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Development of an immobilized P-glycoprotein stationary phase for on-line liquid chromatographic determination of drug-binding affinities

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Abstract

The membrane transporter P-glycoprotein (PGP) has been immobilized on an immobilized artificial membrane (IAM) LC stationary phase. The resulting PGP-IAM phase retained the ability of the native PGP to bind the known PGP-ligand vinblastine. Displacement studies using other known PGP ligands, verapamil and cyclosporin A, demonstrated that there was selective binding between vinblastine and the immobilized PGP transporter. The binding affinity (K_d value) of vinblastine for the PGP-IAM was determined to be 19±20 and 71±11 n*M* on two separate columns. These values are consistent with previously reported values of 9±2, 8±2, and 37±10 n*M*, which were obtained using native membranes. The K_d values obtained on the PGP-IAM for cyclosporin A and verapamil were 492±21 and 172±29 μ*M*, respectively. These results were higher than the corresponding K_d values obtained using native membranes, but the relative affinities vinblastine>cyclosporin A≫verapamil are consistent in both approaches. During several months of experiments and storage, the PGP-IAM was found to be reproducible and stable. The stationary phase appears to be useful in the on-line screening for PGP ligands.

Keywords: Stationary phases, LC; Drug-binding affinities; P-Glycoprotein; Verapamil; Cyclosporin A

1. Introduction

P-glycoprotein (PGP) is a 170–180-kDa membrane transporter [1,2] that acts as an ATP-driven drug efflux pump. The over-expression of PGP has been associated with multidrug resistance (MDR) in tumor cells and the MDR phenotype is a factor in the failure of the chemotherapeutic treatment of breast cancer [3,4]. One approach to the development of therapeutic protocols to overcome MDR in breast cancer patients has concentrated on the inhibition of the PGP-mediated pump. For example, in vitro studies have demonstrated that the presence of verapamil in the incubation media increased the cytotoxicity of vinca alkaloids and anthracycline derivatives in MDR1/PGP tumor cell lines [5]. However, a clinical trial combining verapamil with the vinca alkaloid VP16 and the anthracycline derivative adriamycin was not successful due to the cardiotoxicity of verapamil [6]. Thus, the development of novel agents to reverse MDR1/PGP-mediated drug resistant remains a key objective in breast cancer research.

The functions of PGP have been studied using a

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variety of experimental formats including, detergent solution [7], proteoliposomes [8–12], membrane vesicles [13,14], and native membranes [15–17]. However, the evaluation of ligand-binding parameters and the screening of pools of drug candidates for their PGP binding affinities remain a formidable task.

Our laboratory has recently reported the development of immobilized nicotinic receptor-based liquid chromatographic (LC) stationary phases that can be used for the on-line analysis of drug-receptor interactions [18,19]. In the present work we extend this study to the preparation of PGP-based LC stationary phases for the study of drug–PGP interactions. One PGP-based stationary phase was prepared by embedding PGP in the phospholipid monolayer of an immobilized artificial membrane (IAM) HPLC stationary phase [20] creating the PGP-IAM. In a second approach, PGP was also reconstituted into the phospholipid bilayer of liposomes that were immobilized on Superdex 200 gel beads by using freeze-thawing methods (PGP-LIP). The latter approach was originally developed for the immobilization of liposomes or liposomes containing human red cell glucose transporter in chromatographic stationary phase [21,22].

In this study, the PGP binding affinities of vinblastine, cyclosporin A and verapamil were assessed using the PGP-IAM stationary phase and frontal chromatographic techniques. The rank order of the calculated K_d values, i.e. highest affinity to lowest affinity, were consistent with previously reported values [15]. The PGP-IAM was stable, reproducible and appears to be a useful addition to the study of PGP–ligand interactions and for the rapid on-line screening of new agents for the treatment of MDR1/PGP resistant tumors.

2. Experimental

2.1. Chemicals

L-α-Lecithin [20% phosphatidylcholine (PC)], *E. coli* bulk phospholipid and L-α-phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). IAM.PC particles were obtained from Regis Chemical Co. (Morton Grove, IL, USA). Superdex 200® prep grade (a gel filtration media with a unique composite matrix of dextran and agarose), Sephadex G50 medium and glass column (HR5/5 and HR 5/10) were purchased from Amersham Pharmarcia Biotech (Uppsala, Sweden). [^{3}H]Vinblastine sulphate was from Amersham Life Science Products (Boston, MA, USA). Cyclosporin A, octyl-β-D-glucopyranoside, leupeptin, pepstain A, glycerol, benzamidine, cholesterol (>99%), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT, USA).

2.2. Immobilization of PGP in IAM particles

Cultured MDA435/LCC6^{MDR1} cells which over express the MDR1 gene and PGP [23] were used as the source of the PGP. About 2×10^6 cells were harvested in 18 ml of 50 m*M* Tris–HCl buffer [50 m*M*, pH 7.4] {Buffer A} containing 50 m*M* NaCl, 2 μ*M* leupeptin and 4 μ*M* pepstatin A. The mixture was homogenized for 2×20 s with a Brinkmann Polytron homogenizer, the homogenates were centrifuged at 35 000 *g* for 10 min, the supernatant was discarded and the pellets resuspended in 6 ml solubilization solution [Buffer A containing 250 m*M* NaCl, 0.5% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate}, 2 m*M* DTT (dithiothreitol), 5% glycerol] for 2 h at 4°C. Dried IAM.PC particles (100 mg) were suspend in the PGP-CHAPS solution (7 ml) and stirred for 1 h at 4°C. The mixture was then dialyzed against dialysis buffer [150 m*M* NaCl, 10 m*M* Tris–HCl buffer, pH 7.5, 1 m*M* EDTA (ethylenediamine-teraacetic acid), 1 m*M* benzamidine] for 36 h at 4°C. The obtained PGP-IAM particles were washed with Buffer A by centrifugation and packed in a glass column.

2.3. Reconstitution and immobilization of PGP in Superdex 200 gel beads

The membrane pellet obtained as described above was suspended in 4 ml solubilization solution [50 m*M* Tris–HCl, pH 7.5 containing 1.4% octyl-β-D-glucopyranoside, 20% glycerol, 1 m*M* DTT, 1 m*M* benzamidine and 0.4% phospholipid: *E. coli* bulk phospholipid–PC–PS–cholesterol (60:17.5:10:12.5,

v/v)] by stirring at 0°C for 40 min. Non-soluble material was removed as a pellet by centrifugation. The supernatant was applied on to a Sephadex G50 column (1×80 cm) which equilibrated with elution buffer [150 m*M* NaCl, 10 m*M* Tris–HCl, 1 m*M* EDTA, 1 m*M* Benzamidine]. The liposome fractions (18 ml) were collected and concentrated to 1 ml. The concentrated liposome solution was mixed with 50 mg dried Superdex 200 and kept in room temperature for 2 h. The mixture of liposome and Superdex 200 was frozen at −75°C for 10 min and thawed at 25°C for 10 min. The freezing and thawing was repeated once. The non-immobilized liposomes were removed by washing the pellet with Buffer A until the supernatant was clear in centrifugation. The obtained PGP-Superdex gel beads were packed in a chromatographic column (0.5 cm I.D.).

2.4. Frontal chromatographic analysis of binding affinity of drugs with PGP-IAM stationary phase

The PGP-IAM column was placed in a standard HPLC system and equilibrated with Buffer A. [^{3}H]Vinblastine (10–45 ml of a 0.5 or 1 n*M* solution in Buffer A in the absence or presence of cold vinblastine) was applied to the column and an elution profile with frontal and plateau (see an example of the elution profiles in Fig. 1) is recorded by an on-line flow scintillation detector (Radiomatic™ 525 TR, Packard Instruments). The radioactive signal (CPM) in the outlet eluate were recorded in 6-s intervals, summed up in 1-min intervals and smoothed with a ten-point moving average using the Microsoft Excel program. The retention volumes of [^{3}H]vinblastine (0.5 n*M*) in the absence and presence in different concentration of drugs in the mobile phase were taken as the elution volume corresponding to the half height of plateau. The flow-rate, 0.4 ml/min, was used in all runs of chromatography. Vinblastine and doxorubicin were solubilized in Buffer A, verapamil was solubilized in Buffer A with 20% ethanol and the final concentration of ethanol was adjusted to 1.6% and cyclosporin A was solubilized in Buffer A with 1.6% ethanol.

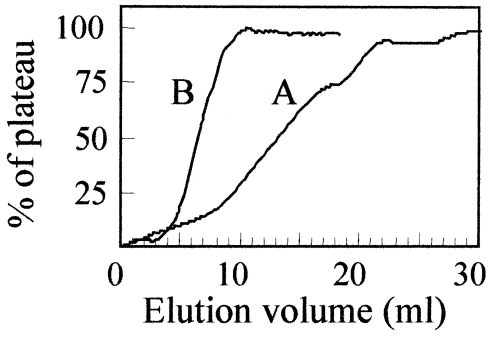

Fig. 1. Elution profiles of [^{3}H]VB [1 nM] in frontal chromatography based on the PGP-IAM column (0.5×0.8 cm) in the absence (profile A) and presence of doxorubicin [200 nM] (profile B) in the mobile phase: Tris–HCl buffer [50 m*M*, pH 7.4]. Flow rate: 0.4 ml/min.

2.5. Bicinchoninic acid (BCA) protein assay

The PGP-IAM particles, PGP-Superdex 200 gel beads, IAM particles, Superdex 200 gel beads were collected. The samples were diluted with 0.1 m of NaOH to 2 ml. A protein standard (0.2–25 μg protein in 50 μl) was prepared with albumin standard (Pierce) and amount of proteins were detected using Pierce BCA protein assay kit (BCA is a trademark of Pierce for protein assays using bicinchoninic acid and the reagents contain sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1 m sodium hydroxide). The standards and samples (50 μl each) were added to triplicate wells in a 96-well plate, 20 ml of reagent A was mixed with 0.4 ml of reagent B and a 200-μl aliquots of the resulting BCA reagents (A+B) were added to each well. The plate was incubated for 2 h at room temperature and read in a spectrophotometer at 570 nm using Softmax program for the calculation of protein amount.

3. Results and discussion

Protein assay showed that for 1 ml of bed volume about 170 mg proteins were immobilized in IAM particles and about 10 mg proteins were immobilized in Superdex 200 gel beads. On a PGP-IAM column (0.5×0.8 cm), [^{3}H]vinblastine (1 n*M*) was retarded and showed a frontal profile with retention volume 13.3 ml (Fig. 1, profile A). When a known displacer, doxorubicin (200 n*M*), was included in the mobile phase, the retention volume of [^{3}H]vinblastine [1

nM] was decrease from 13.3 to 6.5 ml (Fig. 1, profile B). This displacement experiment indicated that the specific binding activity of PGP was retained after immobilization.

The K_d value of vinblastine (K_{VB}) and the number of the active and available binding sites (B_{max}) of immobilized PGP were calculated from the retention volumes, V, of [^{3}H]vinblastine at the different concentration in frontal chromatography according to Eq. (1):

$$[VB](V - V_{min}) = B_{max}[VB]/(K_{VB} + [VB]) \quad (1)$$

This equation was adapted from the rectangular hyperbola equation [24] using a previously described approach (see Eq. (3) in Ref. [25]). Using this approach, K_d and B_{max} can be calculated by the plotting $[VB](V - V_{min})$ vs. [VB] or by one binding site nonlinear regression with program Prism (GraphPad Software), taking [VB] as X values and $[VB](V - V_{min})$ as Y values. V_{min} is the elution volume of vinblastine [0.5 nM] when the specific interaction is completely suppressed. V_{min} can be taken approximately as the retention volume in the presence of high concentration of vinblastine. When a competitive displacer (drug) was included in the mobile phase, V_{min} value can be calculated more precisely by Eq. (2) (below).

The calculated K_d value for vinblastine determined on the initial PGP-IAM column (0.5×0.8 cm) in Buffer A was 19±20 nM with B_{max} 546±60 nmol and on a second PGP-IAM column (0.5×1.5 cm) was 71±11 nM with B_{max} 1073±57 nmol. The retention volume of vinblastine [0.5 nM] on the second PGP-IAM column was 25.0±1.0 ml during the initial chromatographic run and 24.5±0.8 ml after over 1 month of use at room temperature. These results demonstrate that the PGP-IAM is reproducible and stable.

The addition of ethanol [1.6%] was necessary for the solubilization of the hydrophobic drugs in the mobile phase. When 1.6% ethanol was included in the mobile phase the retention volume of vinblastine [0.5 nM] was decreased from 25.0±1.0 to 15±1 ml. Verapamil and cyclosporin A were still able to displace vinblastine and ethanol appears to predominately affect the non-specific retention interactions.

The retention volumes, V, of [^{3}H]vinblastine [0.5 nM] measured in the absence or presence of verapamil and cyclosporin A, respectively, at different concentrations, [drug], were used to calculate the K_{drug}, according to Eq. (2) [18]:

$$(V_{max} - V)^{-1} = (1 + [VB]K_{VB})(V_{min}B_{max}K_{VB})^{-1} + (1 + [VB]K_{VB})^2(V_{min}B_{max}K_{VB}K_{drug})^{-1}[drug]^{-1} \quad (2)$$

By plotting $(V_{max} - V)^{-1}$ vs. $[drug]^{-1}$, V_{min} was obtained when [drug] was extrapolating to infinity, and K_{drug} or B_{max} for drugs were calculated from the ratio between the slop and the ordinate intercept, which equals $(1 + [VB]K_{VB})/K_{drug}$. The obtained K_d values of vinblastine, verapamil and cyclosporine were presented in Table 1. The mean and deviations of the K_d values were obtained using the retention volumes from two runs.

The K_d value of vinblastine measured from two PGP-IAM columns is 19±20 and 71±11 nM. These values are consistent with previously reported values of 9±2 nM [15], 8±2 nM [16] and 37±10 nM [17] that were obtained using native membranes. In another study, the K_d value of vinblastine was found to be 36±55 nM in native membranes and 130±9 nM after the PGP were solubilized in detergent [9]. The authors concluded that solubilization has altered the PGP phospholipid environment reducing its specific capacity.

The K_d values obtained for cyclosporin A (492±21 nM) and verapamil (172±29 μM) are higher than the previously reported values of 17±2 nM [15], 18±3 nM [17] for cyclosporin A and 600±180 nM [15], 452±50 nM [17] for verapamil, respectively. These results may be due to the fact that PGP has at least two allosterically coupled binding sites [17] and that the solubilization of the protein before immobilization on the IAM support

Table 1
K_d values calculated using frontal chromatography on an PGP-IAM column

Drugs	K_d
Vinblastine	71±11 nM
Cyclosporine A	492±21 nM
Verapamil	172±29 μM

may have altered the configuration of one or more of these sites. This possibility is under investigation.

Although the absolute K_d values determined on the PGP-IAM are higher than those obtained by other methods, the relative affinities are the same. This demonstrates that the method previously developed for immobilization of nicotinic receptors in LC stationary phase also can be used to immobilize PGP for study of interaction between PGP and drugs.

The methods to reconstitute into liposomes and immobilize PGP-liposomes in Superdex 200 gel beads were also included in this manuscript as an alternative for the preparation of PGP-LC stationary phase. PGP reconstituted in the phospholipid bilayer of the immobilized proteoliposomes also displayed a similar binding activity as the PGP immobilized in IAM particles (data not shown). Since immobilized PGP-Superdex 200 did not show obvious advantages over PGP-IAM in the study of drug binding on PGP, additional experiments are being performed on PGP-Superdex 200 column and will be reported at a future date.

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