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Profiling of drug binding proteins by monolithic affinity chromatography in combination with liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

A new approach for proteome-wide profiling drug binding proteins by using monolithic capillary affinity chromatography in combination with HPLC–MS/MS is reported. Two immunosuppresive drugs, namely FK506 and cyclosporin A, were utilized as the experimental models for proof-of-concept. The monolithic capillary affinity columns were prepared through a single-step copolymerization of the drug derivatives with glycidyl methacrylate and ethylene dimethacrylate. The capillary chromatography with the affinity monolithic column facilitates the purification of the drug binding proteins from the cell lysate. By combining the capillary affinity column purification and the shot-gun proteomic analysis, totally 33 FK506- and 32 CsA-binding proteins including all the literature reported target proteins of these two drugs were identified. Among them, two proteins, namely voltage-dependent anion-selective channel protein 1 and serine/threonine-protein phosphatase PGAM5 were verified by using the recombinant proteins. The result supports that the monolithic capillary affinity chromatography is likely to become a valuable tool for profiling of binding proteins of small molecular drugs as well as bioactive compounds.

1. Introduction

A large number of small molecular drugs possess multiple targets (polypharmacology), although they were designed to aim at a single target [1]. The "off-target" effect may lead to complicated adverse effects; on the other hands, that may provide an opportunity to repurpose existing drugs [1,2]. Therefore, it is necessary to profile targets of the small molecule drugs at the proteomic level. However, up to date, target identification of small-molecule drugs or small-molecule probes remains a vital and daunting task in the community of pharmacology as well as chemical biology [3,4]. Generally applicable and robust methods for the drug target identification are urgently needed [5].

Affinity chromatography is the most direct approach used to purify target proteins of small molecule drugs [5–8]. The target proteins can be captured by the agarose-based affinity beads on which the studied drug molecules are immobilized covalently through the linker, and subsequently eluted for identification (pull-down experiment) [9]. Up to date, almost all the pull-down experiments were performed manually with the following procedure: Cell lysate was incubated with affinity matrices for a certain period of time at 4 °C, centrifuged, and transferred to spin columns for elution of the target proteins. The columns were then drained and washed with wash buffer. Retained proteins were eluted by SDS–PAGE sample buffer [7]. Subsequently, the bound proteins were subjected to SDS–PAGE separation. The protein bands were excised for an in-gel trypsin digestion followed by the mass spectrometry (MS)-based shotgun proteomic analysis [9]. One disadvantage of such a procedure is that a large amount of starting protein should be used and the stringent wash conditions are required [10]. This is a very

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tedious process. Moreover, the moderate affinity binding proteins may be lost in the stringent wash process.

Alternatively, capillary liquid chromatography with the monolithic column can be an attractive approach for the purification of target proteins. The monolithic bed with high permeability and a relative high surface area which are favorable for a chromatographic separation [11–14]. Most importantly, the monolithic columns can be easily amenable to capillary-HPLC to allow a fast and high performance purification of drug target proteins from minute biological samples. Thus far, a few affinity monolithic columns have been reported[15,16]. However, no one concerning the drug target identification.

FK506 and CsA are immunosuppressant drugs used after organ transplantation. The action of these 2 drugs are due to their interaction with the immunosuppressant-immunophilin complexes [6]. CsA binds the immunophilin cyclophilin and inhibits the calcium/calmodulin dependent protein phosphatase calcineurin, while FK506 interacts with the immunophilin FK506 binding protein (FKBP12) [6,17]. Although their immunosuppressive action mechanism is clear, however, the action mechanisms of acute and chronic nephrotoxicity[18], apoptosis [19], nerve regeneration [20] remain unclear. Profiling of their binding proteins in the proteome level might lead to a better understanding on their "off target" effect and pave the way to get safer immunosuppressant or develop new use of them.

In this study, we reported a new approach for a proteome-wide profiling of drug target proteins by using monolithic capillary affinity chromatography in conjunction with HPLC–MS/MS. The monolithic affinity columns were prepared in a single-step copolymerization of FK506 or CsA derivatives with glycidyl methacrylate and ethylene dimethacrylate inside a fused silica capillary column. With our approach, totally 33 FK506- and 32 CsA-binding proteins were identified. Among them voltage-dependent anion-selective channel protein 1 and serine/threonine-protein phosphatase PGAM5 were verified by using the recombinant proteins.

2. Experimental

2.1. Chemical and materials

Glycidyl methacrylate (GMA), ethylene dimethacrylate [6], dimethyl formamide (DMF), Grubbs 2nd generation catalyst, N-hydroxysuccinamide (NHS), acrylamide, thiourea, toluene, 4,7,10-trioxa-1,13-tridecanediamine, tris (hydroxymethyl) aminomethane (Tris) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO). Triethylamine, phosphoric acid, azodiisobutyronitrile (AIBN) and recombinant human FKBP12 were purchased from Sinopharm (Shanghai, China). Trypsin was from Promega (Madison, WI, USA). Voltage-dependent anion-selective channel protein 1 (VDAC1) was from Abnova (Taiwan, China). Acetonitrile (HPLC grade) was from Merck (Darmstadt, Germany). Vinyltrimethoxysilane (VTMOS) was purchased from Trustchemsilanes (Nanjing, China). FK506 and CsA were purchased from Shanghai Boyle (Shanghai, China). Fused silica capillary with a dimension of 370 μm o.d. and 200 μm i.d. was obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Capillary liquid chromatography

All separations were performed on a homemade capillary liquid chromatography system consisting of a Pu-2080 HPLC pump, a CE 970 UV detector with a capillary flow-cell holder (Jasco, Tokyo, Japan) and a Rheodyne 7725i HPLC injection valve equipped with a 100 μL loop (Rohnert Park, CA, USA). The mobile phase delivered by the HPLC pump at flow rate of 200 μL/min was split with a zero-dead-volume T (VICI, Schenkon, Switzerland). The majority of the mobile phase was split to waste, while the remainder for driving the separation was about 1–5 μL/min depending on the split ratio controlled by the length of the capillary tubing (50 μm i.d.). One end of the monolithic column was directly connected to the injection valve, and another end was connected to the capillary flow cell (50 cm, 100 μm i.d., 365 μm o.d.) by a zero dead volume PCU-360 Picoclear union (New Objective, MA, USA). Data were processed with a HS2000 chromatography workstation (Yingpu, Hangzhou, China). All separations were carried out at ambient temperature (20–25 °C).

2.3. Synthesis of the FK506 and CsA derivatives

The procedure for synthesis of FK506 and CsA derivatives is shown in Supporting Information Fig. S1. Briefly, FK506 was chemically modified by NHS-activated 4-pentenoate (3 molar excess) through a reaction catalyzed by Grubbs 2nd generation catalyst (10% molar) in dry 1,2-dichloroethane. After stirring the reaction mixture overnight at 70 °C, the solvent was evaporated and the residue was purified by flash chromatography on silica gel with a mixture of solvents consisting of dichloromethane/ethylacetate. The resulting FK506 derivative was dissolved in dry dichloromethane, then equal molar 4,7,10-trioxa-1,13-tridecanediamine was added. After stirring for 5 h at room temperature, equal molar NHS-activated 4-pentenoate was added and the solution was stirred for another 5 h. Finally, the FK506 derivative was purified with a RP-C18 semi-preparation column (10 mm × 250 mm, packed with Hypesil ODS 5 μm 100 Å, Thermo Fisher Scientific, Waltham, MA, USA). The purity of the final product was determined as 99% by HPLC analysis. The CsA derivative was synthesized following the identical synthetic procedure (Fig. S1).

2.4. Preparation of the monolithic affinity capillary columns

FK506 monolithic column was prepared in a single step (see Fig. 1). Briefly, the fused silica capillaries were etched with 0.1 M NaOH for 30 min at room temperature, then flushed with 0.1 M HCl, water and methanol for 30 min, respectively. After drying the capillary at 120 °C for 2 h, solution of vinyltrimethoxysilane in dry toluene 10% (v/v) was charged into the capillaries and kept at 45 °C for overnight to functionalize the inner surface. The capillaries were flushed with methanol for 30 min, and then purged with dry nitrogen gas for 30 min for dryness.

The composition of the polymerization mixture was optimized as follows: 60 wt% porogen solvents (54 wt% 1-dodecanol, 6 wt% cyclohexanol), 16 wt% cross-linker EDMA, 23 wt% monomer GMA and 1 wt% affinity ligand (FK506- or CsA derivative), and AIBN (1 wt% with respect to total monomers). The rationale for selecting 1 wt% affinity ligand is that it is their maximum solubility in the polymerization mixture. The polymerization mixture was ultrasonicated in ice bath for 20 min, then degassed by purging with nitrogen gas for another 10 min. After fully filling the vinylized capillary with the polymerization mixture, both ends of the capillary were sealed with rubber septum, and the capillary was placed in an electric heating oven to allow polymerization reaction at 60 °C for 24 h. Finally, the resulting monolithic column was flushed with methanol to remove the porogenic solvent and the unreacted reagents. A control column was also prepared under the identical conditions except in the absence of the affinity ligand. The epoxy groups on the surface of the monolith probably were opened by the treatment with formic acid solution to increase the hydrophilic property of the monolithic bed.