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Characterization of a multiple endogenously expressed adenosine triphosphate-binding cassette transporters using nuclear and cellular membrane affinity chromatography columns☆

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ABSTRACT

Glioblastoma multiforme is an aggressive form of human astrocytoma, with poor prognosis due to multi-drug resistance to a number of anticancer drugs. The observed multi-drug resistance is primarily due to the efflux activity of ATP-Binding Cassette (ABC) efflux transporters such as Pgp, MRP1 and BCRP. The expression of these transporters has been demonstrated in nuclear and cellular membranes of the LN-229 human glioblastoma cell line. Nuclear membrane and cellular membrane fragments from LN-229 cells were immobilized on the IAM stationary phase to create nuclear and cellular membrane affinity chromatography columns, (NMAC(LN-229)) and (CMAC(LN-229)), respectively. Pgp, MRP1 and BCRP transporters co-immobilized on both columns were characterized and compared by establishing the binding affinities for estrone-3-sulfate (3.8 vs. 3.7 μM), verapamil (0.6 vs. 0.7 μM) and prazosin (0.099 vs. 0.033 μM) on each column and no significant differences were observed. Since the marker ligands had overlapping selectivities, the selective characterization of each transporter was carried out by saturation of the binding sites of the non-targeted transporters. The addition of verapamil (Pgp and MRP1 substrate) to the mobile phase allowed the comparative screening of eight compounds at the nuclear and cellular BCRP using etoposide as the marker ligand. AZT increased the retention of etoposide (+15%), a positive allosteric interaction, on the CMAC(LN-229) column and decreased it (−5%) on the NMAC(LN-229), while the opposite effect was produced by rhodamine. The results indicate that there are differences between the cellular and nuclear membrane expressed BCRP and that NMAC and CMAC columns can be used to probe these differences.

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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. Multi-drug resistance to drugs used in cancer treatment carried out by membranes of the ATP-Binding Cassette (ABC) efflux transporters has been associated with poor prognosis. The ABC superfamily consists of 49 members [1], of which P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multi-drug resistance-associated protein 1 and 2 (MRP1 and MRP 2) are the most well-known members. Pgp, MRP1 and BCRP have both individual and overlapping selectivity towards the anticancer agents that are substrates for these efflux transporters. For example, Pgp substrates include vinca alkaloids, anthracyclines, and epipodophyllotoins [2,3], while MRP1 substrates include etoposide, anthracyclines, vincristine, methothrexate, paclitaxel and irinotecan [3], and BCRP plays a role in doxorubicin and methotrexate resistance [4,5].

The role of the ABC transporters in the MDR phenotype has resulted in these proteins being targeted in drug development and drug discovery programs. While ABC transporters are usually considered as cellular membrane proteins, they are also expressed in nuclear and mitochondrial membranes. For example, the expression of BCRP in nuclear and cellular membranes was recently demonstrated in six human-derived glioblastoma and astrocytoma cell lines [5]. The presence of this transporter in two distinct

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membrane environments raises the potential of differences in selectivity between cellular and nuclear BCRP transporters. While classical binding assays, functional assays and surface plasmon resonance are the most common approaches for binding affinity determination [6,7], differences between the cellular and nuclear BCRP would be difficult to determine using these standard membrane binding techniques. These approaches allow quantitative determination of the binding affinity of a ligand for its receptor, however, are limited in their ability to study allosteric sites and determine conformational changes resulting from changes in the lipid environment. One approach that provides for the direct measurement of multiple binding sites including orthosteric and allosteric sites, multiple conformations as well as subtype ligand interactions is cellular membrane affinity chromatography, where the target transmembrane protein is immobilized onto silica based stationary phase [8].

The development and use of cellular membrane affinity chromatography (CMAC) columns have been extensively demonstrated [9] and we have recently reported the development of a nuclear membrane affinity chromatographic (NMAC) column obtained using nuclear membranes from the LN-229 cell line [10]. Initial studies using FTC, a selective BCRP inhibitor, as a marker demonstrated that the BCRP transporter was immobilized within the NMAC(LN-229) column and that the protein retained the ability to bind substrates and inhibitors.

Since nuclear and cellular membranes may contain multiple ABC transporters, this study was designed to prepare NMAC(LN-229) and CMAC(LN-229) columns, to examine the expression and function of Pgp, MRP1 and BCRP transporters in both columns, and to determine if any differences exist between ABC transporters contained within nuclear membranes relative to those contained within cellular membranes.

The data from this study demonstrates that Pgp, MRP1 and BCRP were functionally immobilized within both the CMAC(LN-229) and NMAC(LN-229) columns indicating that these were multi-transporter columns. The immobilization of the respective membranes was also carried out in an open tubular format generating CMAC(LN-229)-OT and NMAC(LN-229)-OT columns. The co-immobilization of the three ABC transporters within the OT columns was also demonstrated. In addition, the OT columns were used for a comparative screening study of eight compounds at the BCRP transporter using the addition of selective Pgp and MRP1 substrates to the mobile phase to selectively study the BCRP transporter. The results indicate that there are differences between the affinity of the BCRP transporter contained within nuclear membranes and the BCRP transporter contained within the cellular membranes.

2. Experimental

2.1. Materials

Ammonium acetate, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), sodium ortho-vanadate, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), glycerol, 2-mercaptoethanol, benzamidine, protease inhibitor cocktail, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), adenosine 5′-triphosphate (ATP), amino propyl trimethoxy silane (APTS), gluteraldehyde aqueous solution, avidin, biotin-X (6-[(biotinoyl)amino]hexanoic acid), etoposide, biochanin A, fumitremorgin C (FTC), verapamil, estrone-3-sulfate, prazosin, zidovudine (AZT), rhodamine 123, quercitin, tamoxifen and sulfasalazine were obtained from Sigma–Aldrich (St. Louis, MO, USA), tris(hydroxymethyl)aminomethane (TRIS) was obtained from Schwarz/Mann Biotech (Cleveland, OH, USA). Dialysis tubing was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Open tubular capillaries (100 μm i.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). De-ionized water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Cell line

The LN-229 astrocytoma cell line was obtained from American Type Tissue Culture (Manassas, VA, USA) and maintained as previously described [10].

2.2.2. Western blot analysis

Cells were lysed in radioimmunoprecipitation (RIPA) buffer containing EGTA and EDTA (Boston BioProducts, Ashland, MA, USA). The lysis buffer contained protease inhibitor cocktail. Protein concentrations were determined using the BCA assay (Thermo Fisher Scientific). Proteins (20 μg/well) were separated electrophoretically on 4–12% Tris-Glycine precast gels (Invitrogen, Grand Island, NY, USA) under reducing conditions and then transferred onto PVDF membrane (Invitrogen). 5% non-fat milk in TBST was used for blocking the non-specific proteins and to incubate with the primary antibody of interest: ABCG2 (M-70), PGP (A-14), and MRP1 (C-20) (Santa Cruz, CA), followed by incubation with a secondary antibody conjugated with the enzyme horseradish peroxidase. The detection of immunoreactive bands was performed by using the ECL Plus Western Blotting Detection System (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The primary antibody for β-actin was from Abcam (Cambridge, MA, USA).

2.2.3. Preparation of nuclear membranes (NM)

The LN-229 nuclear membranes were prepared as previously described [10]. Briefly, 10×10^6 LN-229 cells were re-suspended in CER-1 reagent from NE-PER Kit (Thermo Fisher Scientific) supplemented by 1:100 dilution of protease inhibitor cocktail, 1:100 dilution of Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and 1:1000 dilution of 1 mM sodium ortho-vanadate. After incubation on ice, 110 μL CER-II reagent from NE-PER Kit was added. The cell suspension was centrifuged for 5 min at 4 °C at 16,000 × *g*. The cytoplasmic extract was removed and the resultant pellet was re-suspended in NER. After incubation on ice, the solution was centrifuged at 16,000 × *g* for 10 min at 4 °C and the resulting supernatant contained the nuclear membranes.

2.2.4. Preparation of cellular membrane (CM)

The cellular membranes were prepared following a previously described protocol with slight modifications [11]. Briefly, 10×10^6 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 10 mL of Tris buffer [10 mM, pH 7.4], supplemented with 500 mM NaCl, 5 mM 2-mercaptoethanol, 100 μM benzamidine, 1:100 dilution of protease inhibitor cocktail, 50 μg/mL TPCK, 100 μM PMSF and 100 μM ATP. The cell suspension was homogenized three times for 30 s at 3000 rpm using PRO200 homogenizer (PRO Scientific, Oxford, CT, USA). The homogenized cell suspension was centrifuged for 10 min at 700 × *g* at 4 °C. The supernatant containing the cellular membranes was collected and centrifuged for 30 min at 100,000 × *g* at 4 °C. The supernatant was discarded and the pellet contained the cellular membranes.

2.2.5. Preparation of LN-229 nuclear membrane affinity chromatography (NMAC) and cellular membrane affinity chromatography (CMAC) columns

Nuclear and cellular membranes were suspended in 10 mL of solubilization buffer (Tris buffer [10 mM, pH 7.4], supplemented

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