Talanta 81 (2010) 1477–1481

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

The synthesis and characterization of cellular membrane affinity chromatography columns for the study of human multidrug resistant proteins MRP1, MRP2 and human breast cancer resistant protein BCRP using membranes obtained from *Spodoptera frugiperda* (Sf9) insect cells

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ARTICLE INFO

Article history:
Received 25 January 2010
Accepted 20 February 2010
Available online 25 February 2010

Keywords:
Sf9
Affinity chromatography
Drug discovery
Receptor screening
ABC transporters
P-glycoprotein
MRP1
MRP2
BCRP
Membrane receptors

ABSTRACT

CMAC (cellular membrane affinity chromatography columns) have been developed for the study of the human multidrug transporters MRP1, MRP2 and the breast cancer resistance protein (BCRP). The columns were constructed using the immobilized artificial membrane (IAM) stationary phase and cellular membrane fragments obtained from *Spodoptera frugiperda* (Sf9) cells that had been stably transfected with human Mrp1, Mrp2 or Bcrp cDNA, using a baculovirus expression system. The resulting CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$) columns and a control column produced using membrane fragments from non-transfected Sf9 cells, CMAC(Sf9), were characterized using frontal affinity chromatography using [^{3}H]-etoposide as the marker ligand and etoposide, benzbromarone and MK571 as the displacers on the CMAC($Sf9_{MRP1}$) column, etoposide and furosemide on the CMAC($Sf9_{MRP2}$) column and etoposide and fumitremorgin C on the CMAC($Sf9_{BCPR}$) column. The binding affinities (K_i values) obtained from the chromatographic studies were consistent with the data obtained using non-chromatographic techniques and the results indicate that the immobilized MRP1, MRP2 and BCRP transporters retained their ability to selectively bind known ligands. (S)-verapamil displaced [^{3}H]-etoposide on the CMAC($Sf9_{MRP1}$) column to a greater extent than (R)-verapamil and the relative IC_{50} values of the enantiomers were calculated using the changes in the retention times of the marker. The observed enantioselectivity and calculated IC_{50} values were consistent with previously reported data. The results indicated that the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$) columns can be used for the study of binding to the MRP1, MRP2 and BCRP transporters and that membranes from the Sf9 cell line can be used to prepare CMAC columns. This is the first example of the use of membranes from a non-mammalian cell line in an affinity chromatographic system.

Published by Elsevier B.V.

1. Introduction

Acquired or innate multiple drug resistance (MDR) decreases the therapeutic activity of a number of drugs used in the treatment of cancer, infectious diseases, and central nervous system disorders like epilepsy, schizophrenia and chronic depression. One of the mechanisms associated with the development of MDR is the overexpression of transmembrane multiple drug transporters, which actively export drugs out of cells, thereby decreasing intercellular concentrations and reducing therapeutic effect [1]. One key family of multidrug exporters is the ATP-binding cassette (ABC) transporter family, which includes P-glycoprotein (Pgp/ABCB1), the multidrug resistance proteins MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP6 (ABCC6), MRP7 (ABCC10) [2] and the breast cancer resistance protein, BCRP(ABCG2) [3].

Pgp was the first ABC transporter that was identified and characterized, the cellular biology and pharmacology of this protein has been extensively discussed [4]. MRP1 was identified in a H69AR (Human Small Cell Lung Cancer) cell line [5] and MRP2 in a Hep-G2 (Hepatocellular carcinoma) cell line [6]. BCRP is a half transporter, consisting of a single transmembrane and ATP-binding domain [7], which is functional as a homodimer [8]. BCRP was initially cloned from a drug resistant breast cancer cell line (MCF-7/AdrVp) [3]. These transporters have been shown to play a role in the cellular resistance to a number of anticancer agents such as etoposide, doxorubicin, vinblastin and vincristin [9] and mitoxantrone [10].

In addition to their role in MDR, ABC transporters are also expressed in healthy tissues, including the intestine, liver, kidneys

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0039-9140/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.talanta.2010.02.055

and brain, where they may be involved in drug absorption, distribution and excretion [4]. The role of Pgp in oral bioavailability has been extensively studied and lead drug compounds are routinely screened for interactions with this protein [11]. MRP1 is also expressed in mucosal epithelia of the gut indicating a potential role in oral bioavialibility [12] as well as in the blood brain barrier, blood cerebrospinal fluid and blood tumor barriers indicating a potential role in CNS bioavailability [13]. MRP2 is expressed in the liver, kidneys and intestine, and this transporter is believed to be responsible for the biliary elimination of various endogenous and exogenous anions [14]. Although being similar in function and having various common substrates, it differs from MRP1 in terms of its localization. MRP2 has been reported to be present on the apical plasma membranes of cells as opposed to the basolateral location of the human MRP1 [15]. Owing to its presence in tissues like the liver, intestine and kidney, it is believed MRP2 plays a significant role in active extrusion of endogenous substances and xenobiotics [16]. Functional expression in tumors of the brain and liver [17] make it an important element in most drug development programs.

BCRP is expressed in the blood brain barrier where it is localized on apical side of cells indicating a role in the export of chemotherapeutic agents [18]. The protein is also functionally expressed in the intestine and liver indicating a role in oral and systemic bioavailability [19]. In addition, BCRP has been shown to play a role in protecting hematopoeitic stem cells, and it appears that the expression profile of BCRP in human stem cells is significantly higher than that of Pgp or MRP1 [20].

Since ABC transporters play a role in systemic and CNS bioavailability and in drug resistance, the screening of lead drug compounds for interactions with these proteins has become an important element in drug discovery programs [21]. The primary industrial screen for Pgp substrates and inhibitors is the Caco-2 efflux assay [22]. However, a similar assay has not been established for the screening at MRP1, MRP2 or BCRP. For these proteins, the main experimental approaches involve *in vitro* selected cell lines or stably transfected cell lines and efflux studies [23,24] or transport across isolated membrane vesicles [14] or cellular monolayers [25].

We have recently demonstrated that cellular membrane affinity chromatography (CMAC) can also be used as a screen for Pgp substrates and inhibitors [26]. The screen was based upon a column that contained immobilized membrane fragments obtained from a cell line that stably expressed Pgp, the CMAC(Pgp) column [27]. The CMAC(Pgp) column was used in rapid frontal affinity chromatography and yielded equivalent results and throughput when compared to the Caco-2 assay [28]. The objective of this study was to create and characterize CMAC columns containing membranes from cell lines expressing the MRP1, MRP2 and BCRP. While a number of mammalian cell lines have been used to over-express ABC transporters, we chose to use commercially available membranes obtained from stably transfected insect cell lines, *Spodoptera frugiperda* (Sf9), that expressed MRP1 [29], MRP2 [30] and BCRP [8]. These membranes were used to create the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$) columns. Membranes from the Sf9 cell line was also chosen for this study because they are extensively used in protein expression and due to the fact that it can be used with all baculovirus expression systems [31] and that the cells are robust and efficient in the production of the transfected proteins [32].

The results of these studies are reported below and indicate that the drug transporters immobilized within the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and the CMAC($Sf9_{BCRP}$) columns retain the ability to bind MRP1, MRP2 and BCRP ligands, respectively and can be used to characterize these interactions. In addition, all of the previous CMAC columns have been prepared using mammalian cell lines or tissues [26,33,34]. The data from this study demonstrate that insect-derived cellular membranes can also be used to create CMAC columns, thereby expanding the utility of the technique.

2. Experimental

2.1. Materials

2.1.1. Chemicals

[^{3}H]-etoposide, was obtained from Moravek Biochemicals (Brea, CA, USA). MK571 and fumitremorgin C was obtained from Calbiochem (San Diego, CA, USA). Etoposide, benzbromarone, (R)-verapamil, (S)-verapamil, furosemide, HEPES, NaCl, β-mercaptoethanol, Tris–HCl, protease inhibitor cocktail (Catalog number P8340), adenosine triphosphate (ATP), glycerol, ethylene glycol-bis(2-aminotheylether)-N,N,N′N′-tetra-acetic acid (EGTA), glutathione (GSH), CHAPS and trizma were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The immobilized artificial membrane phospahtidyl choline stationary phase (IAM-PC, 12 μm, 300 Å) was purchased from Regis Technologies Inc. (Morton Grove, IL, USA).

2.1.2. Sf9 membranes

Sf9-human MRP1 membranes (Catalog number 453456), Sf9-human MRP2 membranes (Catalog number 453332) and the Sf9-human BCRP membranes (Catalog number 453270) isolated from Sf9 cell line transfected with Human Mrp1 cDNA, human Mrp2 cDNA and human BCRP cDNA gene using a baculovirus system and control membranes (Catalog number 453200) isolated from non-transfected, native Sf9 cell line were obtained from BD Biosciences (Woburn, MA, USA).

2.2. Methods

2.2.1. Preparation of CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$), CMAC($Sf9_{BCRP}$) and CMAC(Sf9) columns

The CMAC columns were prepared using 750 μg protein in 150 μl of the commercially available membrane solutions and immobilized on 100 mg IAM stationary phase following previously described procedures [26,35]. The CMAC stationary phases were packed into Tricorn 5/20 columns (GE Healthcare, UK) yielding a 75 mm × 5 mm (i.d.) chromatographic beds.

2.2.2. Chromatographic studies

The cellular membrane affinity columns were placed in a frontal affinity chromatography system and competitive displacement studies were carried out using previously described techniques [26,35]. Briefly the system consisted of a manual FPLC injection valve, 50-ml superloop both obtained from (Amersham Biotechnology, Columbia, MD), CMAC column, LC-10AD HPLC pump (Shimadzu Inc.) and an on-line radioactive/scintillation flow detector (IN/US, Tampa, FL, USA). Solutions of the marker and test ligands were prepared in the running buffer, Tris–HCl [10 mM, pH 7.5] containing 1 mM $MgCl_2$, and 5 ml samples were placed in the superloop, pumped across the CMAC column at a flow rate of 0.2 ml/min and monitored through a 250 μl flow cell with the radioflow detector. The scintillation flow rate was 0.6 ml/min while the split ratio was 100. The breakthrough volume of the marker was calculated using the retention times at the midpoint of the chromatographic curves and the effect on the breakthrough volumes produced by increasing displacer concentrations was used to calculate the dissociation constant (K_i) of the displacer as previously described [26,33]. The concentration of the marker ligand, [^{3}H]-etoposide, was 1 nM, and the concentrations of the displacer ligands etoposide and benzbromarone were 10 μM, 25 μM, 50 μM, 100 μM and 250 μM, the concentrations of MK571 were 1 μM, 5 μM, 10 μM, 25 μM and 50 μM, furosemide was injected in the concentrations

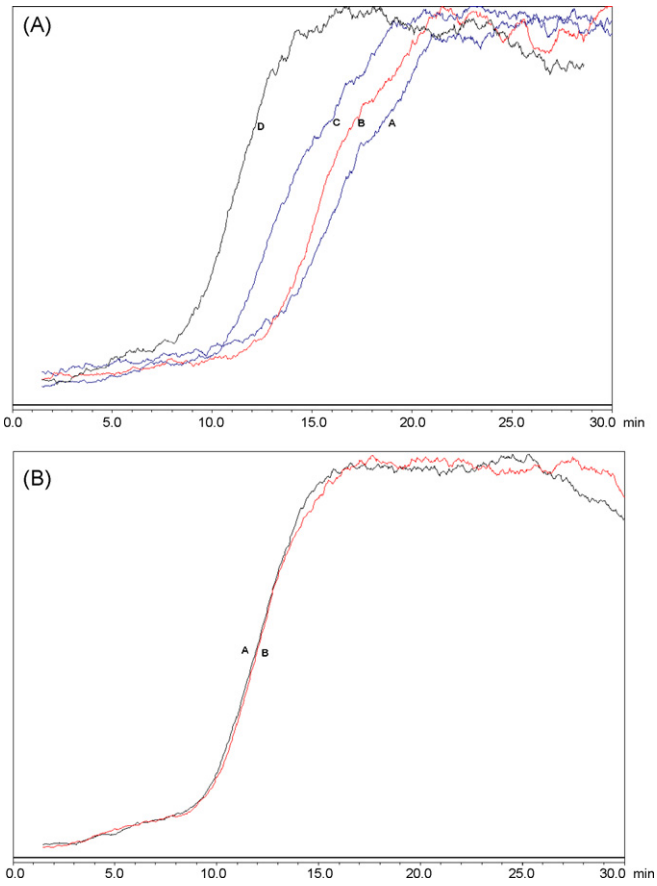

Fig. 1. (A) Chromatographic traces produced by [^{3}H]-etoposide on the CMAC(Sf9$_{MRP1}$) column, where A = [^{3}H]-etoposide alone, B = with 10 μM etoposide, C = 10 μM (R)-verapamil and D = 10 μM (S)-verapamil. (B) Chromatographic traces produced by (A) [^{3}H]-etoposide 1 nM and (B) 1 nM [^{3}H]-etoposide with 100 μM etoposide on the CMAC(Sf9) column.

of 1 μM, 2.5 μM, 5 μM, 15 μM and 30 μM and fumitremorgin C was passed through the column in the concentration of 500 nM, 1 μM, 2.5 μM, 5 μM and 10 μM.

2.2.3. Data analysis

Binding affinities, expressed as K_i values, were calculated using non-linear regression with a rectangular hyperbolic curve as previously described. [26,36], using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA) running on a personal computer.

3. Results

3.1. CMAC(Sf9$_{MRP1}$) column

The presence of the MRP1 protein in the Sf9$_{MRP1}$ membranes and the absence of MRP1 in the Sf9 membranes were confirmed by Western Blotting (data not shown). The membranes were immobilized on the IAM stationary phase to create the CMAC(Sf9$_{MRP1}$) and CMAC(Sf9) columns. The binding activity of the immobilized MRP1 protein was established using [^{3}H]-etoposide as the marker ligand. This compound, also known as VP-16, has been identified as a substrate for the human MRP1 [6,15] and has been previously used in competitive inhibition studies, [37]. When 1 nM [^{3}H]-etoposide was placed in the running buffer, the resulting frontal chromatographic trace obtained on the CMAC(Sf9$_{MRP1}$) column contained an initial flat region, followed by a vertical breakthrough and a plateau region, Fig. 1A, Curve A. These results indicate that the marker bound in a specific and saturable manner on the CMAC column [26]. The addition of 10 μM etoposide produced a significant reduction in the retention volume of the marker indicating that the competitive displacement experiments could be conducted on the CMAC(SF9$_{MRP1}$) column, Fig. 1A, Curve B. When the same experiments were conducted on the CMAC(Sf9) column, a frontal chromatographic trace was also observed, Fig. 1B, Curve A, indicating that [^{3}H]-etoposide also bound specifically and non-specifically to proteins and other components of the membranes obtained from the non-transfected Sf9 cells. However, the addition to the mobile phase of up to 100 μM etoposide produced no significant reduction in the retention volume of the marker, Fig. 1B, Curve B, indicating that etoposide could be used as a marker for specific competitive binding experiments on the CMAC(Sf9$_{MRP1}$) column. This is consistent with previous studies which have demonstrated that specific binding to a target protein can be studied in the presence of additional specific and non-specific interactions with the cellular membrane by the use of control columns [26].

Table 1
The displacement of 1 nM [^{3}H]-etoposide by the addition of 10 μM etoposide, (R)-verapamil and (S)-verapamil to the running buffer represented as ΔT calculated as $\Delta T = T(o) - T(d)$, where $T(o)$ is (16.4 min) the retention time of [^{3}H]-etoposide with no displacer in the running buffer and $T(d)$ is the retention time of [^{3}H]-etoposide after the addition of the displacer. NC = not calculated.

Displacer	Retention time T(d) (min)	$\Delta T = T(o) - T(d)$ (min)	IC_{50} (μM)
Etoposide	15.4	0.9	NC
(R)-verapamil	13.8	2.6	8.6
(S)-verapamil	11.0	5.4	4.1

In order to confirm that specific binding to the MRP1 protein binding could be studied on the CMAC(Sf9$_{MRP1}$) column, we determined the effect of the enantiomers of verapamil; (R)-verapamil and (S)-verapamil, on the retention of [^{3}H]-etoposide. Since (R)-verapamil and (S)-verapamil have essentially equivalent physicochemical properties, any difference in their effect on the retention of [^{3}H]-etoposide will be due to specific interactions between the immobilized membranes and the two isomers.

Verapamil was also chosen on the basis of the report by Gaj et al. [37] which demonstrated that (S)-verapamil was a more efficient MRP1 inhibitor than (R)-verapamil in human nasopharyngeal KB cell lines over-expressing MRP1. In the study by Gaj et al., 10 μM concentrations of each of the verapamil enantiomers were used to determine the changes in the IC_{50} values associated with the anti-proliferative activities of etoposide, vincristine and doxorubicin. In each instance, the presence of (S)-verapamil produced a greater decrease in the IC_{50} value of the anticancer agent than (R)-verapamil. When etoposide was the anti-proliferative agent, the observed IC_{50} was decreased from 28 μM to 5 μM by (S)-verapamil and to 15 μM by (R)-verapamil, a 3-fold difference in enantioselectivity.

In this study, the addition of 10 μM (S)-verapamil to the mobile phase reduced the retention time of [^{3}H]-etoposide by 5.4 min, while the equivalent concentration of (R)-verapamil reduced the retention by 2.6 min, Fig. 1, Curves C and D, respectively, Table 1. The results indicate that the affinity of (S)-verapamil is ~2-fold greater than that of (R)-verapamil, which is consistent with the data reported by Gaj et al. [37]. Both verapamil enantiomers produced a larger displacement than 10 μM etoposide, which reduced the retention of the marker by 0.9 min, Fig. 1A, Curve B, Table 1. This is also consistent with the previously reported difference in IC_{50} values of 48 μM for etoposide [38] and 5–13 μM for racemic verapamil [38,39]. When the same experiments were conducted on the CMAC(Sf9) column, no specific displacements of [^{3}H]-etoposide were produced by the compounds indicating that the competitive displacements observed on the CMAC(Sf9$_{MRP1}$) column were due to interactions at the expressed MRP1 protein.

In a previous study utilizing a CMAC(α3β4) it was demonstrated that relative agonist activities (EC_{50} values) could be ascertained by a single competitive displacement experiment [40]. The experimental approach involved the determination of the change in the retention volume (Δml) of a characterized marker ligand produced by the addition of a test compound to the mobile phase, where Δml = retention volume of the marker alone – retention volume of the marker in the presence of the test compound. When known agonists with established EC_{50} values (standards) were included in the experimental set, the relative EC_{50} values of the test compounds were determined from the relationship between Δml(test) and Δml(standard).

The relationship between the Δml and EC_{50} values as based upon the approach developed by Cheng and Prusoff [41] in which the functional inhibition (IC_{50} values) of enzymes by competitive inhibitors was equated to the binding affinities (K_i values) of these inhibitors. The analysis can be used to determine relative IC_{50} values between different inhibitors if the inhibitors have identical mechanisms of action and the assays are performed under the same conditions. The data obtained in this study was analyzed in the same manner in order to calculate the relative IC_{50} values for (S)-verapamil and (R)-verapamil, although the change in retention time (ΔT) was used in place of Δml. The calculated IC_{50} values were 4.1 μM and 8.6 μM, respectively. The magnitudes of the IC_{50} values are consistent with the previously reported IC_{50} value for racemic verapamil of 5–7 μM [39] and 13.4 μM [38] and the observation that the inhibitory effect of (S)-verapamil is ~2-fold stronger than (R)-verapamil is also consistent with the enntioselecitivity reported by Gaj et al. [37]. It is of interest to note that the experiments conducted by Gaj et al. involved the inhibition of etoposide transport by 10 μM concentrations of the verapamil enantiomers.

The application of the CMAC($Sf9_{MRP1}$) column in the determination of binding affinities, K_i values, to the immobilized MRP1 protein was confirmed using competitive binding experiments in which increasing concentrations of displacer ligands were added to the mobile phase and the effects on the retention of the marker ligand used to calculate the affinity of the displacer to the immobilized protein [26]. Increasing concentrations of the MRP1 ligands etoposide, benzbromarone and MK571 produced significant reductions in the retention of [^{3}H]-etoposide, Supplemental data, Fig. S1. The K_i values were calculated and were consistent with previously reported values calculated using non-chromatographic methods, Table 1. The results indicate that the CMAC approach could be used to determine binding affinities to the MRP1 protein.

3.2. CMAC($Sf9_{MRP2}$)

The presence of the MRP2 protein in the $Sf9_{MRP2}$ membranes was confirmed by Western Blotting (data not shown). The membranes were immobilized on the IAM stationary phase to create the CMAC($Sf9_{MRP2}$) column, and when 1 nM [^{3}H]-etoposide was placed in the running buffer the expected frontal chromatographic trace was observed. Competitive displacement studies were conducted using etoposide and furosemide, a specific MRP2 competitive inhibitor [38], Supplemental data, Fig. S2, and the calculated K_i values were consistent with previously reported values calculated using non-chromatographic methods, Table 1. The results indicate that the CMAC approach could be used to determine binding affinities to the MRP2 protein.

3.3. CMAC($Sf9_{BCRP}$)

The presence of the BCRP protein in the $Sf9_{BCRP}$ membranes was confirmed by Western Blotting (data not shown). The membranes were immobilized on the IAM stationary phase to create the CMAC($Sf9_{BCRP}$) column, and when 1 nM [^{3}H]-etoposide was placed in the running buffer the expected frontal chromatographic trace was observed. Competitive displacement studies were conducted using etoposide and fumitremorgin C, a specific BCRP competitive inhibitor [42], Supplemental data, Fig. S3, and the calculated K_i values were consistent with previously reported values calculated using non-chromatographic methods, Table 2. The results indicate that the CMAC approach could be used to determine binding affinities to the BCRP protein.

Table 2
The binding affinities (K_i values) of selected MRP1, MRP2 and BCRP ligands calculated from competitive displacement binding studies performed using the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$) columns. See text for experimental details.

	K_i (μM)	IC_{50} (μM)
CMAC($Sf9_{MRP1}$)		
Etoposide	23.8 ± 6.6	48 [38]
Benzbromarone	21.0 ± 2,2	4 [30]
MK571	4.8 ± 1.9	1.2 [45]
CMAC($Sf9_{MRP2}$)		
Etoposide	17.9 ± 9.2	48 [38]
Furosemide	18.0 ± 5.1	24 [38]
CMAC($Sf9_{BCRP}$)		
Etoposide	2.9 ± 1.5	8 [10]
Fumitremorgin C	1.7 ± 1.2	3 [19]

4. Discussion

The data from this study indicates that membranes from stably transfected Sf9 cell lines expressing the MRP1, MRP2 or BCRP transporters have been successfully immobilized on the IAM stationary phase to create a series of cellular membrane affinity chromatography columns; CMAC(Sf9), CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$). The results demonstrate that the resultant columns can be used to study the interactions of small molecules with the expressed transporters and to determine K_i values, relative IC_{50} values and enantioselectivities. The successful development and characterization of the Sf9 columns represents the first CMAC columns produced using membranes obtained from an insect cell line. Since the Sf9 and related insect cell lines are popular and highly productive expression systems, these results represent a potential expansion of the CMAC approach.

The CMAC(Sf9) columns produced in this study were stable for up to one month and could be used for ~100 samples during this period. As has been previously discussed, there was no attempt to purify the immobilized membranes and the amount of immobilized protein on the column, and thereby the column to column reproducibility, was determined using the number of active binding sites, B_{max} [26]. The calculated B_{max} values had less than 10% variation between columns for the two CMAC($Sf9_{MRP1}$) columns, indicating that the columns can be reproduced.

Currently, there are several approaches that are used to screen substrates/inhibitors for interactions with Pgp, MRP1, MRP2 and BCRP. MRP1 transport, for example, has been studied using isolated membrane vesicles [43,44] inside-out vesicles [45], substrate toxicity [19] and intracellular accumulation of transported substrate [38]. Similar studies with membrane vesicles [46] and inside-out vesicles [24] were also carried out for the MRP2 and BCRP [14] transporters. Thus most of the standard methods employed to screen compounds involve culture of native or transfected cells and isolation of membranes.

The data from this study indicate that the CMAC columns produced and characterized in this study represent a potential alternative approach to the screening of compounds for their binding to and activity at MRP1, MRP2 and BCRP. However, while

the average time needed for the frontal experiment was 25 min, the average washout period was 2 h, which limited the potential use of these columns in large screening programs. We have previously demonstrated that CMAC-based screening is hampered by the long washout periods required with the IAM stationary phase and that this problem can be overcome by moving to an open tubular chromatographic format [47]. This is illustrated by the studies with the CMAC(Pgp) columns in which moving to an open tubular format reduced the total time required for the frontal experiment and washout to 30 min and resulted in a throughput that was equivalent to the rate obtained with the Caco-2 screen [28]. The demonstration that the Sf9 membranes used in this study can be immobilized on the IAM support to create functional CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$) columns suggests that these membranes can also be immobilized in the open tubular format. These studies are currently underway and the results will be reported elsewhere.

5. Conclusion

The data from this study indicates that membranes from insect cell lines like the Sf9, expressing a target protein can be immobilized on an IAM (immobilized artificial membrane) stationary phase. The resultant columns can be used to study the interactions of target proteins with specific ligands using displacement frontal chromatography. Binding affinities so calculated have been shown to correlate well with reported literature values calculated using cell based assay systems. Moreover the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$), CMAC($Sf9_{BCRP}$) and the CMAC(Sf9) columns reported in this study can be constructed in 48 h as the cellular membranes are commercially available with the target protein expressed. The columns are fairly easy to assemble, reproducible and efficient. The CMAC columns reported here are an initial step in the development of screens for lead candidates as these efflux proteins play a crucial role in oral drug bioavailability and drug–drug interactions. The functional over-expression of MRP1, MRP2 and BCRP in neoplastic tumors also makes these screens essential in the development of anticancer agents.

Acknowledgement

This work was supported by funds from the Intramural Research Program of the National Institute on Aging/NIH.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.02.055.

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