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Development and characterization of an open tubular column containing immobilized P-glycoprotein for rapid on-line screening for P-glycoprotein substrates

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Abstract

Cellular membranes from a cell line expressing P-glycoprotein (Pgp(+)) and from a cell line that does not express Pgp (Pgp(−)) were immobilized on the surface of glass capillaries (25 cm × 100 μm i.d.) by non-covalent interactions using the avidin–biotin coupling system to create two open tubular columns, Pgp(+)-OT and Pgp(−)-OT. Frontal displacement chromatography on the Pgp(+)-OT demonstrated that the immobilized Pgp retained its ability to specifically bind the known Pgp substrates vinblastin and ketoconazole. The calculated affinities, expressed as K_d, for vinblastin and ketoconazole were 97 nM and 12.1 μM, which were comparable with previously reported K_d values of 37 nM and 8.6 μM, respectively. The results confirm that the Pgp(+)-OT can be used to quantitatively estimate binding affinities for the Pgp. Frontal displacement chromatography on the Pgp(−)-OT demonstrated that the immobilized membranes retained the ability to bind some Pgp substrates, but that the binding was not due to specific binding to Pgp. A cohort of compounds containing high affinity Pgp substrates (vinblastin, prazosin) and moderate-low affinity Pgp substrates (doxorubicin, verapamil, ketoconazole) and a non-substrate (nicotine) were chromatographed on the Pgp(+)-OT and Pgp(−)-OT using fast frontal analysis and mass spectrometric detection. The results demonstrated that when the retention on the Pgp(+)-OT was corrected by subtraction of the retention on the Pgp(−)-OT, the test compounds could be accurately sorted into high, moderate-low and non-substrate categories. The data from the study indicates that a single 30-min parallel chromatographic experiment can be used to rank a compound based upon its relative affinity for the immobilized Pgp.

Keywords: Substrates; ABC transporters; Screening; P-glycoprotein

1. Introduction

P-glycoprotein (Pgp) is a 170 kDa integral membrane glycoprotein that contains 12 transmembrane regions and two ATP-binding sites [1]. Pgp is a product of the MDR1 gene and a member of the ABC transporter protein superfamily, which is composed of 48 members, grouped into seven subfamilies (ABC-A to ABC-G) [1]. In addition to Pgp, this family includes the multidrug resistance-associated protein (MRP1), the canalicular multispecific anionic transporter (cMOAT, or MRP2), the organic cation transporter (OCT) the breast cancer resistance protein (BCRP) and the cystic fibrosis conductance regulator (CFCR).

Pgp is an efflux drug transporter expressed in the gastrointestinal epithelium, the kidney, capillary endothelial cells in the central nervous system (blood brain barrier) and tumor cells [1]. Transport by Pgp has been associated with reduced or quite variable bioavailability of orally administered drugs and the overexpression of Pgp in tumor cells has been linked to multiple-drug resistance in cancer chemotherapy [1]. The transporter is involved in the efflux of a number of substrates including the anticancer agents doxorubicin, daunorubicin, vinblastin, and vincristin, as well as actinomycin-D, steroids, verapamil, peptides, quinolines, and cyclosporin.

The rapid determination of a compound's affinity for Pgp and whether it is an inhibitor/substrate of the transporter is

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a key element in drug discovery and drug development programs. This determination has been carried out using a variety of in vitro assays including: the Caco-2 monolayer efflux assay, the membrane-based ATPase activity assay and uptake studies using Calcein-AM or rhodamine-123 fluorescence assays, which have been previously reviewed [2,3]. Some key issues are that the cell-based assays depend on specific culture conditions and passage number, and the primary cells grow very slowly. The Caco-2 Pgp efflux assay typically requires support by a triple quadrupole mass spectrometer. The fluorescent markers are sensitive and adaptable to moderate-throughput screening, but they have relatively low affinity for Pgp, which results in an excessively permissive assay. These assays provide surrogate measures of Pgp interaction such as apparent permeability (Papp) or fold difference from controls in fluorescence signal. Futhermore, cell viability needs to be monitored when test concentrations approach 30–50 μM. The membrane-based assay is relatively rapid and permits the estimation of the V_{max} of Pgp-mediated ATP hydrolysis. However, after a decade of use, there is no standard protocol for the design of the thousands of Caco-2 efflux experiments conducted each year.

We have previously reported an alternative chromatographic approach to the determination of Pgp substrates, which utilizes stationary phases containing membranes from cells expressing the Pgp transporter [4,5]. The cellular membranes were immobilized on a liquid chromatographic stationary phase containing an "immobilized artificial membrane" (IAM stationary phase) to form Pgp-IAM phases. The Pgp-IAM phases were used in frontal affinity chromatographic studies to determine the binding affinities (K_d values) of Pgp substrates and inhibitors and to identify

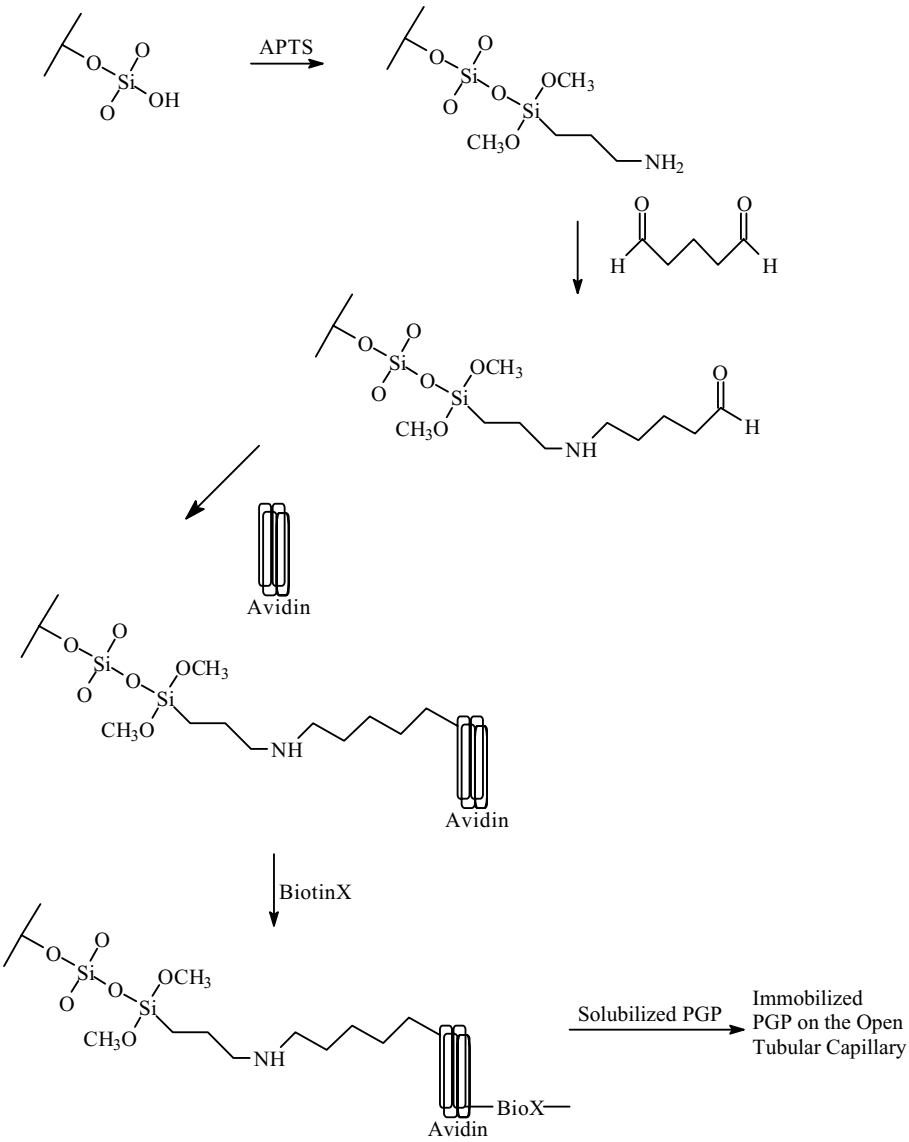

Fig. 1. Scheme used for the immobilization of cellular membranes in the synthesis of the Pgp(+)-OT and Pgp(−)-OT.

competitive, allosteric, and enantioselective interactions in ligand binding to Pgp [4–6].

While the Pgp-IAM stationary phase produced accurate and reproducible data, the chromatographic run times often ranged from 1 to 3 h. A significant portion of the chromatographic retention was the result of strong lipophilic interactions between the substrates/inhibitors and the IAM backbone. Due to long retention times, the Pgp-IAM stationary phase was not suitable for use in the rapid determination and characterization of Pgp binding interactions.

The objective of this project was to reduce the non-specific interactions arising from the chromatographic backbone. This was approached through the immobilization of cellular membranes from Pgp expressing cells (Pgp(+) membranes) onto the inner surface of an open tubular capillary to create a Pgp(+)-OT column. The immobilizations were accomplished using biotin–streptavidin coupling, an experimental approach which was initially described by Berger and Wood [7] and Hofmann and Kiso [8]. Biotin–streptavidin coupling has also been used to immobilized lipid membranes using biotinylated liposomes and streptavidin-coupled gel beads [9,10]. The approach described in the latter papers, i.e. [9,10], were used in this study, Fig. 1.

In order to differentiate between specific binding to Pgp and non-specific binding to the components of the cellular membranes, membranes from a cell line that does not express Pgp (Pgp(−) membranes) were also immobilized to create a Pgp(−)-OT. The results demonstrate that the combination of Pgp(+)-OT and Pgp(−)-OT can be used to correctly sort compounds with high affinity, low affinity and no affinity to Pgp. The Pgp-OT columns are stable, reusable and can be adapted to standard chromatographic equipment and mass spectrometers.

2. Experimental

2.1. Materials

Vinblastin (Vb), verapamil, doxorubicin, ketoconazole, prazosin, (−)-nicotine, benzamidine, dithiothreitol (DTT), 3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulfate (CHAPS), glycerol, leupeptin, pepstatin, phenyl methyl sulfonyl fluoride (PMSF), EDTA, sodium hydroxide, tris[hydroxymethyl]aminomethane (Trizma) and tris[hydroxymethyl]aminomethane hydrochloride (Tris–HCl) (used to prepare the Tris buffer), avidin, glutaric dialdehyde and aminopropyltrimethoxy silane (APTS) were purchased from Sigma (St. Louis, MO, USA). [^{3}H]-Vb was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Biotin-X (6-[(biotinoyl)amino] hexanoic acid) was purchased from Molecular Probes (Eugene, OR, USA). Open tubular capillaries (100 μm i.d.) were purchased from Polymicro Technologies (Pheonix, AZ, USA).

2.2. Preparation of Pgp(−) and Pgp(+) membranes

2.2.1. Cell lines

Pgp(−) membranes were obtained from the MDA435/LCC6 cell line. The Pgp(+) membranes were obtained from the MDA435/LCC6MDR1 cell line prepared from the MDA435/LCC6 cell line by transduction with a retroviral vector carrying MDR1 cDNA (Pgp) as previously described by Leonessa et al. [11].

2.2.2. Solubilization of the membranes

The MDA435/LCC6 or MDA435/LCC6MDR1 cells (52×10^6 cells) were placed in 10 ml of homogenization buffer (Tris–HCl [50 mM, pH 7.4] containing 50 mM NaCl, 8 μM leupeptin, 2 μM PMSF, and 4 μM pepstatin). The suspension was homogenized for 3×10 s at the setting of 15 on a model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland). The homogenate was centrifuged at $450 \times g$ for 7 min and the pellet containing the nuclear proteins was discarded. The supernatant was centrifuged at $100{,}000 \times g$ for 35 min and the resulting pellet containing the cellular membranes was collected, resuspended in 2 ml of solubilization buffer (Tris–HCl [50 mM, pH 7.4] containing 500 mM NaCl, 15 mM CHAPS, 2 mM DTT, 10% glycerol), and the resulting mixture was rotated at 150 rpm using an orbit shaker (Lab-line model 3520, Melrose Park, IL, USA) for 18 h at 4 °C. The resulting solution was centrifuged at $50{,}000 \times g$ for 25 min, the pellet was discarded and the supernatant containing the solubilized Pgp(−) or Pgp(+) membranes was used for the immobilization onto the open tubular capillary.

2.3. Immobilization of the Pgp membranes on the open tubular capillary

2.3.1. Preparation of the capillary

A peristaltic pump (Minipulse 2, Rainin, Woburn, MA, USA) set at 50 (a flow rate of ≈10 μl/min) was used throughout the procedure. A solution of 1 M NaOH was passed through a glass capillary (25 cm × 100 μm i.d.) for 30 min followed by water for 15 min and then air for 5 min. The capillary was incubated at 95 °C for 1 h. An aqueous solution of 90:10 (v/v) water: APTS was passed through the capillary for 5 min then incubated in the oven at 95 °C for 30 min, this was repeated a second time and the capillary was left open to air overnight. The following morning, a 1% gluteraldehyde solution in phosphate buffer (50 mM, pH 7.4) was passed through the capillary for 60 min followed by a 250 μl of water and then a solution of avidin (10 mg in 2 ml of phosphate buffer [50 mM, pH 7.4]) was run through the capillary for 30 min, after which both tips of the capillary were submerged in the avidin solution for 5 days at 4 °C. Subsequently, the capillary was washed for 3 h with Tris buffer (10 mM, pH 7.4) prior to continuing to the next stage.

2.3.2. Immobilization using biotin-X

A solution of biotin-X (14 mM in 3:1 (v/v) DMSO: phosphate buffer [50 mM, pH 7.4]) was run through the capillary for 1 h with the peristaltic pump set at 50 and both tips of the capillary were then submerged in the solution overnight. The following day the solution containing the solubilized Pgp(+) or Pgp(−) membranes was run through the column for 15 min.

2.3.3. Detergent removal

The open tubular capillary was capped on both ends with dialysis membrane, secured using copper wires and placed in a glass beaker containing the dialysis buffer (Tris buffer [10 mM, pH 7.4] containing 150 mM NaCl, 1.0 mM EDTA, 1.0 mM benzamidine). The beaker was placed in a cold room at 4 °C and rotated overnight at 110 rpm using the orbit shaker. The following day, solubilized Pgp(+) or Pgp(−) membranes were again passed through the capillary for 3 h at 10 μl/ml and the dialysis was repeated. On the third day, the column was attached to a Shimadzu LC-10Advp pump (Shimadzu Corp., Columbia, MD, USA) and equilibrated by rinsing the column with ammonium acetate (10 mM, pH 7.4) for 1 h at 50 μl/min.

2.4. Frontal chromatography with radio-labeled markers

2.4.1. Chromatographic system

The chromatographic system was composed of a Shimadzu LC-10Advp pump, 50 ml superloop (Amersham Pharmacia, Uppsala, Sweden), an IN/US system β-Ram model 3 radioflow detector (IN/US, Tampa, FL, USA) with a dwell time of 2 s and the output data was analyzed using Laura lite 3 (IN/US) running on a PC.

2.4.2. General procedures

In these studies, the mobile phase consisted of the marker ligand [^{3}H]-Vb in 3 ml of ammonium acetate (10 mM, pH 7.4) delivered at a flow rate of 50 μl/min. At the beginning of each experimental series, the mobile phase was run through the Pgp(+)-OT column until the observation of an elution profile showing both front and plateau regions. Increasing concentrations of the unlabeled marker or unlabeled displacer were then added to the mobile phase and the frontal chromatographic experiments were repeated. When Vb was studied, the mobile concentration of [^{3}H]-Vb was 0.65 nM and the added concentrations of Vb were 50, 125, 250, and 500 nM. When ketoconazole was studied, the mobile concentration of [^{3}H]-Vb was 0.33 nM and the added concentrations of ketoconazole were 200 nM, 500 nM, and 10 μM.

2.4.3. Calculation of dissociation constants

Using the frontal chromatograms, the association constants of the competitive ligands (CL), K_{CL}, as well as the number of the active and available binding sites of immobilized receptors, P, were calculated using the following equations (Eqs. (1) and (2)) [13,14]:

$$(V_{max} - V)^{-1} = (1 + [M]K_M)(V_{min}[P]K_M)^{-1} + (1 + [M]K_M)^2 \times (V_{min}[P]K_M K_{CL})^{-1}[\text{drug}]^{-1} \quad (1)$$

$$(V - V_{min})^{-1} = (V_{min}[P]K_{CL})^{-1} + (V_{min}[P])^{-1}[M] \quad (2)$$

where V is the retention volume of the marker ligand (M); V_{max} the retention volume of M at the lowest concentration and in the absence of drugs; V_{min} the retention volume of M when the specific interaction is completely suppressed. The value of V_{min} is determined by running M in a series of concentration of drugs and plotting $1/(V_{max} - V)$ versus 1/[CL] extrapolating to infinite [CL]. From the above plot and a plot of $1/(V - V_{min})$ versus [M], dissociation constant values, K_d, for M and CL can be obtained as can the number of active binding sites on the immobilized protein [P].

2.5. Rapid-frontal chromatography with mass spectrometric detection

2.5.1. Chromatographic system

The chromatographic system consisted of a series 1100 liquid chromatography/mass selective detector (LC/MSD) (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum de-gasser (G 1322 A), a binary pump (1312 A), a manual seven-port injector with a 500 μl loop (model no. 7010, Rheodyne, Rohnert Park, CA, USA), a mass selective detector (MSD) (G1946 B) supplied with atmospheric pressure ionization electrospray (API-ES) and an on-line nitrogen generation system (Whatman, Haverhill, MA, USA). The chromatographic system was interfaced to a 250 MHz Kayak XA computer (Hewlett-Packard, Palo Alto, CA, USA) using ChemStation software (Rev B.10.00, Hewlett-Packard).

2.5.2. Chromatographic conditions

The mobile phase was composed of ammonium acetate (10 mM, pH 7.4) and the experiments were carried out at a flow rate of 50 μl/min at ambient temperature. The parameters on the MSD were set at 11 Lpm for the drying gas flow, 285 °C for the drying gas temperature, 30 psig for nebulizer pressure, and 60 V for the fragmenter.

2.5.3. General procedures

The test ligands were Vb, verapamil, doxorubicin, ketoconazole, prazosin, and (−)-nicotine, Fig. 2. Each compound was prepared as a 1 nM solution and a 500 μl aliquot was applied using the injection loop on the manual injector. The compounds were monitored using single ion monitoring ($M + 1$) at the following m/z values: 455.6 (verapamil); 385.1 (prazosin); 812 (Vb); 532.0 (ketoconzaole); 163.2 (nicotine); 543.6 (doxorubicin). The studies were carried out in triplicate.

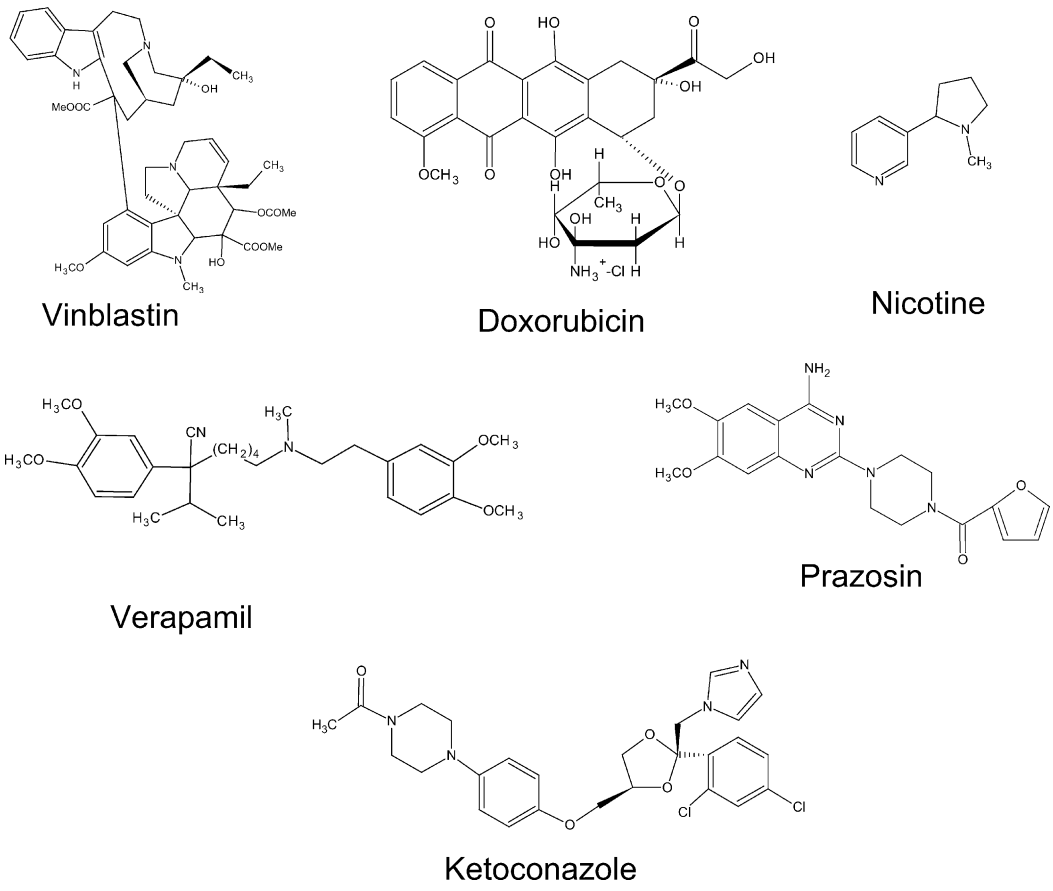

Fig. 2. Structures of the compounds used in this study.

3. Results and discussion

3.1. Characterization of the immobilized Pgp(+)-OT

The activity of the Pgp immobilized in the Pgp(+)-OT was determined using frontal displacement chromatography with [^{3}H]-Vb as the marker ligand, and Vb and ketoconazole as the displacers. Vb was chosen as a representative high affinity ligand with a K_d in the nM range and ketoconazole as a low affinity ligand with a K_d in the μM range.

Elution profiles showing both front and plateau regions were obtained with the marker alone and after the addition of the displacers, Fig. 3. Using this approach, the affinity of the displacer for the immobilized Pgp, expressed as the dissociation constant (K_d), and number of available binding sites [P] were determined for Vb and ketoconazole, Table 1. The K_d values obtained on the Pgp-OT were calculated using linear regression analyses and correlation coefficients were used to determine the correctness of fit rather than standard deviations from the norm.

The K_d value obtained for Vb, 97 nM ($r^2 = 0.902$) was comparable to the values previously obtained on the Pgp-IAM (71 nM [4] and 24 nM [5]) and from membrane filtration studies (37 nM [12] and 36 nM [13]). The K_d obtained for ketoconazole, 12.1 μM ($r^2 = 0.845$), was also equivalent to the K_d value of 8.6 μM obtained using ATP hydrolysis rate studies [14]. For both substrates, there was less than a 10-fold difference in the K_d values determined using different experimental techniques. Thus, the results demonstrate that the immobilized Pgp retained its ability to specifically bind known Pgp substrates, and could discriminate between a solute with nM affinity for the transporter (Vb) and one with a μM affinity (ketoconazole).

In this study, the membranes from 52×10^6 cells were solubilized and used to prepare the open tubular column. This was equivalent to the amount of cells used in the preparation of the Pgp-IAM stationary phases, 20×10^6 [4] and 80×10^6

Table 1
Comparison of K_d values determined on the Pgp-OT column with those previously obtained on the Pgp-IAM and with those obtained using membrane binding studies (literature)

	Pgp-OT		Pgp-IAM		Literature: K_d (nM)
	K_d (nM)	P (nmol)	K_d (nM)	P (nmol)	
Vinblastin	97	3	71 [4], 24 [5]	546 [4]	37 [12], 36 [13]
Ketoconazole	12140	1.5	NR	NR	8600 [14]

NR: not reported.

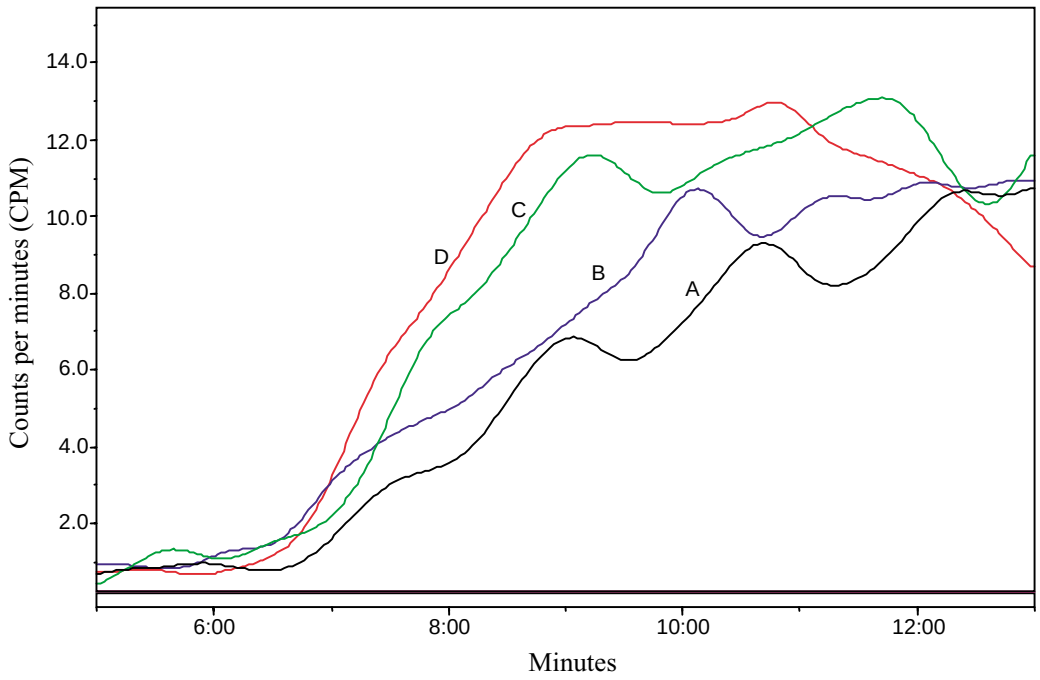

Fig. 3. Frontal chromatographic study of the displacement of [^{3}H]-vinblastin by ketoconazole on the Pgp(+)-OT column using a mobile phase containing 0.3125 nM [^{3}H]-vinblastin: (A) mobile phase; (B) after the addition of 200 nM ketoconazole to the mobile phase; (C) after the addition of 500 nM ketoconazole to the mobile phase; and (D) after the addition of 10,000 nM ketoconazole to the mobile phase. Radioactive decay was measured in counts per minute (cpm) and the chromatograpm was summed up in 1-min intervals and smoothed using the Laura lite program.

[5]. However, the number of the calculated available binding sites (P) on the Pgp(+)-OT were 200-fold less than the number calculated for the Pgp-IAM column produced from 20×10^6 cells, 3 nmol versus 546 nmol, respectively. The large disparity in the P-values most probably reflects the reduction in the total amount of membranes immobilized on the surface of the open tubular column relative to the amount immobilized on the IAM particles, and thereby, a reduction in the total amount of Pgp.

Since the immobilization process utilizes crude membrane preparations and not the purified (or even semi-purified) Pgp protein, it is difficult to assess the total amount of material that has been immobilized on the support. One way of addressing this issue is to measure the total amount of immobilized protein as a reflection of the extent of membrane immobilization. In the previous studies with the Pgp-IAM support, the results from a bicinchoninic acid protein assay (micro-BCA) established that 17 μg of protein were immobilized per mg of support [4]. In this study, the total amount of immobilized protein could not be measured by the micro-BCA, which has a sensitivity of 0.5 μg protein/ml. This result is consistent with the assumption that a significantly reduced amount of Pgp-containing membranes had been immobilized within the open tubular column.

One consequence of the reduced amount of available Pgp binding sites on the Pgp(+)-OT was a significant reduction in the relative capacity of the column. On the Pgp(+)-OT, the half-height of the saturation curve was reached after the application of 500 μl of a 0.625 nM solution of [^{3}H]-Vb, Fig. 4A, while it took 15 ml of a 1 nM solution to reach the same break-through on the Pgp-IAM column [5]. Since both studies used a flow rate of 50 μl/min, the experiments took 10 and 38 min, respectively.

Frontal studies with the Pgp(−)-OT using the same conditions as the Pgp(+)-OT, i.e. 0.625 nM [^{3}H]-Vb, and a flow rate of 50 μl/min, produced elution profiles showing both front and plateau regions, Fig. 4B. However, the addition of Vb or ketoconazole to the mobile phase did not displace the curve. These results indicated that Vb is retained on the immobilized Pgp(−) membranes at specific sites other than Pgp (i.e. at other transporters or receptors), at non-specific sites or at a combination of specific and non-specific sites, and that these sites were saturated under the conditions used in frontal studies.

3.2. *Rapid-frontal chromatography using the Pgp(+)-OT and Pgp(−)-OT*

The results from the frontal chromatographic studies with the Pgp(−)-OT demonstrated that the retention on Pgp(+)-OT was the sum of specific interactions with the Pgp transporter and other specific and non-specific interactions arising from the components of the cellular membrane. The data from the displacement chromatographic studies with Vb and ketoconazole indicated that the background binding did not significantly effect the determination of K_d values and thus the determination of specific binding to the immobilized Pgp. However, while the background binding

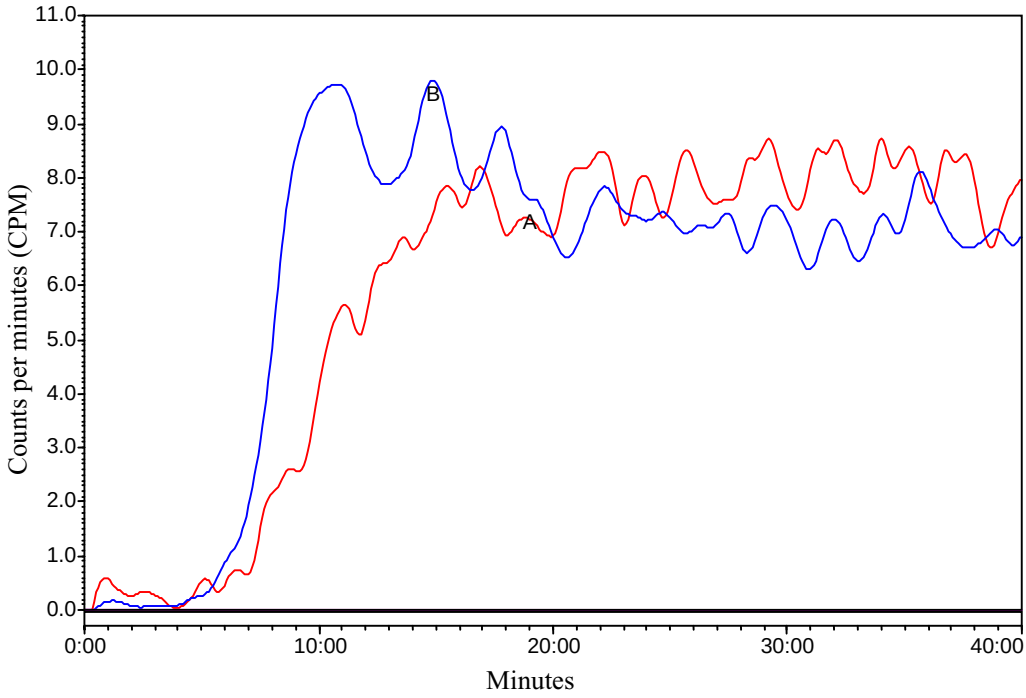

Fig. 4. Frontal chromatographic study of the retention of [^{3}H]-vinblastin on the Pgp(+)-OT column (chromatogram A) and Pgp(−)-OT column (chromatogram B) using a mobile phase containing 0.3125 nM [^{3}H]-vinblastin. Radioactive decay was measured in counts per minute and the chromatograpm was summed up in 1-min intervals and smoothed using the Laura lite program.

to the immobilized membranes did not pose a problem for displacement frontal chromatography, it clearly created a obstacle to the direct determination of relative K_d values from a single experiment on the Pgp(+)-OT.

One approach to correct for background membrane binding is to use the Pgp(−)-OT as a control for the Pgp(+)-OT. Since the Pgp(+) and Pgp(−) membranes are obtained from essentially the same cell lines, the only significant difference between them is the presence of the expressed Pgp in the Pgp(+) membranes. Thus, with the exception of interactions with Pgp, a compound chromatographed on the Pgp(+)-OT and Pgp(−)-OT should experience the same specific interactions with surface receptors and transporters and non-specific interactions with membrane components. Thus, in order to correct for the background binding, a parallel screening protocol was developed in which the test compounds were chromatographed on both the Pgp(+)-OT and Pgp(−)-OT and the differential retention times Δ (min)were determined.

The parallel approach was explored using five known Pgp substrates with relatively high affinity (Vb [4], prazosin [15]), moderate affinity (verapamil [4]), and low affinity (ketoconazole [14], doxorubicin [4]) and a compound that was not a Pgp substrate (nicotine). The experiments were conducted without a marker ligand and the injected compounds were directly detected using a mass spectrometer running in the single ion monitoring ($M + 1$) mode.

The results from these experiments are presented in Table 2 and representative parallel elution profiles are presented in Fig. 5. The data clearly indicates the necessity of using the Pgp(−)-OT as a control. Using only the results from the Pgp(+)-OT, the relative ranking of the affinities of the six test compounds (as reflected by their retention times) was Vb, prazosin, doxorubicin, verapamil, nicotine,

Table 2
Parallel screening of compounds for relative affinities to immobilized Pgp using differential retention, where Δ (min) is calculated from Pgp(+) retention time − Pgp(−) retention time and compared to previously reported K_d values obtained by other methods. The retention times are expressed as mean ± SD where $n = 3$

Compounds (1 nM)	Pgp(+)-OT retention time (min)	Pgp(−)-OT retention time (min)	Δ (min)	K_d literature (nM)
Vinblastin	14.138 ± 0.456	1.550 ± 0.181	12.588	23.5 [4]
Prazosin	10.402 ± 0.111	3.107 ± 0.354	7.295	46 [15]
Verapamil	4.089 ± 0.356	2.947 ± 0.009	1.142	450 [4]
Ketoconazole	1.717 ± 0.017	1.579 ± 0.042	0.138	8600 [14]
Doxorubicin	10.349 ± 0.174	9.737 ± 0.205	0.612	31000 [4]
Nicotine	2.327 ± 0.147	2.361 ± 0.411	−0.034	–

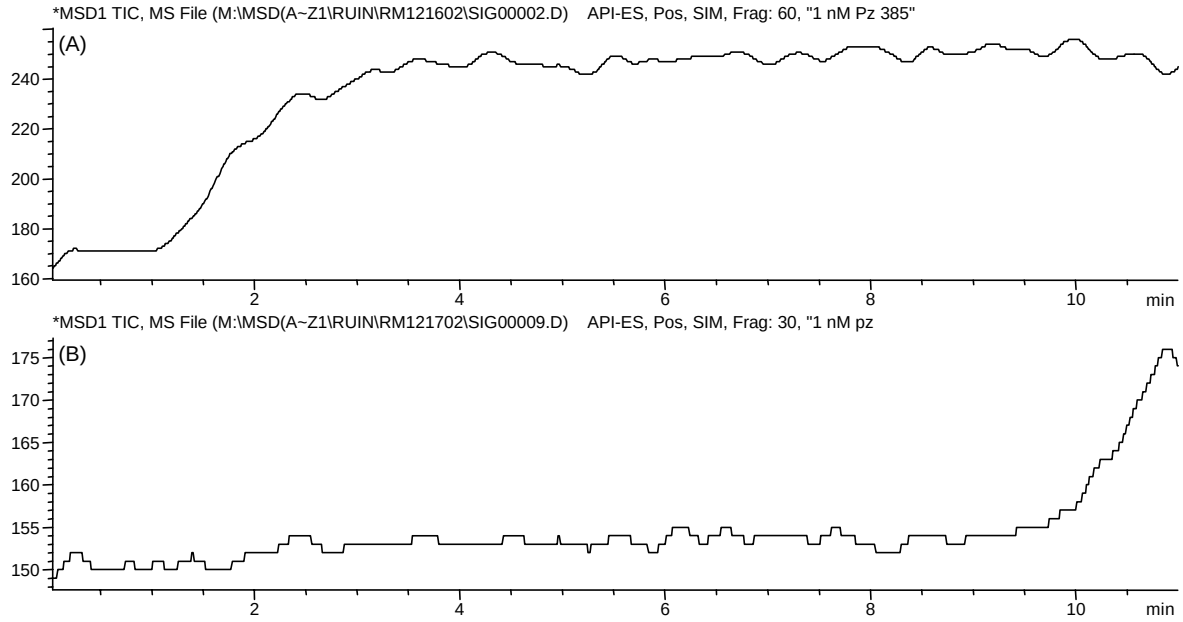

Fig. 5. Parallel rapid-frontal chromatography of prazosin on the Pgp(−)-OT column (chromatogram A) and Pgp(+)-OT column (chromatograpm B) after a 500 μl injection of 1 nM prazosin onto both columns and using mass spectrometric detection. The chromatograms were smoothed using the Agilent LC-MSD smoothing program.

ketoconazole. Based upon the previously published K_d values, both doxorubicin and nicotine were misplaced. When the observed retention on the Pgp(−)-OT was subtracted from the data obtained on the Pgp(+)-OT, it was clear that nicotine was not retained by the immobilized Pgp and the relative differential retention of doxorubicin places its apparent K_d value between those of verapamil and ketoconazole.

The results from the chromatographic experiments indicate that the parallel use of the Pgp(+)-OT and Pgp(−)-OT columns was able to differentiate between compounds with high, moderate-low, and no affinity for the immobilized Pgp. This is clearly a qualitative assessment, but one achieved with a single injection on each column. Using a parallel screening system, which permits simultaneous chromatography on two columns [16], the relative affinity of a compound could be determined in 30 min (15 min frontal chromatography, 15 min wash period).

4. Conclusions

The data from this study indicate that membranes from a cell line expressing a target receptor or transporter can be immobilized on the surface of glass capillaries with retention of the target's binding activity, and that this activity can be measured using displacement frontal chromatographic techniques. The results form this study also demonstrate that since cellular membranes are immobilized, instead of the purified target, the membranes contribute specific and non-specific off-target binding to the observed chromatographic retention. This necessitates the construction of a second open tubular capillary column using membranes from a cell line that does not express the target receptor or transporter. The target(−) column can then be used as a control for the target(+) column. This approach should be applicable for use with any membrane bound target where "knock-in" or "knock-out" cell lines are available.

In this study, Pgp(+)-OT and Pgp(−)-OT columns were used in 30-min parallel frontal chromatographic experiments to qualitatively rank compounds based upon their relative affinities for the immobilized Pgp transporter. The Pgp(+)-OT/Pgp(−)-OT approach permits one to rank up to 200 compounds per week and, as the columns are active for between 3 and 4 weeks, each pair could be used to screen a minimum of 800 compounds. The chromatographic approach should be a significant advance over the current approach to the determination of a Pgp substrate/inhibitor, which involves the use of multiple Pgp assays and a complex classification system of transported and non-transported substrates and inhibitors [17]. A comparative inter-assay assessment is currently being conducted with a larger cohort of compounds and will be reported elsewhere.

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