Journal of Chromatography A, 1436 (2016) 91–99

Contents lists available at ScienceDirect

Journal of Chromatography A

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

# Screening of minor bioactive compounds from herbal medicines by *in silico* docking and the trace peak exposure methods

Bin Wu, Hui-Peng Song, Xu Zhou, Xin-Guang Liu, Wen Gao, Xin Dong, Hui-Jun Li, Ping Li*, Hua Yang*

*State Key Laboratory of Natural Medicines (China Pharmaceutical University), Nanjing 210009, China*

ARTICLE INFO

*Article history:*
Received 7 October 2015
Received in revised form 9 January 2016
Accepted 26 January 2016
Available online 29 January 2016

*Keywords:*
Ginkgo
Ultrafiltration
LC-QTOF MS
Docking
Trace peak exposure
Minor bioactive compounds

ABSTRACT

Screening of high potent enzyme inhibitors from herbal medicines is always lacking of efficiency due to the complexity of chemicals. The constituents responsible for the holistic effect may be trace-level chemicals, but these chemicals were covered by highly abundant compositions. To challenge this bottleneck, a strategy for screening minor bioactive compounds was proposed. It generally included four steps, (1) preliminarily find the enzyme binders by ultrafiltration; (2) optimise and predict the potential inhibitors by docking analysis; (3) selectively identify and prepare trace compounds by segment and exposure approach; (4) validate the activity and summarize the structure-activity relationship. As a case study, α-glucosidase (AGH) and *Ginkgo biloba* extract were used as the experimental materials. By comprehensive screening, 11 trace flavones were screened out and identified as strong AGH inhibitors. Among them, AGH inhibitory activities of syringetin and sciadopitysin were reported for the first time. Their $IC_{50}$ values were 36.80 and 8.29 μM, respectively, which were lower than that of a positive control acarbose. In addition, the AGH inhibitory activities of the flavonoids could be ranked, based on a decreased order, as biflavone, flavone, flavone glycoside, flavone biglycoside. The strategy is expected to be practical and useful for screening bioactive molecules from herbal medicines, especially for the minor compounds, which will definitely accelerate the discovery of new drug candidates.



## 1. Introduction

Screening of high potent enzyme inhibitors from herbal medicines (HMs) has been a difficult problem for the past decades, though some successful discoveries of bioactive compounds from HMs were reported [1–3]. There is a common phenomenon that many crude HM extracts have powerful activities, but the major constituents of them could not be responsible for the holistic effect since HMs are complex mixtures [4–6]. It means that some potent inhibitors of HMs are not discovered and most of them may be trace-level constituents [7,8]. Moreover, the presence of highly abundant compositions could mask the detection of lower abundance constituents. Therefore, screening of potent trace inhibitors was a huge challenge for researchers.

Conventional method to discover the enzyme inhibitors from complex mixtures is dependent on the isolation of compounds one by one. By using this method, many trace components could be easily ignored or lost during the multi-step separation and purification. In recent years, with the development of modern analytical techniques, many new screening methods have appeared, such as immunoassay biosensor [9], microfluidic enzymatic chip [10] and Capillary electrophoresis [11]. Nevertheless, due to the requirement for complex operations or high experimental conditions, these methods could not be widely applied. Thus, rapid and efficient identification of minor enzyme inhibitors from HMs remained a technical challenge.

In this work, a strategy was established and developed to comprehensively screen high-quality enzyme inhibitors from HMs by *in silico* docking and the trace peak exposure methods. Ultrafiltration liquid chromatography QTOF mass spectrometry (UF-LC-QTOF MS), a high-throughput screening (HTS) method for discovering the enzyme inhibitors, could be used to identify the binders that showed high signal response in the chromatogram [12,13]. *In silico* docking method was then performed to optimise and predict the candidate compounds in the natural-product derived combinational library, which is obtained by the trace peak exposure method. To fish out the high-quality trace inhibitors from HMs efficiently, high content peaks that may interfere the screening result were

* Corresponding authors. Fax: +86 25 8327 1379.
*E-mail addresses:* liping2004@126.com (P. Li), 104yang104@163.com (H. Yang).

http://dx.doi.org/10.1016/j.chroma.2016.01.062
0021-9673/

removed, and then the signals of trace components in complex samples were enhanced. By optimising the diversity of compounds, improving the quality of the library, and increasing the efficiency and sensitivity of the screening method, the strategy is useful and practical to screen the high-quality enzyme inhibitors from complex mixtures.

As an illustrative case study, α-glucosidase (AGH) and *Ginkgo biloba* extract (GBE) were used as the model enzyme and the real biological sample, respectively. Recently, as the interests into natural products increased, naturally occurring AGH inhibitors are considered as therapeutic alternatives to reduce the produce of glucose [14–16]. Our preliminary experiments showed that GBE had good AGH inhibitory activity with $IC_{50}$ value of 21.42 μg/mL (Fig. S1). Thus, GBE was used as the natural compound library for screening. By using the proposed strategy, eleven flavones were identified as high-quality α-glucosidase inhibitors, and two of them, syringetin and sciadopitysin, were discovered as novel AGH inhibitors with excellent activities. Enzymatic activity assays showed that their $IC_{50}$ values were 36.80 and 8.29 μM, respectively.

## 2. Experimental

### 2.1. Materials and reagents

The reference standards of quercetin-3-*O*-β-D-glucoside, quercetin-3-*O*-α-L-rhamnoside, quercetin, kaempferol, isorhamnetin, apigenin, ginkgetin, bilobetin, isoginkgetin, and amentoflavone were purchased from Chengdu Must Bio-technology Co., Ltd. (98%). Syringetin, myricein, sciadopitysin, quercetin-3-*O*-α-L-rhamnopyranosyl-2"-(6"'-*p*-coumaroyl)-β-D-glucoside (QRCG) and kaempferol-3-*O*-α-L-rhamnopyranosyl-2"-(6"'-*p*-coumaroyl)-β-D-glucoside (KRCG) were previously isolated from GBE in the authors' laboratory. The *G. biloba* extract used in this work was purchased from Jiangsu Shenlong Pharmaceutical Co., Ltd. (Nanjing, China). The extract with 6% ginkgolides and 24% flavonids was extracted by the standardized process according to the Chinese Pharmacopoeia [17] 4-nitrophenyl-α-D-glucopyranoside (PNPG) and the α-glucosidase (EC 3. 2. 1. 20) obtained from Saccharomyces cerevisiae were purchased from Sigma (St. Louis, MO). Centrifugal ultrafilters (Omega Nanosep, 10 K) were purchased from Pall (Ann Arbor, MI). Distilled-deionized water was provided by a Milli-Q water purification machine from Millipore (Bedford, MA, USA). Acetonitrile of HPLC grade were purchased from Merck (Darmstadt, Germany).

### 2.2. Screening binders to AGH by UF-LC-QTOF MS

A 10 μL aliquot of 10 mg/mL GBE was incubated with 80 μL of AGH solution (at a final concentration of 5 μM) in 110 μL of phosphate buffer (67 mM, pH 6.8) for 30 min at 37 °C, and then the mixture was filtered through a 10 kDa ultrafiltration filter followed by centrifugation at 16,200 × *g* for 20 min. The complexes of AGH and ligand were retained in the ultrafiltration tube, and washed three times by 200 μL aliquots of phosphate buffer. Another centrifugation at 16,200 × *g* for 20 min was performed to remove the unbound compounds. The ligands were dissociated from AGH using 100 μL 50% methanol aqueous solution with a 15 min ultrasonication. After centrifugation, the ultrafiltrates containing the released ligands were analyzed by Agilent 1290 UHPLC system. The control groups were carried out in a similar manner without AGH. All experiments were done in triplicate.

### 2.3. Structural assignment of screened binders to AGH

A 20 μL aliquot of ultrafiltrate was injected to an Agilent 1290 UHPLC system equipped with an online degasser, a binary pump, an auto-sampler and a thermostatically controlled column compartment. Chromatographic separation was conducted at room temperature using a Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm; Agilent) and a C18 guard column. The mobile phase was consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B), and the flow rate was 1.0 mL/min. The gradient program was as follows: 0–60 min,14–14% B; 60–80 min, 14–24% B; 80–90 min, 24–27% B; 90–110 min, 27–27% B; 110–111 min, 27–30% B; 111–124 min, 30–30% B; 124–135 min, 30–35% B; 135–139 min, 35–35% B; 139–142 min, 35–40% B; 142–145 min, 40–40% B; 145–148 min, 40–47% B; 148–154 min, 47–47% B; 154–156 min, 47–60% B; 156–162 min, 60–80% B; 162–164 min, 80–80% B; 164–167 min, 80–100% B; 167–172 min, 100–100%. The UV wavelength of detection was 350 nm.

Qualitative analysis was carried out by a 6530 QTOF mass system (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization (ESI) interface. The ESI source worked in negative mode. The operating parameters were as follows: drying gas temperature, 325 °C; drying gas ($N_2$) flow rate, 10.0 L/min; shealth gas temperature, 350 °C; shealth gas ($N_2$) flow rate, 10.0 L/min; fragmentor voltage, 120 V; skimmer voltage, 65 V; nebulizer, 35 psig; capillary, 3500 V; OCT RF V, 250 V; collision energy, 10 V and 30 V. The mass range was at *m*/*z* 100–1100. Acquisition and analysis of data were performed under Masshunter Workstation Software (version B.02.00).

### 2.4. Activity test of AGH

The related compounds were assessed for the AGH inhibitory activity according to the modified method [18,19]. An assay mixture composed of 10 μL of test solution and 40 μL of 0.6 U/mL AGH solution was pre-incubated at 37 °C for 30 min. After pre-incubation, the reaction was started by the addition of 100 μL of 1.0 mM *p*-NPG, and then incubated for another 30 min. The enzyme-substrate reaction was stopped by adding 50 μL of 0.2 mol/L $Na_2CO_3$ to the mixture. Phosphate buffer solution (67 mM, pH 6.8) was used as a blank control. All the reagents were dissolved in the solution of phosphate buffer (67 mM, pH 6.8). The AGH activity was calculated by testing the *p*-nitrophenol released from *p*-NPG at 405 nm using a microplate reader. Acarbose, a known AGH inhibitor, was used as the positive control. The AGH inhibition activity (%) of test sample on AGH could be calculated as

$$\text{Inhibition activity}(\%) = 100\% \times \left[\left(\frac{A_S - A_{SB}}{A_C - A_{CB}}\right)\right]$$

where $A_S$, $A_{SB}$, $A_C$ and $A_{CB}$ are the absorbance of sample, sample blank, control and control blank, respectively. All assays were done in triplicate and the inhibition percentages were the means of triplicate observations. The IC50 value for each tested sample was determined on three replicates of several concentrations. Statistical analyses and the $IC_{50}$ values were calculated using GraphPad Prism version 6.02 (GraphPad Software Inc.).

### 2.5. The segment and exposure strategy by LC-QTOF MS

HPLC separation of crude methanol extract and fractions was conducted by an Agilent 1290 UHPLC system. A 1 μL aliquot of 20 mg/mL sample was injected to HPLC and used as a control without usage of the series of segments in this study. In order to avoid the detection of high-abundance peaks, series of the segments were designed at 2.2–3.2 min, 6.0–7.0 min, 21.0–22.5 min, 30.8–33.0 min, 34.0–36.3 min, 37.0–39.5 min,