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Development of a mitochondria-based centrifugal ultrafiltration/liquid chromatography/mass spectrometry method for screening mitochondria-targeted bioactive constituents from complex matrixes: Herbal medicines as a case study

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ABSTRACT

Mitochondria are an important intracellular pharmacological target because damage to this organelle results in a variety of human disorders and because mitochondria are involved in complex processes such as energy generation, apoptosis and lipid metabolism. To expedite the search for natural bioactive compounds targeting mitochondria, we initially developed an efficient mitochondria-based screening method by combining centrifugal ultrafiltration (CU) with liquid chromatography/mass spectrometry (LC/MS), which is called screening method for mitochondria-targeted bioactive constituents (SM-MBC) and is compatible with the search of mitochondria-targeted compounds from complex matrixes such as herbal medicines extracts. Functionally active, structurally intact and pure mitochondria were obtained from rat myocardium using an optimized protocol for mitochondrial isolation comprising organelle release followed by differential and Nycodenz density gradient centrifugation. After evaluating the reliability of the method using thiabendazole (TZ), rotenone (RN), amiodarone (AR) and trimetazidine (TD) as positive controls, this method was successfully applied to screen bioactive constituents from extracts of Polygoni Cuspidati Rhizoma et Radix (PCRR) and Scutellariae Radix (SR). Nineteen active compounds were detected and identified by LC/MS, of which 17 were new mitochondria-targeted compounds. The activity of 9 of the 19 hit compounds was confirmed by *in vitro* pharmacological trials. These results demonstrate that SM-MBC can be used for the efficient screening of mitochondria-targeted constituents in complex preparations used to treat mitochondrial disorders, such as PCRR and SR. The results may be meaningful for an in-depth understanding of drug mechanism of action and drug discovery from medicinal herbs.

1. Introduction

Mitochondria are the powerhouses of cells and serve as the primary energy source. Mitochondria also have many other important roles, including adjustment of apoptotic cell death, reactive oxygen species generation, maintenance of calcium homeostasis and regulation of lipid metabolism [1]. Accordingly, mitochondrial dysfunction leads to a number of human diseases, including cancer, neurodegenerative diseases, ischemia–reperfusion injury, diabetes and obesity [2]. Mutations to mitochondrial DNA can also cause a number of human disorders [2]. Thus, strategies to prevent mitochondrial damage or to manipulate mitochondrial functions in a clinically useful manner may provide effective therapies for a variety of human diseases [2]. A large number of therapeutically applied substances have been specifically designed to affect mitochondrial functions to exert their therapeutic effects as antitumor agents, neuroprotective agents, immunosuppressants, antiviral drugs, potassium channel openers, sulfonylureas and anesthetics [3]. Therefore, mitochondria have become a prominent intracellular pharmacological target for new drug development [4–7].

Herbal medicines (HMs) are increasingly of interest as sources of bioactive compounds for the discovery of promising new drugs [8]. A rapidly expanding body of literature suggests that many HMs affect mitochondrial function to exert their therapeutic effects, including anticancer [9], antiaging [10], anti-diabetes [11],

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anti-obesity [12], neuroprotection [13], cardioprotection [14] and hepaticprotection [15]. The bioactive constituents in HMs that interact with mitochondria have not been identified, severely limiting an understanding of their mechanisms and hindering drug development from medicinal herbs. Hence, screening of the bioactive constituents in HMs that act on mitochondria is urgently required.

Because of the large number of substances in HMs, bioactive constituent screening and analysis are extremely difficult, even for well-documented formulations for specific diseases. The conventional approach for screening mitochondria-targeted bioactive constituents includes extraction and isolation of the HM constituents and pharmacological tests of the purified compounds [16]. This method is inefficient for the direct screening of bioactive compounds from complex agents in addition to being labor-intensive, expensive, and time- and sample-consuming. High-throughput screening [17,18] and high-content screening [19] methods have also been used to efficiently identify mitochondria-targeted compounds. However, these methods are designed to screen pure compounds and are not suitable for the direct detection of multiple bioactive compounds from complex matrixes. Consequently, further development of screening approaches to efficiently detect mitochondria-targeted bioactive constituents from natural complex matrixes such as HMs is required.

Centrifugal ultrafiltration (CU) employs centrifugal force and a semi-permeable membrane to retain suspended solids and high-molecular-weight solutes while liquid and low-molecular-weight solutes are allowed to permeate the membrane, depending on the nominal molecular weight cut-off of the membrane. Thus, due to its simple operation, high speed and high dependability, CU has become a useful technique for screening bioactive compounds from complex objects bound to biomacromolecules such as bovine serum albumin [16], *Plasmodium falciparum* thioredoxin reductase [20], α-glucosidase [21], quinone reductase-2 [22], DNA [23], retinoid X receptors [24], β-amyloid protein [25] and liposomes [26]. Liquid chromatography/mass spectrometry (LC/MS) has been widely applied for the simultaneous separation and identification of active constituents in complex mixtures [20–23]. The combination of LC/MS with CU permits the efficient screening and identification of active constituents in HMs. However, no screening method has been reported for the direct identification of mitochondria-targeted constituents from complex matrixes.

This study was initially designed to develop a rapid and efficient screening method coupling CU with LC/MS to discover potential mitochondria-targeted bioactive constituents from HMs used to treat mitochondrial disorders, exemplified by two Chinese traditional medicines, Polygoni Cuspidati Rhizoma et Radix (PCRR) and Scutellariae Radix (SR). Using this new method, called the screening method for mitochondria-targeted bioactive constituents (SM-MBC), fractions containing bioactive constituents that specifically bind the mitochondria were isolated by the CU technique. The ultrafiltrates were then collected, evaporated and injected into the LC/MS system for separation and identification. A pharmacological verification trial confirmed that SM-MBC is an effective protocol for the efficient screening of potential bioactive constituents that bind mitochondria from complex mixtures (Fig. 1). SM-MBC may be useful for an in-depth comprehension of drug mechanism of action and drug discovery using medicinal herbs as sources of lead compounds.

2. Experimental

2.1. Chemicals and materials

Nifedipine (NP, lot no. 100338-201103), captopril (CP, lot no. 100318-200602), baicalin (lot no. 110715-200514), baicalein (lot no. 11595-201306) and wogonin (lot no. 11514-200403) were obtained from the National Institute for Food and Drug Control (Beijing, China). Thiabendazole (TZ, lot no. 5838), rotenone (RN, lot no. 031201AG-AC), trimetazidine (TD, lot no. JLISA-BE), amiodarone (AR, lot no. 77290) and rhodamine 123 (Rh123, lot no. LK80N08) were purchased from J&K Scientific Ltd. (Beijing, China). Piceid (lot no. 131121), emodin-1-*O*-glucoside (lot no. 140510), resveratrol (lot no. 131021), emodin-8-*O*-glucoside (lot no. 130719), emodin (lot no. 130410), oroxylin A-7-*O*-glucuronide (lot no. 131122), wogonoside (lot no. 131214) and oroxylin A (lot no. 130323) were provided by Chengdu Pufeide Biological Technology Co., Ltd. (Chengdu, China). HPLC grade formic acid (lot no. 2K3004) was purchased from ROE Scientific Inc. (Newark, DE, USA). Trypan blue (lot no. C1803130), neutral red (lot no. 608A046) and the Bicinchoninic Acid (BCA) Protein Determination Kit (lot no. D1106140) were obtained from M&C Gene Technology (Beijing) Ltd. (Beijing, China). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 mM, lot no. 1108191310) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Cytochrome c oxidase-IV (COX-IV) rabbit polyclonal antibody (lot no. 00020194) and calnexin rabbit polyclonal antibody (lot no. 00000625) were purchased from Proteintech Group, Inc. (Chicago, IL, USA). GAPDH mouse monoclonal antibody (lot no. A1713) and β-action mouse monoclonal antibody (lot no. E1012) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). LAMP-2 rabbit monoclonal antibody (lot no. 00018269) was provided by Epitomics, Inc. (an Abcam Co., Burlingame, CA, USA). C-Jun mouse monoclonal antibody (lot no. 20131108) was purchased from EnoGene Biotech Co., Ltd. (Nanjing, China). HRP AffiniPure Goat Anti-Mouse IgG (H + L) (lot no. OG2701) and HRP AffiniPure Goat Anti-Rabbit IgG (H + L) (lot no. MO1401) were purchased from EarthOx, LLC (San Francisco, CA, USA). Complete protease inhibitor cocktail (lot no. 14339100) was purchased from Roche Diagnostics (Penzberg, Germany). SuperSignal West Pico luminal chemiluminescence substrate (lot no. P1010-250) was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). Janus green B was provided by Sigma–Aldrich (St. Louis, MO, USA). PVDF membranes were purchased from Millipore (Bedford, MA, USA). Nycodenz was purchased from Axis-Shield PoC AS (Oslo, Norway). HPLC grade methanol and acetonitrile were supplied by Fisher Scientific (Fairlawn, NJ, USA). Deionized water was purified using a Milli-Q Water Purification System (Millipore, Billerica, MA, USA). All other reagents were of analytical grade or higher. Polygoni Cuspidati Rhizoma et Radix (PCRR, purchase date July 20, 2014) and Scutellariae Radix (SR, purchase date Nov. 13, 2010) were purchased from the Tianheng Drug Store (Beijing, China) and the Xijingou Collection Station of Materia Medica (Luanping, Hebei, China), respectively. All samples were authenticated by Professor Shao-Qing Cai, and voucher specimens of PCRR (No. 7545) and SR (No. 6624) have been deposited in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, Peking University (Beijing, China).

2.2. Experimental animals

Experimental schemes involving conscious animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the Biomedical Ethical Committee of Peking University (No. SYXK2011-0039). Efforts were made to minimize the number of animals used and their suffering. Healthy male Sprague-Dawley rats (300 ± 50 g) were used. Animals were provided by the Department of Laboratory Animal Science, Peking University Health Science Center (Beijing, China), and the animals had access to pellet food and tap water *ad libitum*. Rats were maintained in an environmentally controlled breeding room throughout the experiments.