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The development of mitochondrial membrane affinity chromatography columns for the study of mitochondrial transmembrane proteins

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Abstract

Mitochondrial membrane fragments from U-87 MG (U87MG) and HEK-293 cells were successfully immobilized on to Immobilized Artificial Membrane (IAM) chromatographic support and surface of activated open tubular (OT) silica capillary resulting in mitochondrial membrane affinity chromatography (MMAC) columns. Translocator protein (TSPO), located in mitochondrial outer membrane as well as sulfonylurea and mitochondrial permeability transition pore (mPTP) receptors, localized to the inner membrane, were characterized. Frontal displacement experiments with multiple concentrations of dipyridamole (DIPY) and PK-11195 were run on MMAC-(U87MG) column and the binding affinities (K_d) determined were 1.08 ± 1.49 and 0.0086 ± 0.0006 μ M respectively, which was consistent with previously reported values. Further, binding affinities (K_i) for DIPY binding site were determined for TSPO ligands, PK-11195, mesoporphyrin IX, protoporphyrin IX and rotenone. Additionally, the relative ranking of these TSPO ligands based on single displacement studies using DIPY as marker on MMAC-(U87MG) was consistent with the obtained K_i values. The immobilization of mitochondrial membrane fragments was also confirmed by confocal microscopy.

Keywords

Mitochondrial membrane affinity chromatography; translocator protein

1. Introduction

Mitochondria are unique cell organelles with multiple functions including energy transduction, amino acid and lipid metabolism, cell division and growth, and programmed

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apoptosis [1]. Mitochondria are organelles composed of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), Fig. 1 [2,3]. The OMM has a relatively simple structure based upon a smooth phospholipid bilayer containing protein structures, porins, which make the membrane permeable to molecules of up to 10 kDa. The IMM is a more complex highly lipophilic structure due to the high content of cardiolipin. Both membranes contain surface and transmembrane receptors and transporters.

Mitochondrial dysfunction has been associated with a broad heterogeneous group of disorders including the mitochondrial diseases and neurodegenerative disorders [1,4,5]. The etiology of mitochondrial diseases as well as their treatment have been related to specific receptors and transporters expressed in the OMM and IMM and these proteins have become important therapeutic targets in drug development [1]. A key OMM expressed receptor is the translocator protein (TSPO), an 18 kDa protein, previously called peripheral benzodiazepine receptor (PBR), which differs in ligand selectivity from the central type benzodiazepine receptor [6,7,8]. The TSPO is believed to play a key role in steroid synthesis [9,10], heme biosynthesis [11], immunomodulation [12] and has also been associated with the growth control of various cancers [13]. It has also been reported to be involved in regulating mitochondrial membrane potential [14], modulating voltage-dependent calcium channels, attenuation of oxidative stress and ischemia-reperfusion, and apoptosis [9,10,12,14,15]. As a result, it has been considered as an important clinical and therapeutic target [1,6,7].

The TSPO has been shown to be part of the mitochondrial permeability transition pore (mPTP) as it is functionally associated with the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocase (ANT) [7,8,16-20]. The mPTP is a multiprotein complex, also known as mega-channel, which is formed at the contact sites between IMM and OMM under certain pathological conditions or stress and which is responsible for the non-selective permeability state of the IMM [1,21]. The molecular composition of mPTP has not been fully established but it was recently confirmed that the c subunit of the mitochondrial F_1/F_0 ATP synthase is a fundamental component of mPTP [21].

Several methods are currently used for characterizing TSPO including competitive ligand binding assays [22], functional studies that measure caspase activity and reactive oxygen species (ROS) production [15,17,23]. While these methods are currently in use, they are not ideal for the development of a screening approach. As a result, we have established a novel *in vitro* approach to characterize TSPO receptor and to develop a ligand screening method would help identification of potential drug leads for TSPO related disorders.

In this work, we present an alternative approach to the characterization of receptors and transporters expressed on the OMM and IMM. The approach is based upon the immobilization of cellular membrane fragments onto a silica based stationary phase, cellular membrane affinity chromatography (CMAC) [24]. These stationary phases have been used to study the cellular and pharmacological functions of a wide range of transmembrane proteins: ligand gated ion channels, G-protein receptors, nuclear receptors and drug transporters. A recent review gives a comprehensive overview on the different applications of CMAC columns [25].

In addition to CMAC column, more recently, the development and characterization of immobilized nuclear membrane fragments from the LN-229 cell line has been carried out to study multiple ATP binding cassette (ABC) transporters, breast cancer resistance protein (BCRP), P-glycoprotein (Pgp) and multidrug resistance protein 1 (MRP1) [26]. Similar to the mitochondria, nuclear membranes are also comprised of an inner and outer membranes. The resulting nuclear membrane affinity chromatography column (NMAC), was characterized and the presence of the three transporters was demonstrated [26,27].

In current study the mitochondrial membrane fragments were immobilized onto a stationary phase resulting in mitochondrial membrane affinity chromatography (MMAC) columns. The approach provides for the direct measurement of multiple binding sites of the target protein including orthosteric and allosteric sites. In order to determine whether both the OMM and IMM were immobilized, proteins that are localized to each membrane were targeted. To this end, the characterization of TSPO, localized to the OMM, and mPTP and sulfonylurea receptor (SUR), which are expressed in the IMM (Fig 1), was carried out. The results indicate the functional presence of these receptors in MMAC and MMAC-open tubular (MMAC-OT) columns.

2. Materials and methods

Materials

Ammonium acetate, sodium chloride (NaCl), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2-mercaptoethanol, benzamidine, protease inhibitor cocktail, N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), adenosine 5'-triphosphate (ATP), amino propyl trimethoxy silane (APTS), glutaraldehyde aqueous solution, avidin, N-(+)-Biotinyl-6- aminohexanoic acid (Biotin-X), dipyrindamole (DIPY), mesoporphyrin IX (MIX), protoporphyrin IX (PIX), PK-11195 (PK), rotenone (Rot), flunitrazepam (Flu), glipizide, glibenclamide and diclofenac were obtained from Sigma-Aldrich (St. Louis, MO, USA or Munich, Germany), tris(hydroxymethyl)aminomethane (Tris) and glycerol were obtained from Applichem (Darmstadt, Germany), ethylenediaminetetraacetic acid (EDTA) was obtained from Scharlau (Barcelona, Spain). Dialysis tubing was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Open tubular capillaries (100 μ m i.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). De-ionized water was obtained from a Milli-Q system (Millipore, Molsheim, France). All other chemicals used were of analytical grade.

Cell line maintenance

The U-87 MG (U87MG) human glioblastoma and HEK-293 (HEK) human embryonic kidney cell lines were received as a gift from Karolinska Institutet in Sweden. The cells were seeded in either CELLSTAR T-75 culture flasks or on tissue culture dishes (greiner bio one, Frickenhausen, Germany) with Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine, Na-Pyruvate and 4.5 g/L glucose (Naxo, Tartu, Estonia) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), penicillin (100 U/ml, PAA The Cell Culture Company, Pasching, Austria), streptomycin (100 μ g/ml, PAA The Cell Culture Company) and maintained at 37 °C in a humidified atmosphere containing

5% CO₂. The cells were sub-cultured or collected for experiments at 90% confluence and the medium was replaced when needed.

Preparation of mitochondria

The mitochondria was isolated using Mitochondria Isolation Kit for Cultured Cells (abcam, Cambridge, UK). Briefly, 40×10⁶ frozen cells (20×10⁶ in case of HEK cell line) were thawed and re-suspended to 5 mg/ml (whole cell protein) in Reagent A. After incubation on ice for 10 min, the cells were homogenized using a dounce homogenizer (30 strokes). The resulting suspension was centrifuged for 10 min at 1000 ×g at 4 °C. The supernatant was saved (#1) and the pellet was re-suspended in Reagent B. Homogenization and centrifugation steps were repeated and the supernatant was saved (#2). Supernatants #1 and #2 were mixed thoroughly and centrifuged for 15 min at 12 000 ×g at 4 °C. The resulting pellet contained mitochondria.

Mitochondria were solubilized using the previously described protocol for the synthesis of CMAC and NMAC columns [24,26]. The solubilization buffer was Tris buffer [10 mM, pH 7.4], supplemented with 2% (w/v) CHAPS, 10% glycerol, 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 with 10 ml utilized in the synthesis of the MMAC columns and 3 ml of solubilization in the preparation of the MMAC-OT columns. The resulting mixtures were mixed at 4 °C for 18 h using a tube roller.

Western Blot Analysis

Western blot analysis was performed according to previously reported method [26], which involved equal protein loading of 20 μg/well, separation using SDS-PAGE, blocking in 5% non-fat milk and incubated with the primary antibody, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The detection of immune-reactive bands was performed by using the ECL Plus Western Blotting Detection System (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

As recommended by the Mitochondrial Quality Analysis section of the Abcam's mitochondrial isolation kit protocol, MitoProfile® Total OXPHOS Human WB Antibody Cocktail (MS601) (cat. # ab110411, Abcam, Cambridge, MA) was used for determination of Complex I, Complex II, Complex III core 2, Complex IV and ATP synthase subunits; whereas MitoProfile® Membrane Integrity WB Antibody Cocktail (MS620) (cat. # ab110414, Abcam) was used for determination Complex III core 1, Complex V, Porin, Cyclophilin D and cytochrome c.

Preparation of MMAC and MMAC-OT columns

The columns were prepared following previously reported protocols [24,27]. In brief:

MMAC columns—The solubilized mitochondria was mixed with 150 mg Immobilized Artificial Membrane (IAM) particles (Regis Technologies, Morton Grove, IL, USA) and rotated at 4 °C for 1 h using a tube roller. The suspended particles were then dialyzed against Tris buffer [10 mM, pH 7.4] containing 500 mM NaCl, 1 mM EDTA and 100 nM of

benzamidine, for 1 day, and repeated. Next, the suspension was centrifuged for 3 min at 4 °C at 700 × g. The obtained pellet was then washed two times with ammonium acetate [10 mM, pH 7.4] by centrifuging again 3 min at 4 °C at 700 xg. Final pellet was re-suspended in 1–2 ml ammonium acetate [10 mM, pH 7.4] and packed into a Tricorn 5/20 column (GE Healthcare Life Sciences, Uppsala, Sweden) to yield a 15 × 5 mm (i.d.) chromatographic bed.

MMAC-OT columns—An open tubular capillary (25 cm × 100 µm i.d.) was primed and then a 10% aqueous solution of APTS was passed through the capillary followed by 30 min incubation at 95 °C twice. After 18 h, a 1% aqueous solution of glutaraldehyde was passed through the capillary for 1 h followed by water and 25 mM avidin. Both tips of the capillary were submerged in the avidin solution for 4 days at 4 °C. Then 14 mM biotin-X was run through the capillary for 1 h. The solubilized mitochondria were recycled through the column for 60 min. The open tubular capillary was then dialyzed against Tris buffer [10 mM, pH 7.4] containing 500 mM NaCl, 1 mM EDTA and 100 nM benzamidine for 1 day, and repeated.

Chromatographic studies

The mitochondrial columns were attached to the Series 6200 Accurate-Mass TOF LC/MS chromatographic system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Series 1200 Infinity binary pump (G1312B), a mass selective detector (G6230A) supplied with atmospheric pressure ionization electrospray. The chromatographic system was interfaced to a 2.66 GHz Intel® Xeon® CPU computer (Hewlett-Packard Company, Palo Alto, CA, USA) running MassHunter Workstation Software – LC/MS Data Acquisition (Rev B.05.00, Agilent).

In the chromatographic studies, mobile phase consisted of ammonium acetate [10 mM, pH 5.9] unless stated otherwise, delivered at 0.4 ml/min for the MMAC columns and 0.05 ml/min for the MMAC-OT columns. Pump B was used to apply series of ligands. In the first set of experiments, K_d 's were determined for: PK (0.005, 0.01, 0.02, 0.04, 0.08 and 0.1 µM), DIPY (0.0625, 0.25, 0.5, 1, 2, 3, 5 and 10 µM), Flu (0.0125, 0.025, 0.05, 0.1, 0.2, 0.5, 1 and 2 µM), glibenclamide (0.125, 0.25, 0.5, 1, 2, 5, 10 and 20 µM), glipizide (0.125, 0.25, 0.5, 1, 2, 5 and 10 µM) and diclofenac (0.125, 0.25, 0.5, 1, 2 and 20 µM). In the second set of experiment K_i for DIPY binding site was determined for series of ligands: PK (0.1, 0.2, 0.5, 1, 2.5, 5, 7.5 and 10 µM), MIX (0.125, 0.5, 1, 2 and 10 µM), PIX (0.125, 0.5, 2, 4, 5 and 7.5 µM) and Rot (0.25, 1, 2.5, 5, 7.5, 20 and 25 µM); wherein 0.5 µM DIPY was used as a marker. DIPY, PK, Flu, glibenclamide, glipizide and diclofenac, were monitored in the positive ion mode at $m/z = 505.32, 353.86, 314.3, 494.14, 445.18$ and $295.02 [MW + H]^+$ ion, respectively, with the capillary voltage at 3500 V and the nebulizer pressure at 60 psig. In case of DIPY, the fragmentor was at 100 V and the drying gas flow was 9 L/min at a temperature of 320 °C. In case of glibenclamide, glipizide, PK and Flu, the fragmentor was at 110 V and the drying gas flow 11 l/min at a temperature of 350 °C. In case of diclofenac, the fragmentor was at 90 V and the drying gas flow 9 l/min at a temperature of 350 °C. The ion of ligand under study was extracted from TIC chromatogram in MassHunter Workstation Software – Qualitative Analysis (Rev B.05.00, Agilent).

Data analysis

The dissociation constants, K_d 's, for the displacer ligands were determined using a previously reported approach [28]. 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 TSPO, B_{max} , can be calculated using equation (1):

$$[D] (V - V_{min}) = P [D] = (K_d + [D])^{-1} \quad (1)$$

where: V is the retention volume of ligand, V_{min} is the retention volume of ligand when the specific interaction is completely suppressed and P is the product of the B_{max} (number of active binding sites) and (K_d/K_{dM}) where K_{dM} is the dissociation constant for the marker. The K_d for D is obtained from the plot of $[D] (V - V_{min})$ versus $[D]$. The data was analyzed by nonlinear regression with a sigmoidal response curve using Prism 4 software (GraphPad Software, Inc., San Diego, CA, USA) running on a personal computer.

Single displacement study for ranking TSPO ligands using MMAC

MMAC-(U87MG) was utilized for this study using ammonium acetate [10 mM, pH 5.9] as mobile phase. The change in the retention volume of 0.125 μ M DIPY in the presence of 2.875 μ M of the following compounds was determined: Flu, Rot, PIX, MIX, PK and DIPY. The data was normalized to the change in retention volume observed in 3 μ M DIPY.

Confocal microscopy

The presence of TSPO in the isolated mitochondria and its immobilization onto IAM particles was confirmed utilizing confocal microscopy. The isolated mitochondria from U87MG cells were suspended in 1ml of ammonium acetate [10 mM, pH 5.9] buffer and vortex mixed for 1 min. This suspension was aliquoted equally in ten 1.5 ml centrifuge tubes. The tubes were centrifuged for 5 min at 10,000 $\times g$ at 4 $^{\circ}C$ and the supernatant was discarded. These mitochondrial fractions were mixed with series of 1 ml solutions containing 1 μ M PIX; 1 μ M PIX + 1 μ M DIPY; 1 μ M PIX + 0.5 μ M DIPY; 1 μ M PIX + 0.25 μ M DIPY; 1 μ M PIX + 0.125 μ M DIPY; 1 μ M PIX + 0.0625 μ M DIPY; 1 μ M PIX + 0.03125 μ M DIPY. This mixture was incubated by stirring at 1000 rpm for 15 min at room temperature. Later, these mixtures were centrifuged for 1 min at 10,000 $\times g$ at 4 $^{\circ}C$ and the supernatant was discarded. These mitochondrial fractions were suspended in 0.1 ml of ammonium acetate [10 mM, pH 5.9] buffer and then transferred in to 35-mm glass bottom culture dishes (MatTek Corp., Ashland, MA). In the next series of experiment 150 mg of IAM particles with immobilized mitochondrial fragments was utilized instead of bare isolated mitochondria. Similar procedure as described above was conducted to study the effect of increasing concentration of DIPY (0.03125, 0.0625, 0.125, 0.25, 0.5 and 1 μ M) on PIX (1 μ M) binding to IAM particles with immobilized mitochondrial fragments. Further, these plated samples were imaged with a Zeiss LSM 710 confocal microscope (Thornwood, NY) equipped with a temperature-controlled and humidified CO₂ chamber and a definite focus system. A 405 nm laser was used for the excitation of the PIX. The Zeiss Zen software was used to collect images with a 40/1.3 NA objective for each samples, with all confocal

settings remaining the same throughout the experiments. Experiments were repeated two times.

3. Results and Discussion

In this study, mitochondrial membranes from U87MG and HEK cells were immobilized onto the IAM stationary phase as well as to the surface of an OT silica capillary to produce MMAC and MMAC-OT, respectively. In order to demonstrate the purity of the isolated mitochondria, western blot analysis of the isolated mitochondria was carried out (Figure S1). The presence of mitochondria in the isolated fraction was demonstrated by using the MitoProfile® Total OXPHOS Human WB Antibody Cocktail, where the presence of Complex I, Complex II, Complex III core 2, Complex IV and ATP synthase was observed (Fig S1a). Further the mitochondrial integrity was demonstrated by western blot analysis using MitoProfile® Membrane Integrity WB Antibody Cocktail, where the presence of Complex III core I, Complex V, Porin, Cyclophilin D and cytochrome c was observed (Fig S1b). It was demonstrated that membrane fragments from both the inner and outer membrane were immobilized, with the characterization of proteins localized to each membrane. For the OMM, the TSPO receptor was fully characterized, while for the IMM mPTP and SUR were characterized.

3.1. Characterization of OMM receptor TSPO

3.1.1. Determination of optimal mobile phase composition—Frontal affinity chromatography - was carried out on MMAC-(U87MG) using multiple concentrations of DIPY, a TSPO specific high-affinity ligand [29-31]. The calculated binding affinity (K_d) was $1.12 \pm 0.15 \mu\text{M}$ (Table 1), which is consistent with the previously reported K_i value of $0.68 \mu\text{M}$ (Table 2) [29]. However, the breakthrough time for DIPY under this condition was ~200 min with a total run time of ~400 min for each sample, indicating interactions not only with the target receptor but also nonspecific interactions with the IAM backbone. Previous studies have shown that changing the mobile phase composition, such as pH and/or addition of organic modifier, could reduce the interaction of the ligand with the backbone by altering ligand partitioning between the IAM bonded phase and the mobile phase [24]. The pK_a of DIPY is 6.1 [32] and the isoelectric point of TSPO is 9.1 [7], thus the net charge of TSPO will not change if the pH is reduced below 7.4. However, at pH close to 6.1, DIPY will shift from an uncharged state (Scheme 1) to a charged state, and as a result will reduce the hydrophobic interactions with the stationary phase. Thus, changing the pH of mobile phase from 7.4 to 5.9 decreased the breakthrough time by ~ 50% (~ 100 min) (Fig. 2A) with no effect on the binding affinity ($1.08 \pm 0.49 \mu\text{M}$) (Fig. 2B). Moreover, the addition of 10% organic modifier, methanol, to the mobile phase further decreased the breakthrough time by an additional ~ 50% (Fig. 2B), without changing the binding affinity ($0.92 \pm 0.47 \mu\text{M}$) (Table 1). However the MMAC had a shorter lifetime, when 10% methanol was used as a part of mobile phase composition. This could possibly be due to the change in the lipid aggregate structure of the transmembrane receptor/protein caused the organic modifier, leading to reduction in the lifetime of the column [24]. Therefore all the further studies were conducted utilizing ammonium acetate [10 mM, pH 5.9] as mobile phase.

3.1.2. Characterization of TSPO receptor—Binding affinities (K_d/K_i) calculated for TSPO ligands PK, Flu, MIX, PIX and Rot on MMAC-(U87MG) based on the frontal displacement experiments were $0.0086 \pm 0.0006 \mu\text{M}$, $0.29 \pm 0.06 \mu\text{M}$, $1.47 \pm 0.12 \mu\text{M}$, $3.33 \pm 1.35 \mu\text{M}$ and $3.40 \pm 1.27 \mu\text{M}$, respectively (Table 2). Interestingly, the K_d of PK was significantly stronger than the K_i ($1.14 \pm 0.38 \mu\text{M}$), determined using $0.5 \mu\text{M}$ DIPY as the marker ligand, indicating that PK binds to two distinct sites, a high affinity site and a low affinity site that competes with DIPY. Previous studies have shown that TSPO has multiple binding sites [23], and it has been previously reported that PK had two independent sites on TSPO, one high and another low affinity binding site [33], which is consistent with the frontal results obtained (Table 2).

Another interesting finding was the concentration dependent effect of Flu on the retention of DIPY on the MMAC-(U87MG). Flu did not significantly displace DIPY at concentrations lower than $0.5 \mu\text{M}$, whereas at higher concentrations (greater than $0.5 \mu\text{M}$) it increased breakthrough time of DIPY by ~10-15%. For example, $2.875 \mu\text{M}$ of Flu increased retention of $0.125 \mu\text{M}$ DIPY by ~15% (Fig. 3), indicating a concentration independent positive allosteric effect [34].

In order to determine if the MMAC approach could be extended to other cell lines, the MMAC-(HEK) was synthesized and characterized. The calculated K_d for DIPY on MMAC-(HEK) column was $0.68 \pm 0.27 \mu\text{M}$ which is consistent with the K_d for DIPY on MMAC-(U87MG) column (Table 1).

3.1.3. Single displacement study for ranking TSPO ligands—In order to increase the throughput of the analysis, it was determined whether a single displacement study could be used to rank compounds for their affinity for TSPO. In this study, a single concentration of six compounds ($2.875 \mu\text{M}$) (DIYP, PK, MIX, PIX, Rot and Flu) was run individually with $0.125 \mu\text{M}$ DIPY on the MMAC-(U87MG) (Fig. 3). Previously, it was demonstrated that a single displacement experiment could be used to correctly rank known ligands for nicotinic acetylcholine receptor (nAChR) [35]. In this study, the displacement observed with $2.875 \mu\text{M}$ of the tested compounds was normalized to the displacement observed with $2.875 \mu\text{M}$ DIPY, c.f. Fig. 3. The ranking order of displacement observed in case of DIPY, PK, MIX, PIX and Rot was in close agreement with the binding affinities K_i determined (Table 2), i.e. DIPY > PK > MIX > PIX > Rot.

Flu, on the other hand, increased the retention of DIPY (Fig. 3), which is consistent with our results obtained in the frontal displacement experiments. Therefore, the results demonstrate that membranes from the OMM were immobilized and that the resulting MMAC can be used to characterize mitochondrial receptors and to study ligand-receptor affinities.

3.2. Characterization of IMM receptors

In order to determine whether the IMM were co-immobilized with the OMM, the presence of SUR and mPTP was investigated. Glibenclamide, an antidiabetic sulfonyl urea, was used as the marker ligand for SUR [36], while diclofenac was used as the marker ligand for mPTP [1, 37]. The binding affinities (K_d values) obtained for glibenclamide and diclofenac, based on the frontal displacement experiments carried out on MMAC-(U87MG) using

ammonium acetate [10 mM, pH 7.4] was $0.79 \pm 0.39 \mu\text{M}$ and $1.20 \pm 0.19 \mu\text{M}$ respectively (Table 3), which is consistent with previously reported values.

The results confirm that both the OMM and IMM were immobilized from the isolated mitochondria.

3.3. MMAC-OT columns

An alternative approach to the development of membrane affinity columns using the IAM support is the immobilization of the membranes onto the surface of OT capillaries [24]. The advantages of the OT format include increased throughput resulting from a decrease in non-specific binding to the stationary phase, as well as a reduced amount of compound needed for the studies. As a result, this format may be more amenable to multiple compound screening. In order to create a screening method for the TSPO, mPTP and SUR, mitochondrial fragments isolated from U87MG and HEK were immobilized onto the surface of an OT capillary, generating MMAC-OT columns. Binding affinities (K_d) obtained for DIPY, diclofenac and glibenclamide, based on the frontal displacement experiments carried out on MMAC-OT (HEK) were $0.76 \pm 0.25 \mu\text{M}$, $0.78 \pm 29 \mu\text{M}$ and $0.99 \pm 0.52 \mu\text{M}$ respectively (Table 4). The calculated binding affinities on MMAC-OT was similar to the values obtained on MMAC (Table 1, 3 and 4), and, as expected the breakthrough time of these ligands on MMAC-OT were ~80% lower than that observed on MMAC. Further, the MMAC-OT (HEK) column was able to selectively bind TSPO ligands after 6 months of storage at 4 °C, while the MMAC-OT (U87MG) did not result in a functional column. This may be a result of the lower number of binding sites (B_{max}) compared MMAC-(U87MG). It is known that due to the difference in the available surface area for immobilization of receptors, OT columns have significantly lower B_{max} compared to IAM columns [24]. For example, B_{max} on the Pgp-OT were 200-fold less than the number calculated for the Pgp-IAM column, 3 nmol versus 546 nmol, respectively [38].

3.4. Confocal microscopy studies

The presence of TSPO on the mitochondrial membrane and its successful immobilization on IAM particles was confirmed by competitive binding study using confocal microscopy using the MMAC-(U87MG) stationary phase. In this study a low affinity TSPO fluorescent ligand PIX was used as a marker and DIPY, a high affinity TSPO ligand, was used as displacer. An emission wavelength of 636 nm was used to monitor DIPY as it had minimal background interference, as the auto-fluorescence contributed by IAM and mitochondrial fragments was negligible (data not shown). Fig. 4 shows binding of PIX (1 μM) to isolated mitochondrial fragments and mitochondrial fragments immobilized on IAM particles. In addition to the literature report [29], we have found that DIPY has 3-4 fold higher affinity than PIX for TSPO receptor [MMAC-(U87MG)] (Table 2). Similar difference in the binding affinity to the non-immobilized and immobilized mitochondrial fragments (U87MG) was observed between PIX and DIPY in the confocal studies (Fig. 4). Co-incubation of mitochondrial fragments and IAM with immobilized fragment with a series of increasing concentration of DIPY (0.03125, 0.0625, 0.125, 0.25, 0.5 and 1 μM) with 1 μM PIX, showed competitive displacement of PIX by DIPY. The inhibition of PIX binding in presence of DIPY was clearly observed as the exhibited fluorescence of PIX was dramatically decreased with

increasing concentration of DIPY. Maximum visible inhibition of 1 μ M PIX was observed with 0.125 μ M DIPY clearly indicating that DIPY has a higher binding affinity than PIX (Fig. 4). Similar results were also observed when PK was used to block the binding of PIX (data not shown).

4. Conclusion

This synthesis of first mitochondrial membrane affinity chromatography (MMAC) column enabled to create a new tool for the ligand binding and drug discovery studies. The immobilization of mitochondrial membrane fragments, consisting of outer and inner mitochondrial membrane receptors onto IAM and OT surface was successfully demonstrated. Confocal experiments confirmed the presence of TSPO on the MMAC stationary phase. The binding studies with DIPY, confirmed that TSPO was immobilized in a functional confirmation. Single displacement studies confirmed that the more rapid method could be used to screen for TSPO ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

OMM	outer mitochondrial membrane
IMM	inner mitochondrial membrane
TSPO	translocator protein
mPTP	mitochondrial permeability transition pore
CMAC	cellular membrane affinity chromatography
MMAC	mitochondrial membrane affinity chromatography
SUR	sulfonylurea receptor
OT	open tubular
DIPY	dipyridamole
MIX	mesoporphyrin IX
PIX	protoporphyrin
PK	PK-11195
Rot	rotenone

Flu	flunitrazepam
IAM	Immobilized Artificial Membrane

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Figure 1.

A schematic figure of mitochondrial membranes showing the localization of TSPO, mPTP and SUR. (Source for image of mitochondria: <http://micro.magnet.fsu.edu/cells/mitochondria/mitochondria.html>)

Figure 2.

(A) Breakthrough curve of 1 μ M DIPY using (I) ammonium acetate [10 mM, pH 7.4], (II) ammonium acetate [10 mM, pH 5.9], (III) ammonium acetate [10 mM, pH 5.9] containing 10% methanol, had breakthrough time ~200 min, ~100 min and ~50 min respectively. (B) Representative frontal elution profiles of 0.0625 μ M (I), 0.25 μ M (II), 0.5 μ M (III), 1 μ M (IV), 2 μ M (V), 3 μ M (VI), 5 μ M (VII), 7.5 μ M (VIII) and 15 μ M (IX) DIPY on the MMAC(U87MG) column (0.531 cm $\times$ 2 cm) on the Agilent TOF LC/MS. Mobile phase: ammonium acetate [10 – 1 mM, pH 5.9] at 0.4 ml min⁻¹.

Figure 3.

Single frontal displacement studies of 6 compounds (2.875 μM), DIPY (I), PK (II), MIX (III), PIX (IV), Rot (V), Flu (VI), carried out on the MMAC(U87 MG) column (0.531 cm $\times$ 2 cm) using ammonium acetate [10 mM, pH 5.9] in the presence 0.125 μM DIPY as the mobile phase. Additionally, breakthrough volume for 0.125 μM DIPY (VII) was also determined. The data was normalized to the change in breakthrough volume observed with DIPY and the relative changes from DIPY (100.0%) are reported.

Figure 4.

Confocal microscopic images showing the presence of TSPO in the isolated mitochondria from U87MG cells, and its immobilization onto IAM particles (12 μ m, 300 $\text{\AA}$).

Mitochondrial fragments and/or IAM particles with immobilized mitochondrial fragments were suspended in ammonium acetate [10 mM, pH 5.9] buffer prior to confocal imaging.

A – Mitochondrial fragments; B – Mitochondrial fragments after incubation with 1 μ M PIX; C – Mitochondrial fragments after incubation with 1 μ M PIX and 0.25 μ M DIPY; D – IAM particles with immobilized mitochondrial fragments; E – IAM particles with immobilized mitochondrial fragments after incubation with 1 μ M PIX; F – IAM particles with immobilized mitochondrial fragments after incubation with 1 μ M PIX and 0.125 μ M DIPY.

Scheme 1.

Structures of ligands for translocator protein (TSPO), sulfonyleurea receptor (SUR) and mitochondrial permeability transition pore (mPTP).

Table 1

Binding affinities of DIPY determined by FAC-MS at different mobile phase compositions on MMAC.

Mobile phase buffer	DIPY K_d values
Ammonium acetate [10 mM, pH 7.4]	$K_d = 1.12 \pm 0.25 \mu\text{M}$, $r^2 = 0.99$ [MMAC (U87MG)]. Breakthrough time: 200 min
Ammonium acetate [10 mM, pH 5.9]	$K_d = 1.08 \pm 0.49 \text{ pM}$, $r^2 = 0.92$ [MMAC (U87MG)]. Breakthrough time: 100 min $K_d = 0.68 \pm 0.27 \text{ pM}$, $r^2 = 0.96$ [MMAC (HEK)].
Ammonium acetate [10 mM, pH 5.9 with 10% methanol]	$K_d = 0.92 \pm 0.47 \mu\text{M}$, $r^2 = 0.89$ [MMAC (U87MG)] Breakthrough time: 50 min

Table 2

Binding affinities (μM) of DIPY, PK, Flu, MIX, PIX and Rot determined by frontal affinity chromatography on MMAC (U87MG) column.

TSPO ligand	K_d on MMAC(U87MG)	K_i for DIPY binding site on MMAC(U87MG)	Reported K_i using two different marker ligand [29]	
			PK	4'-Chlorodiazepam
PK	0.0086 ± 0.0006 $r^2 = 0.99$	1.14 ± 0.38 $r^2 = 0.99$	0.011	0.012
Flu	0.29 ± 0.06 $r^2 = 0.98$	-	0.211	0.138
DIPY	1.08 ± 0.49 $r^2 = 0.92$	-	0.679	0.156
MIX	-	1.47 ± 0.12 $r^2 = 0.99$	0.650	0.578
PIX	-	3.33 ± 1.35 $r^2 = 0.99$	2.92	2.14
Rot	-	3.40 ± 1.27 $r^2 = 0.95$	20.9	10.5

Table 3

Binding affinities (μM) of diclofenac and glibenclamide determined by frontal affinity chromatography on MMAC-(U87MG) column.

Ligand	K_d on MMAC-(U87MG)	Reported K_d
Diclofenac (mPTP ligand)	1.20 ± 0.19 $r^2 = 0.99$	induces mPTP at low micromolar range [1, 37]
Glibenclamide (SUR ligand)	0.79 ± 0.39 $r^2 = 0.80$	0.36 ± 0.05 [36]

Table 4

Binding affinities (μM) of DIPY, diclofenac and glibenclamide determined by frontal affinity chromatography on MMAC-OT (HEK-293) column.

Ligand	K_d on MMAC-OT (HEK-293)	Reported K_d
DIPY (TSPO ligand)	0.76 ± 0.25 $r^2 = 0.97$	0.679 and 0.156 [29]
Diclofenac (mPTP ligand)	0.78 ± 29 $r^2 = 0.91$	induces mPTP at low micromolar range [1, 37]
Glibenclamide (SUR ligand)	0.99 ± 0.52 $r^2 = 0.87$	0.36 ± 0.05 [36]