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The Synthesis and Characterization of a Nuclear Membrane Affinity chromatography Column for the study of human breast cancer resistant protein (BCRP) using nuclear membranes obtained from the LN-229 cells.

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Keywords

Frontal affinity chromatography; nuclear BCRP; glioblastoma

1. Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive forms of human astrocytoma as only ~10% of patients survive 5 years post diagnosis. A major factor in the poor prognosis is multi-drug resistance (MDR) to drugs used in cancer treatment, and a key component in MDR is drug export by ATP Binding Cassette (ABC) efflux transporters. While P-glycoprotein (Pgp) is the best characterized member of the ABC transporter family, the breast cancer resistance protein (BCRP) has recently gained importance due to its widespread tissue distribution and role in the clinical MDR phenotype [1-3].

BCRP expression has been reported in GBM cell lines and clinical specimens. The ABC transporters were initially identified as located within the cellular membrane. Recent studies have demonstrated that BCRP is also expressed in the nuclear membranes of human-derived glioblastoma and astrocytoma cell lines [4]. Western blot analysis showed that BCRP was expressed both in nuclear and cytoplasmic membranes of 6/7 experimental cell lines and which was confirmed by confocal microscopy studies of the LN229 cell line. The functional export activity of nuclear BCRP was demonstrated by chemical inhibition and siRNA knock down of the nuclear BCRP, which resulted in increased sensitivity to the anti-cancer agent mitoxantrone. The data from this study suggested that nuclear expression of BCRP is a potential new mechanism for MDR. Therefore, the characterization of nuclear BCRP is an important task in order to determine whether the nuclear BCRP has different binding characteristics and inhibitory properties than its cytoplasmic counterpart.

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This laboratory has previously developed an online chromatographic approach for the study of binding interactions between ligands and proteins, cellular membrane affinity chromatography (CMAC) [5, 6]. CMAC is based upon the immobilization of cellular membranes containing the target protein on an immobilized artificial membrane (IAM) liquid chromatography stationary phase. Previous CMAC studies in our laboratory have investigated the interactions of substrates and inhibitors with the ABC transporters Pgp, BCRP and , MRP1 [7]. For example, the CMAC-Pgp column was used in frontal affinity chromatographic studies to determine the binding affinities { K_d values} of Pgp substrates and inhibitors and was able to identify competitive, allosteric and enantioselective interactions between the ligand and the Pgp transporter [7, 8].

In the current study, we have immobilized the nuclear membrane fragments from the LN-229 astrocytoma cell line on the IAM stationary phase to produce a nuclear membrane affinity chromatography (NMAC) column. The presence of functional BCRP within the NMAC column was established using [^3H]-etoposide, a BCRP substrate, radio flow detection and frontal displacement chromatography techniques,. Frontal displacement experiments conducted with multiple concentrations of etoposide and the calculated K_d value was 4.54 μM , which is consistent with the previously reported value of 2.9 μM [7]. The BCRP was fully characterized on the NMAC-BCRP column and this is the first example of an immobilized nuclear membrane affinity column.

2. Materials and Methods

2.1. Materials

Etoposide, Biochanin A, Fumitremorgin C (*FTC*), Ammonium acetate, sodium chloride (NaCl), Ethylenediaminetetraacetic acid (EDTA), sodium ortho-vanadate, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2-mercaptoethanol, benzamidine, N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), Phenylmethanesulfonyl fluoride (PMSF), Adenosine 5'-triphosphate (ATP) were obtained from Sigma-Aldrich (St. Louis, MO), Tris(hydroxymethyl)aminomethane (TRIS) was obtained from Schwarz/Mann Biotech (Cleveland, OH). De-ionized water was obtained from a Milli-Q system (Millipore, Billerica, MA). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Cell lines—The LN-229 glioblastoma cell line was obtained from American Type Tissue Culture (Manassas, VA, USA). The cells were seeded in either T-150 culture flasks or glass bottom wells (Mat Tek Corp, Ashland, MA, USA) with Dulbecco's modified Eagle's medium (DMEM) containing 4 mM glutamine and 4.5 g l⁻¹ glucose (Mediatech, Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (Fetal Clone III, Hyclone, Logan, UT), penicillin (100 U/ml, Mediatech), streptomycin (100 $\mu\text{g}/\text{ml}$, Mediatech) and maintained at 37 °C in a humidified atmosphere containing 5% CO₂. In T-150 flasks, cells were sub-cultured or harvested for experiments at 90% confluence and the medium was replaced at 3-4 day intervals. In glass bottom wells, the cells were seeded at 2×10⁵ cells/well and used at ~ 50% confluence

2.2.2. Preparation of LN-229 NMAC column—10×10⁶ LN-229 frozen cell pellet was washed once with PBS and centrifuged for 5 min at 1000 rpm. The cell pellet was re-suspended in 2 mL of CER-1 reagent from NE-PER Kit (Nuclear Protein Extraction Kit) (Thermo, Middletown, VA, USA) supplemented by 1:100 dilution of protease inhibitor cocktail (Sigma, St. Louis, MO, USA), 1:100 dilution of Halt™ Phosphatase Inhibitor Cocktail (Thermo, Middletown, VA, USA) and 1:1000 dilution of 1 mM sodium ortho-

vanadate. The pellet was vortexed hard for 15 seconds and incubated on ice for 10 min. 110 μ L CER-II reagent from NE-PER Kit (Thermo, Middletown, VA, USA) was added, vortexed for 5 seconds and incubated on ice for 1 min. The cell-suspension was divided into 2 eppendorf tubes, vortexed for 5 seconds and centrifuged in the micro-centrifuge at max speed (16,000 g) for 5 min at 4°C. The cytoplasmic extract was removed and the resultant pellet (nuclear membranes) was resuspended in ice cold 500 μ L of NER, vortexed for 15 seconds every 10 minutes over 40 minutes. The solution was centrifuged in the micro-centrifuge at max speed (16,000 g) for 10 min at 4°C. To the supernatant 10 mL of Tris buffer [10 mM, pH 7.4] supplemented with 2 % (w/v) CHAPS, 500 mM NaCl, 5 mM 2-mercaptoethanol, 100 μ M benzamidine, 1:100 dilution of protease inhibitor cocktail, 50 μ g/ml TPCK, 100 μ M PMSF, 100 μ M ATP and 10 % glycerol. The resulting mixture was mixed overnight using an orbital shaker at 150 rpm for 18 h at 4 °C and centrifuged at 100,000 g for 30 min, and the resultant supernatant was mixed with 150 mg Immobilized Artificial Membrane (IAM) particles and rotated at room temperature using orbital shaker for 1 h at 150 rpm. The suspended particles were then dialyzed against HEPES buffer [20 mM, pH 8.0] containing 500 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) for 1 day and centrifuged for 3 min at 4 °C at 700g. The pellet obtained was then washed with ammonium acetate [10 mM, pH 7.4], and centrifuged 3 min at 4 °C at 700g. This step was repeated twice, The pellet was re-suspended in 10 mL ammonium acetate [10 mM, pH 7.4] and packed into an HR 5/2 column (Amersham Pharmacia Biotech, Uppsala, Sweden) to yield a 150 $\times$ 5-mm (i.d.) chromatographic bed.

2.3. Frontal chromatography studies

The NMAC-BCRP column was attached to the chromatographic system Series 1100 Liquid Chromatography/Mass Selective Detector (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum de-gasser (G 1322 A), a binary pump (1312 A), an autosampler (G1313 A) with a 20 μ L injection loop, a mass selective detector (G1946 B) supplied with atmospheric pressure ionization electrospray 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) running ChemStation software (Rev B.10.00, Hewlett-Packard).

In the chromatographic studies, the mobile phase consisted of ammonium acetate [10 mM, pH 7.4] delivered at 0.4 ml min⁻¹ at room temperature. Pumps A, C and D were used to apply a series of ligands in the mobile phase: Etoposide (1 μ M, 2 μ M, 5 μ M, 10 μ M, 20 μ M), FTC (0.125 μ M, 0.25 μ M, 0.5 μ M, 1 μ M, 2 μ M, 7 μ M) and Biochanin A (1 μ M, 2 μ M, 5 μ M, 10 μ M, 20 μ M). The column was checked weekly for stability, by running 1 μ M Etoposide in the mobile phase, to confirm that the retention time did not change greater than 5%.

Etoposide was monitored in the negative ion mode using single ion monitoring at m/z = 587.20 [MW - H]⁻ ion for etoposide, with the capillary voltage at 3000 V, the nebulizer pressure at 35 psi, and the drying gas flow at 11 L/min at a temperature of 350°C. FTC was monitored in the positive ion mode using single ion monitoring at m/z = 380.50 [MW + H]⁺ ion for FTC, with the capillary voltage at 3000 V, the nebulizer pressure at 35 psi, and the drying gas flow at 11 L/min at a temperature of 350°C. Biochanin A was monitored in the positive ion mode using single ion monitoring at m/z = 288 [MW + H]⁺ ion for Biochanin A, with the capillary voltage at 3000 V, the nebulizer pressure at 35 psi, and the drying gas flow at 11 L/min at a temperature of 350°C.

Radioflow studies—The NMAC columns were placed in a chromatographic system consisting of a Shimadzu LC-10 AD pump (Shimadzu, Columbia, MD) isocratic HPLC

pump, a 50-mL sample superloop (Amersham Pharmacia Biotech) and an IN/US system b-ram model 3 on-line scintillation detector (IN/US, Tampa, FL, USA) with a dwell time of 2 s and running Laura Lite 3 software. The mobile phase consisted of ammonium acetate [10 mM, pH 7.4] delivered at 0.4 ml min⁻¹ at room temperature. The marker ligand for all of the frontal chromatographic studies was [³H]-etoposide which was present in the mobile phase at a concentration of 2 nM. In the studies, the 50-ml sample superloop was used to apply 10-ml solutions containing the marker ligand and multiple concentrations of unlabeled etoposide.

2.4. Data Analysis

The dissociation constants, K_d , for the displacer ligands were determined using a previously reported approach [9]. The experimental paradigm is based upon the effect of escalating approach of a competitive binding ligand on the retention volume. For example, the displacer ligands (D) dissociation constant, K_D , as well as the number of the active binding sites of the immobilized BCRP, B_{max} , can be calculated using equation (1):

$$[D] (V - V_{min}) = B_{max} [D] (K_D + [D])^{-1} \quad (1)$$

where: V is the retention volume of ligand, V_{min} is the retention volume of ligand when the specific interaction is completely suppressed. The K_D for D is obtained from the plot of $[D] (V - V_{min})$ versus $[D]$. The data were analysed by nonlinear regression with a sigmoidal response curve using Prism 4 software (Graph pad Software, Inc., San Diego, CA, USA) running on a personal computer.

3. Results and Discussion

The presence of BCRP on the nuclear membrane of the LN-229 cell line was previously demonstrated by western blot analysis and confocal microscopy [4]. To date, the function and selectivity of nuclear BCRP have not been fully characterized. To this end, the LN-229 nuclear membranes were immobilized on the IAM stationary phase to create the nuclear membrane affinity chromatography column containing immobilized BCRP (NMAC-BCRP). The initial immobilization of the nuclear membranes was carried out with a solubilization buffer that contained sucrose and magnesium sulfate. In order to test for specific BCRP binding activity, [³H]-etoposide was used as the marker ligand. Etoposide has been shown to bind to Pgp, MRP1 and BCRP in micromolar range [7]. The resulting NMAC-BCRP column did not display etoposide displacement expected with the BCRP protein. Therefore, the immobilization method was modified in an attempt to optimize the on column activity of the immobilized BCRP protein. The addition of β-mercaptoethanol, ATP and glycerol, in addition to the removal of sucrose and magnesium sulfate, resulted in an NMAC-BCRP column that had specific displacement of etoposide.

As etoposide has been reported to have micromolar affinity for the BCRP protein, all frontal studies were subsequently carried out on an Agilent LC-MSD system. The elution profile of a subsaturating concentration of etoposide contained an initial flat portion representing specific binding to the column followed by a breakthrough curve and plateau representing saturation of the column, Fig. 1. The midpoint of the breakthrough curve occurred at 11.73 min on the NMAC-IAM column, representing breakthrough volume of 4.7 ml. Increasing concentrations of unlabeled etoposide was added to the mobile phase, resulting in a decrease in the retention volume, representing specific binding to the NMAC column, Fig. 1. The calculated K_d value for etoposide on the NMAC-BCRP column was 4.5 μM, which is consistent with previously reported K_d of 2.9 μM, determined by chromatographic methods [7] and 5 μM determined by etoposide accumulation assays [3].

In order to confirm the activity of the immobilized BCRP on the NMAC-BCRP column, the K_d values of the BCRP inhibitors fumitremorgin C (FTC) and biochanin A were also determined using frontal displacement chromatography, where increasing concentrations of the drug was added to the mobile phase and the change in retention volume was used to calculate the affinity of the drug for BCRP. The K_d values calculated for etoposide, FTC and biochanin A were 4.5 μ M, 1.6 μ M and 1 μ M, respectively (Table 1). These results correlated with previously reported values of 3 μ M, 1.7 μ M and 1.0 μ M, respectively, with an r^2 of 0.9526 (Table 1).

The results indicate that nuclear membranes from the LN229 glioblastoma cell line expressing nuclear BCRP transporter has been successfully immobilized onto the IAM liquid chromatography stationary phase to create a nuclear membrane affinity chromatography column; NMAC-BCRP. The results demonstrate that the resulting column can be used to study and characterize the interaction of substrates and inhibitors to the immobilized BCRP. The successful creation and characterization of the NMAC-BCRP represents the first time nuclear membranes have been immobilized within a chromatographic system and used in affinity chromatography studies. The NMAC-BCRP column lays the groundwork for future work on the characterization of this transporter as well as other nuclear membrane proteins that are therapeutically targeted; for example, the nuclear envelope protein, TMEM209, which has been recently demonstrated to be a critical component in lung cancer proliferation [10]

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Highlights

1. Nuclear membranes from the LN229 glioblastoma cell line expressing nuclear BCRP transporter has been successfully immobilized
2. The NMAC-BCRP column can be used to study and characterize the interaction of substrates and inhibitors to BCRP.
3. The successful creation and characterization of the NMAC-BCRP represents the first time nuclear membranes have been immobilized within a chromatographic system and used in affinity chromatography studies.
4. The NMAC-BCRP column lays the groundwork for future work on the characterization of this transporter as well as other nuclear membrane proteins that are therapeutically targeted.

Figure 1.
Frontal elution profiles 1 μ M (A), 2 μ M (B), 5 μ M (C), 10 μ M (D) and 20 μ M (E)
Etoposide on the NMAC_{BCRP}-IAM column (0.531 $\times$ 2cm) on the agilent LC-MSD. Mobile
phase: Ammonium acetate [10mM, pH 7.4] at 0.4ml/min.

Table 1

The binding affinities (K_d) of BCRP ligands calculated using frontal displacement chromatographic binding studies performed on the NMAC-BCRP column.

	K_d (μM)	K_d (μM, lit)
Etoposide	4.548; $r^2=0.918$	2.9 [7]; 5 [3]
Fumitremorgin C (FTC)	1.626; $r^2=0.9377$	1.7 [7], 3 [2]
Biochanin A	1.053; $r^2=0.8230$	0.5-1.0 [1]