from binding measurements. *Trends Pharmacol Sci* **4:**253–255.

Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ and Tracy TS (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: Evidence that multiple substrates can simultaneously bind to cytochrome P450. *Biochemistry* **37:**4137–4147.

Kusuhara H, Suzuki H, Terasaki T, Kakee A, Lamaire M and Sugiyama YP (1997) P-glycoprotein mediates the efflux of quinidine across the blood-brain barrier. *J Pharmacol Exp Ther* **283:**574–580.

Leonessa F, Green D, Licht T, Wright A, Wingate-Legette K, Lippman J, Gottesman

MM and Clarke R (1996) MDA435/LCC6 and MDA435/LCC6MDR1: Ascites models of human breast cancer. *Br J Cancer* **73:**154–161
Litman T, Zeuthen T, Skovgard T and Stein WT (1997) Competitive, non-competitive and cooperative interactions between substrates of P-glycoprotein as measured by its ATPase activity. *Biochim Biophys Acta* **1361:**169–176.
Loe DW, Deeley RG and Cole SPC (1996) Biology of the multidrug resistance-associated protein, MRP. *Eur J Cancer* **32A:**945–957.
Orlowski S, Mir IM, Belehradek J Jr and Garrigos M (1996) Effect of steroids and verapamil on P-glycoprotein ATPase activity: Progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators. *Biochem J* **317:**515–522.
Pascaud C, Garrigos M and Orlowski S (1998) Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic drugs and reversing agents. *Biochem J* **333:**351–358.
Raviv Y, Pollard HB, Bruggemann EP, Pastan I and Gottesman MM (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. *J Biol Chem* **265:**3975–3980.
Rosenberg MF, Callaghan R, Ford RC and Higgins CF (1997) Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *J Biol Chem* **272:**10685–10694.
Shapiro AB and Ling L (1994) ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. *J Biol Chem* **269:**3745–3754.
Sharom FJ (1995) Characterization and functional reconstitution of the multidrug transporter. *J Bioenerg Biomembr* **27:**15–22.
Sonveaux N, Shapiro AB, Goormaghtigh E and Ling V (1996) Secondary and tertiary structure changes of reconstituted P-glycoprotein. *J Biol Chem* **271:**24617–24624.
Spoelstra EC, Westerhoff HV, Pinedo HM, Dekker H and Lankelma J (1994) The multidrug resistance-reverser verapamil interferes with cellular P-glycoprotein-mediated pumping of daunorubicin as a non-competing substrate. *Eur J Biochem* **221:**363–373.
Tamai I and Safa AR (1991) Azidopine noncompetitively interacts with vinblastine and cyclosporin binding to P-glycoprotein in multidrug resistant cells. *J Biol Chem* **266:**16796–16800.
Trock BJ, Leonessa F and Clarke R (1997) Multidrug resistance in breast cancer: A meta analysis of MDR1/gp170 expression and its possible functional significance. *J Natl Cancer Inst* **89:**917–931.
Tsuji A, Terasaki T, Takabatake Y, Tenda Y, Tamai I, Yamashima T, Moritani S, Tsuruo T and Yamashita J (1992) P-glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. *Life Sci* **51:**1427–1437.
Wandel C, Kim RB, Kajiji S, Guengerich FP, Wilkinson GR and Wood AJJ (1999) P-glycoprotein and cytochrome P-450 3A inhibition: Dissociation of inhibitory potencies. *Cancer Res* **59:**3944–3948.
Winzor DJ (1985) Quantitative characterization of interactions by affinity chromatography in affinity chromatography, in *A Practical Approach* (Dean PDG, Johnson WS and Middle FA eds) pp 149–168, IRL Press, Oxford.
Yusa K and Tsuro T (1989) Reversal mechanism of multidrug resistance by verapamil: Direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res* **49:**5002–5006.
Zhang Y, Leonessa F, Clarke R and Wainer IW (2000) Development of an immobilized P-glycoprotein stationary phase for on-line liquid chromatographic determination of drug-binding affinities. *J Chromatogr B* **739:**33–37.
Zhang Y, Xiao Y, Kellar KJ and Wainer IW (1998) Immobilized nicotinic receptor stationary phase for on-line liquid chromatographic determination of drug-receptor affinities. *Anal Biochem* **264:**22–25.

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