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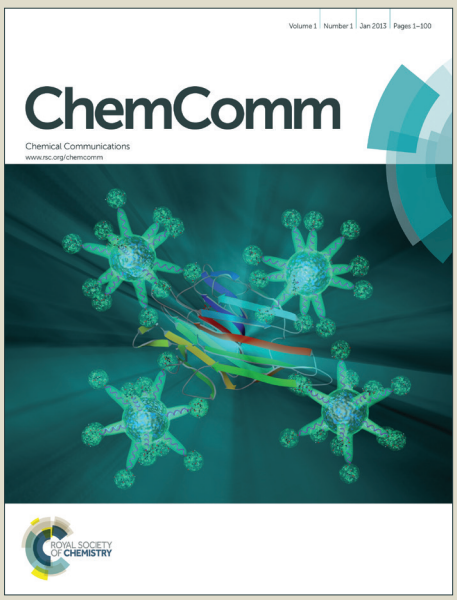

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A strategy for screening of high-quality enzyme inhibitors from herbal medicines based on ultrafiltration LC–MS and *in silico* molecular docking†

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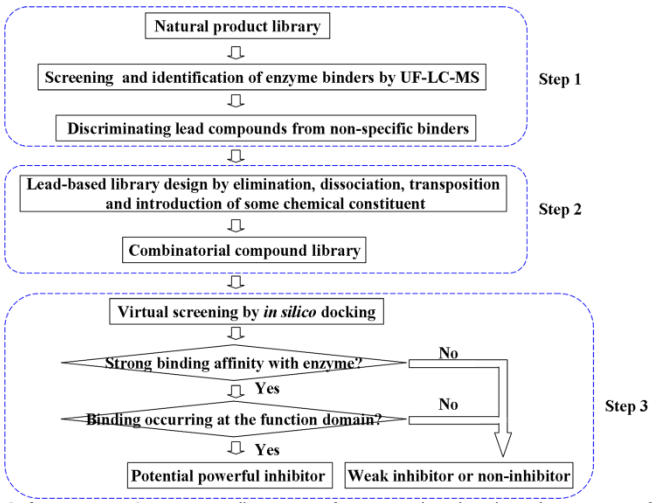
A novel strategy of ultrafiltration LC-MS and *in silico* molecular docking was proposed to discover high-quality enzyme inhibitors from herbal medicines. Using this strategy, two compounds were predicted and finally demonstrated as potent xanthine oxidase inhibitors, whose *in vitro* IC_{50} values were lower than that of a positive control allopurinol.

Screening potent bioactive compounds as drug candidates is one of the major goals of chemical and biological research. To achieve this aim, combinatorial chemistry, a newly rising technology which can construct new chemical entities by semi-synthesis, synthesis or biosynthesis, has been proposed.[1] It is believed that combinatorial chemistry will play a highly important role in multiple processes of drug discovery. However, the biggest achievements of combinatorial chemistry so far have come from the improvement of an existing lead, rather than from the discovery of an initial lead.[2] If a starting point is available and determined, it is undoubtedly that combinatorial chemistry is sufficiently powerful to produce a vast number of compounds for screening.[3] Therefore, there is now a rekindling of interest in natural products, especially herbal medicines, as a generator of leads, in which the chemical scaffolds are more varied and more drug-like than synthetic compounds.

Herbal medicines are complex mixtures containing multi-components. The conventional method of screening drug leads is to isolate chemical compounds from an herbal extract and test their bioactivity one by one. Nevertheless, the method requires complex isolation steps of the chemical compounds and professional staffs for structure identification, which are time-consuming and labor-intensive. To overcome the limitations of conventional procedures, a method of ultrafiltration liquid chromatography/mass spectrometer (UF-LC-MS) which can assess the binding of candidate molecules to target proteins in a high-throughput manner was established and developed.[4] Up to now, considerable studies have reported the successful discovery of bioactive small molecules from complex mixtures through this technique. However, a major problem of the method is the false positives caused by non-specific binding of small molecules to non-functional sites of the enzymes.[5] Although some researchers have attempted to solve this problem by introducing a known ligand or denatured macromolecules in the control experiment, the successful reports were merely restricted to a very small range of target proteins.[5,6]

Herein, a strategy based on UF-LC-MS and *in silico* molecular docking was proposed for screening high-quality enzyme inhibitors from herbal medicines. To the best of our knowledge, we firstly integrated UF-LC-MS and Autodock in the same small molecule-enzyme interaction study to search for potent enzyme inhibitors in a high-throughput way. In the integrated method, UF-LC-MS can facilitate the rapid detection and identification of binders to enzymes, and *in silico* molecular docking can predict their binding sites. By combining the two methods, small molecules binding to the active sites of enzymes with strong affinities could be screened out. To increase the chance of encountering small molecules with excellent activities, we introduce a natural product-derived combinatorial compound library from herbal medicines in the strategy. A natural compound library from herbal medicines was used to screen active components as the lead compounds, and a combinatorial library generated from the lead compounds was used to search for enzyme inhibitors with higher activities. By improving both the screening method and the screening library, the present strategy can obviously increase the possibility of discovering high-quality enzyme inhibitors.

The general procedures of our strategy for screening of high-quality enzyme inhibitors are summarized into a diagram as shown in Scheme 1. The first step of this strategy is to screen and identify small molecule binders to enzyme (xanthine oxidase) from a natural product library (Mai-Luo-Ning injection) by UF-LC-MS. Lead compounds which bind to the function domain of XOD and exhibit XOD inhibition activities can be discriminated from non-specific binders by *in silico* molecular docking and *in vitro* enzymatic activity assay. The second step is to generate a combinatorial compound library based on the lead compounds through elimination, dissociation, transposition or introduction of some chemical constitution. The third step is to conduct *in silico* docking research of the compounds generated in step 2. If an enzyme-ligand complex model generated by docking indicates that the ligand possesses strong binding affinity with enzyme and the binding occurs at the function domain, the ligand can be regarded as a potential powerful enzyme inhibitor. Otherwise, it will be considered as a weak inhibitor or a non-inhibitor. Once the potential powerful inhibitor is targeted, binding displacement experiment and enzymatic activity



Scheme 1. Summary diagram of presently developed strategy for screening of high-quality enzyme inhibitors from herbal medicines based on ultrafiltration LC–MS and *in silico* molecular docking.

assay will be performed to verify the prediction.

As an illustrative case study, xanthine oxidase (XOD) and Mai-Luo-Ning (MLN) injection were used as the experimental materials. XOD, which has the function to oxidize a wide variety of substrates such as hypoxanthine and xanthine to uric acid,[7] was employed as the target protein for screening. Numerous researches suggest that XOD is a potential target in the prevention of cardiovascular disease.[8] MLN injection is a famous herbal preparation widely used in China for nearly 30 years for the treatment of coronary disease and vascular occlusion of angeitides.[9] Enzymatic activity assay showed that the IC_{50} value of lyophilized MLN powder was 78.87 μg/ml, indicating it contained potential XOD inhibitors. Thus, MLN injection was used as the natural compound library for screening.

The principle of the UF-LC-MS screening method is described as follows. A solution of target protein is incubated with a mixture of compounds such as an herbal extract. During the incubation, ligands in the mixture are allowed to bind to the target protein. The solution is injected into an ultrafiltration cell which facilitates to separate the protein-ligand complexes from unbound compounds with low-molecular weight. Subsequently, the protein-ligand complexes are disrupted through the addition of an organic solvent, and then the released ligands are detected by LC-MS. In the present study, there were 15 components separated and detected at 254 nm in MLN injection (Fig. 1a). Four compounds (2, 12, 13 and 14) were found to be XOD binders by comparing the chromatogram with the negative control (without XOD) as shown in Fig. 1b. The above results were validated by using febuxostat, a known XOD inhibitor as the positive control, and loganin, a non-inhibitor as the negative control, which are spiked into the MLN injection before the incubation with XOD. Obviously, febuxostat was screened out from the complex mixture, and loganin was not detected (Fig. 1b), suggesting that the results obtained by ultrafiltration LC-MS were reliable.

In order to identify the binders screened from MLN injection, HPLC-Q-TOF-MS analysis and NMR experiments were conducted. By comparing retention time (t_R), characteristic fragmentation ions, UV spectra and NMR data with those of the corresponding reference compounds and the literature,[10,11] compounds 2, 12, 13 and 14 were unambiguously identified as 3-CQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, respectively (Table S1 and Table S2, ESI†).

A compound binding to the enzyme does not necessarily mean that it is an enzyme inhibitor, owning to non-specific binding of the compound to non-functional sites of the enzyme.[5,6] To discriminate XOD inhibitors from non-specific

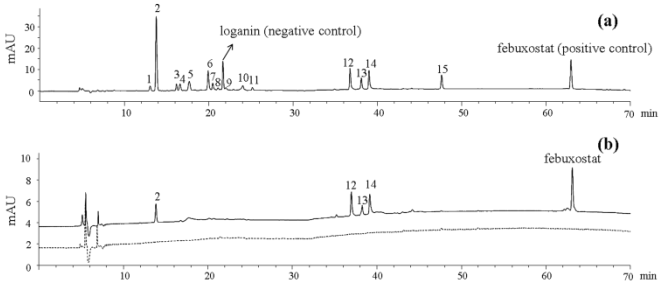


Fig. 1 (a) The chromatogram of MLN injection spiked with the positive control febuxostat and the negative control loganin monitored at 254 nm. (b) The ultrafiltration chromatograms of compounds bound to XOD (the solid line) and negative control without XOD (the dotted line) from MLN injection monitored at 254 nm.

XOD binders, an *in silico* molecular docking and an *in vitro* enzyme inhibition assay were performed. On one hand, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA inhibited XOD in a concentration-dependent manner (Fig. S1, ESI†), with IC_{50} values of 310.00, 159.03 and 68.47 μM, respectively. However, the inhibition rate of 3-CQA was merely 6.06% at concentration of 125 μM, which suggested it did not possess XOD inhibitory ability. On the other hand, the binding orientation ratios of 3,4-diCQA, 3,5-diCQA and 4,5-diCQA (50-65%) were significantly higher than that of 3-CQA (30%) (Fig. S2b, ESI†), indicating that the former three compounds were more likely to bind with the function domain of XOD than the later. Detailed information about the parameter binding orientation ratio could be seen in Section 2 of ESI†. In conclusion, all the above results demonstrated that 3,5-diCQA, 3,4-diCQA and 4,5-diCQA were XOD inhibitors and 3-CQA was a non-specific XOD binder. Thus, the three diCQAs were chosen as the lead compounds for structure optimization.

Lead-based combinatorial compound library design was performed by elimination, dissociation, transposition or introduction of some chemical constitution as shown in Fig. 2. In detail, 4-CQA (d), 3-CQA (e) and 5-CQA (f) were obtained by elimination of a caffeoyl group from the three leads a, b and c respectively. Caffeic acid (g) and quinic acid (h) were the common dissociated products of compounds d, e and f. 1,4-diCQA (i), 1,3-diCQA (j) and 1,5-diCQA (k) were obtained by transposition of the two caffeoyl groups on the quinic core. 3,4-diCQA-ME (l), 3,5-diCQA-ME (m) and 4,5-diCQA-ME (n) were obtained by introduction of a methyl group into leads a, b and c respectively. Finally, a second library containing 14 compounds was generated for screening.

To reasonably evaluate the binding of compounds in Fig. 2 to the active site of XOD, an *in silico* molecular docking was applied. Herein, we introduce two parameters of binding energy and binding orientation ratio as the evaluation criteria according to the previous experience.[5,12] The detailed discussion about docking results was shown in Section 2 of ESI†. Finally, based on the screening strategy described in Scheme 1, 3,4-diCQA-ME and 3,5-diCQA-ME were the candidate high-quality XOD inhibitors which could bind to the functional site of XOD with a high affinity, and the other CQA derivatives were weak inhibitors or non-inhibitors because of the low affinities or non-effective bindings.

To verify the docking results of 3,4-diCQA-ME and 3,5-diCQA-ME binding to the Mo-pt domain of XOD, a binding displacement experiment was performed using a recently FDA-approved drug febuxostat, which was known to bind to Mo-pt domain of XOD from the crystal structure.[13] The reason for choosing febuxostat rather than the classic inhibitor allopurinol was that the latter inhibited XOD through a complex mechanism of the transformation of allopurinol to oxypurinol,

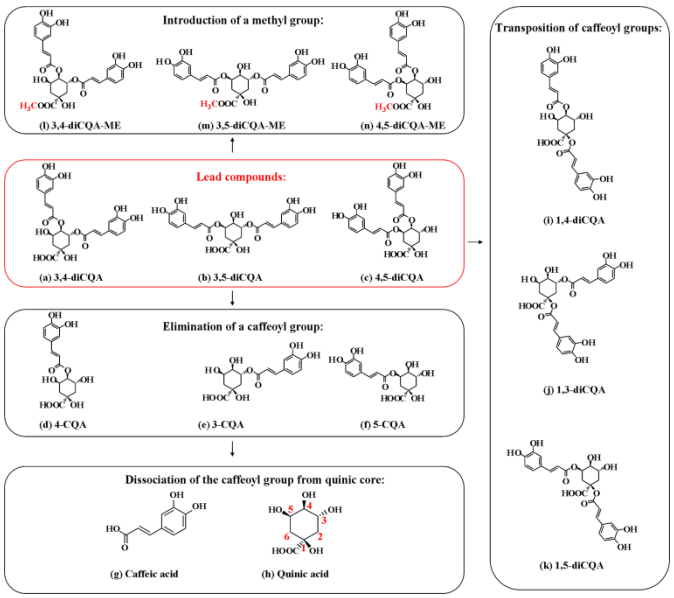


Fig. 2 The combinatorial compound library derived from three bioactive core molecules by elimination, dissociation, transposition or introduction of some chemical constitution.

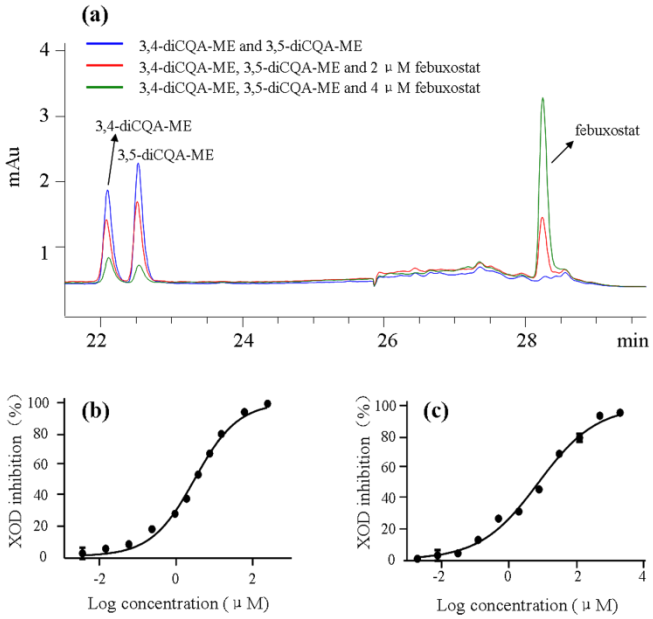


Fig. 3 (a) Determination of binding specificity of 3,4-diCQA-ME and 3,5-diCQA-ME to the function domain of XOD using a binding displacement experiment. (b) Dose-response curve of 3,4-diCQA-ME against XOD. (c) Dose-response curve of 3,5-diCQA-ME against XOD.

which was not suitable as a competitor in the binding displacement experiment. Fig. 3a shows the results obtained by titrating a binding mixture between XOD and the two diCQA-MEs with increasing concentrations of febuxostat. The peak areas of the 3,4-diCQA-ME and 3,5-diCQA-ME diminished as the concentration (peak area) of the competitor febuxostat increased, clearly suggesting that they compete with febuxostat for the Mo-pt domain of XOD.

To demonstrate that the two compounds were high-quality XOD inhibitors, we conducted *in vitro* enzymatic activity assays. As shown in Fig. 3b and Fig. 3c, they inhibited XOD in a dose-dependent manner, and their IC_{50} values were 3.16 and 7.54 μM respectively, which were even lower than that of allopurinol (IC_{50} = 8.36 μM) indicating that they were potent XOD inhibitors. To explore whether the other CQA derivatives were weak inhibitors or non-inhibitors, seven reference compounds, namely, 4-CQA, 3-CQA, 5-CQA, caffeic acid, quinic acid, 1,3-diCQA and 1,5-diCQA, were obtained to assay. Their IC_{50} values were significantly higher than those of 3,4-diCQA-ME and 3,5-diCQA-ME (Table S4, ESI†), showing that their XOD inhibitory activities were weak. The results demonstrated that the proposed strategy was useful in searching for powerful XOD inhibitors.

In conclusion, a novel strategy based on ultrafiltration LC-MS (UF-LC-MS) and *in silico* molecular docking was proposed to discover potent enzyme inhibitors from herbal medicines. Based on the strategy, two compounds 3,4-diCQA-ME and 3,5-diCQA-ME were finally identified as high-quality XOD inhibitors. The *in vitro* IC_{50} values were 3.16 and 7.54 μM respectively which were lower than that of a positive control allopurinol (IC_{50} = 8.36 μM). The proposed strategy was expected to be a universal and promising approach to discovery of powerful enzyme inhibitors.

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