Journal of Pharmaceutical and Biomedical Analysis 104 (2015) 67–74

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Rapid screening natural-origin lipase inhibitors from hypolipidemic decoctions by ultrafiltration combined with liquid chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 3 November 2014
Accepted 11 November 2014
Available online 18 November 2014

Keywords:
Lipase inhibitors
Hypolipidemic decoctions
TCM formulae
HPLC–MS
Ultrafiltration

ABSTRACT

Lipase inhibitors generate hypolipidemic effect that is helpful to control or treat some obesity diseases by inactivating catalytic activity of human pancreatic lipase, a key enzyme involved in triglyceride hydrolysis in vivo. Many traditional Chinese medicine (TCM) formulae have been effectively used to treat obesity and other fat related diseases for centuries and modern biological experiments demonstrate therapeutic effect of these formulae can be linked to their lipid-lowering capability in blood. These observations suggest that these hypolipidemic decoctions (HDs) could be a promising resource of natural-origin lipase inhibitors. This work described a rapid approach for screening lipase inhibitors from four widely used HDs, including Wu-Ling-San (WLS), Ze-Xie decoction (ZX), Xiao-Xian-Xiong decoction (XXX) and Xiao-Chai-Hu decoction (XCH), by ultrafiltration combing with high performance liquid chromatography–mass spectrometry (HPLC–MS). Our results showed sixteen natural-origin lipase inhibitors were discovered and identified by high resolution and multistage mass spectrometry. Inhibitory activities of two compounds were confirmed by a functional assay of lipase, which validated the reliability of our approach. Molecular docking simulation was then performed to investigate potential mechanism of action for these compounds. Together we present an efficient method for rapid screening lipase inhibitors from complex natural products, which can be easily accommodated to other important enzymatic system with therapeutic values.

1. Introduction

In human digestive system, triglyceride from diet is hydrolyzed to produce free fatty acids and monoglycerides that may accumulate in body then be re-absorbed by the intestine, therefore leading to many kinds of obesity diseases [1,2]. Human pancreatic lipase (lipase is used through the text for simplicity) plays an essential role in this important biological process, which makes it a promising therapeutic target to treat lipid-related diseases. Many synthetic compounds have been designed and developed as therapeutic drugs to treat obesity disease by inactivating the lipolytic activity of lipase, such as Orlistat [3,4]. These lipase-inhibiting drugs are useful weapon to control or treat obesity and many kinds of relevant problems that has already been recognized as a global healthy concern [5,6]. In addition to chemical synthesis, natural product is a promising origin of active components as many herbs have been used as traditional medicines or food supplements to treat many diseases for quite a long period [7–9]. Many kinds of traditional Chinese medicine (TCM) formulae and decoctions have been described to control or treat fat problems with few side effects in several famous ancient medical books, e.g., *Treatise on Cold Damage* and *Concise Essential of the Golden Cabinet* (Zhang Zhongjing's herbal formulae, about 300 A.D.) [10]. Therapeutic effects of most of these TCM formulae have been attributed to their ability to lower lipid in blood and it is very likely that some chemicals in the formulae are lipase inhibitors. Identification of these active components would be important to improve our understanding on hypolipidemic property of TCM formulae, which also provide novel opportunity for drug development.

Most natural-origin active components were discovered by a conventional phytochemical approach, in which the systematic isolation, purification and identification of target compounds are necessary steps before we can finally evaluate their activities biologically [11–15]. This traditional research protocol is effective to screen potential ligands in plants or medical herbs, however it is

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http://dx.doi.org/10.1016/j.jpba.2014.11.022
0731-7085/

Table 1
Composition of hypolipidemic TCM decoctions.

Herbs (g)	TCM decoctions			
	WLS	ZX	XXX	XCH
Rhizoma Alismatis	7	34.6		
Rhizoma Atractylodes Macrocephala	12	13.5		
Poria Cocos	7			
Polyporus Umbellatus	7			
Ramulus Cinnamomi	4.7			
Rhizoma Coptidis			7	
Rhizoma Pinelliae			33.3	5.6
Fructus Trichosanthis			1.7	
Radix Bupleuri				11
Radix Scutellariae				4.2
Radix Ginseng				4.2
Radix Et Rhizoma Glycyrrhizae				4.2
Rhizoma Zingiberis				4.2
Jujubae Fructus				2

rather time-consuming and possesses a relatively high risk of failure [9,16,17]. Combination of ultrafiltration with high performance liquid chromatography–mass spectrometry (HPLC–MS) is a rapid method and powerful tool for screening bioactive compounds from complex mixtures [18,19]. It can avoid unnecessary isolation or purification of inactive compounds and direct more focus on target compounds more likely to exhibit desired biological effects, which would greatly improve the screening and analysis efficiency especially for complex mixtures, e.g., TCM decoctions. Until now, many novel methods have been developed for LC–MS based analysis or screening for TCM and its formulae [20–22]. Especially for ultrafiltration, many active compounds for several enzymes, including tyrosinase [23], thioredoxin reductase [24], shikimate kinase [25], α-glucosidase [26], quinone reductase-2 [25], and aromatase [27], have been found by this method over years.

This work described a rapid approach for characterizing and screening lipase inhibitors from four TCM decoctions, including Wu-Ling-San (WLS), Ze-Xie decoction (ZX), Xiao-Xian-Xiong decoction (XXX) and Xiao-Chai-Hu decoction (XCH), by combing the ultrafiltration with HPLC–MS. All of these decoctions were widely used for obesity and other relevant diseases, e.g., diabetes. Sixteen natural-origin lipase inhibitors was discovered and identified, and two of them were tested by a functional assay to prove their inhibition activities. Results showed the TCM decoctions with clinical efficacy are indeed hopeful source for active compounds discovery, and the combination of the ultrafiltration and LC–MS could rapidly screen certain ligands from complex natural products.

2. Material and methods

2.1. Chemicals and materials

The composition of Wu-Ling-San (WLS), Ze-Xie decoction (ZX), Xiao-Xian-Xiong decoction (XXX) and Xiao-Chai-Hu decoction (XCH) were shown in Table 1. All herbs were obtained from the Hangzhou traditional Chinese herbal medicine factory (Hangzhou, Zhejiang province, PR China). Lipase (from porcine pancreas) and 4-methylumbelliferyl oleate were purchased from Sigma–Aldrich Co., Ltd. (St. Louis, MO, USA). Baicalin and Wogonoside were provided by Winherb Medical Technology Co., Ltd. (Shanghai, PR China). Centrifugal ultrafiltration filters (AIMCO Ultra-0.5) were purchased from Millipore Co., Ltd. (Bedford, MA, USA). HPLC-grade acetonitrile (Merck, Darmstadt, Germany), formic acid (ROE Scientific Inc., Newark, DE, USA), and ultrapure water (Milli-Q Plus, Millipore Co. Ltd., Billerica, MA, USA) were used in all experiments. All other chemicals and solvents were of analytical grade.

2.2. TCM decoctions preparation

Herbs of four TCM formulae were extracted with pure water for 1 h under reflux (the amount of the solvent was six and four times of the total weight of the formula, respectively). Extracts were first dried under 70 °C, and then concentrated to total dryness by vacuum freeze-drying. Samples for ultrafiltration experiments were prepared by dissolving the extracts in water to a final concentration of 5 mg/mL. The solutions were centrifuged at 10,000 rpm for 10 min before analysis.

2.3. Incubation and ultrafiltration

The screening experiments were similar to reported works with some modifications [23,25,28]. The main process was as follows (Fig. 1). Adding 100 μL sample solution (5 mg/mL) into the 200 μL lipase solution (5 mg/mL, dissolved in water, pH 7.2, 25 °C). The potential ligands would fully bind with enzymes by incubating the mixture for 60 min at 37 °C, 500 rpm, in a 96-well plate. Transfer the incubated liquid into a centrifugal ultrafiltration filter, which contained regenerated cellulose ultrafiltration membrane with 10 kDa molecule weight cut-off. Ultrafiltrates were injected directly into the HPLC–MS after centrifuged for 30 min, 13,400 rpm. Three groups were deployed for this experiment: the sample group that containing the active lipase; the blank group that using the water instead of the enzyme and the denatured group using the denatured enzyme (30 min boiling water bath). The denatured group was set for non-specific binding control. Binding strength was measured by the binding degree (%) which was calculated by the following formula: Binding degree (%) = (Ad − As)/Ac*100, where Ad, As and Ac are the peak areas of a certain compound in the selective ion monitoring (SIM) chromatogram of denatured group, sample group and blank group, respectively. We also investigated influence of different lipase concentrations (3 mg/mL, 5 mg/mL and 10 mg/mL) and incubation time (30 min, 60 min and 120 min). ZX was used as tested sample and two inhibitors' ions (*m*/*z* 552, *m*/*z* 961) were chosen as observation targets. All experiments were repeated three times.

2.4. LC–MS analysis

Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with an LCQ Deca XP^{plus} ion trap mass spectrometer (IT-MS) (Thermo Finnigan, San Jose, CA, USA) was used for LC–MS experiments. Zorbax SB-C_{18} column (5 μm, 4.6 × 250 mm; Agilent Technologies, Santa Clara, CA, USA) was used in all the chromatographic separations. The mobile phases were 0.05% formic acid – water (A) and 0.05% formic acid – acetonitrile (B). The linear gradient programs were as follows, 0/5, 50/30, 65/50, 70/95, 90/95 (min/B%); sample injection volume was 40 μL; column oven temperature was set to 30 °C; flow rate was 0.6 mL/min; and the photodiode array (PDA) detection range was set from 190 nm to 400 nm. The optimized instrument settings of MS were as follows: positive-ion mode, 4 kV source voltage, 350 °C capillary temperature, 60 arb sheath gas (N_2) and 20 arb auxiliary gas (N_2). Full scan was first used for preliminary ligands searching, and then selective ion monitoring (SIM) was employed for the peak area integration and binding degree calculation after we confirmed potential lipase inhibitors. Multistage MS spectra were also acquired using the same conditions above. The multistage MS data was analyzed by Xcalibur 2.1, Qual Browser (Thermo Fisher Scientific Inc., San Jose, CA, USA). High resolution MS (HR-MS) data was acquired on a Waters ACQUITY UPLC (Waters Corp., Milford, MA, USA) equipped with an AB Triple TOF 5600^{plus} System (AB SCIEX, Framingham, MA, USA). The optimal MS conditions were as follows: the scan range was set