Structure-based multi-ligand molecular modeling to predict the synergistic effects of limonin and obacunone from Simiao pill against nitric oxide synthase 3 associated with hyperuricemia

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Competing interests
The authors declare no conflicts of interest.

Acknowledgements
This work was funded by the Key Project of National Natural Science Foundation of China [grant number 81830117], Joint Funds of National Natural Science Foundation of China [grant number U22A0056], National Natural Science Foundation of China [grant numbers 822040209, 82274499], Guangdong Basic and Applied Basic Research Foundation [grant number 2022A1515110080], Guangdong Provincial Science Foundation [grant number 2022KJ151151], and Science & Technical Plan of Guangzhou, Guangdong, China [grant number 201903010069]. We thank the National Supercomputing Center in Wuxi for providing resources and services to enable our computational work in this study.

Peer review information
Precision Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations
-SMP: Simiao pill; HUA: hyperuricemia; NOS3, nitric oxide synthase 3; ND: nitric oxide; PAINS, pan-assay interference compounds; RMSD, root mean square deviation of backbone Ca atoms; RMSF, root mean square fluctuation.

Citation

Abstract
Hyperuricemia (HUA) mainly occurs because of purine metabolism disorders. We recently proposed that limonin from Simiao pill may have therapeutic effects on nitric oxide synthase 3 (NOS3) that is related to HUA. Concurrently, our previous work employed a new method, structure-based multi-ligand molecular modeling, to identify potential agents from a herbal formula that may produce synergistic effects and may have the potential to develop combination drugs. Therefore, we employed multi-ligand modeling to seek compounds with potential synergistic effects with limonin against NOS3. We obtained 403 multi-ligand docking results between 403 compounds and the limonin-NOS3 complex (average affinity ≈ 8.297 kcal/mol). Then we selected the top 10 highest binding affinity compounds for virtual pharmacokinetic and toxicity screening and we found that only obacunone passed all filters. We further subjected obacunone, bound to limonin and NOS3, to molecular dynamics simulations. We found that the NOS3-limonin-obacunone complex was more stable than the NOS3-limonin complex, based on the root mean square deviation of backbone Ca atoms and root mean square fluctuation, which suggests that synergistic effects may exist between limonin and obacunone. Further cell and animal experimental research is required to verify our results.

Keywords: hyperuricemia; synergistic mechanism; Chinese herbal medicine; complementary and alternative medicine; molecular docking; molecular dynamics simulations; in silico analysis
Recently, we published a paper in the *Journal of Molecular Liquids*, to investigate the therapeutic effects of compounds identified from Simiao pill (SMP) for the management of hyperuricemia (high uric acid (HUA)) [1]. HUA mainly occurs because of purine metabolism disorders [2] and the incidence of HUA is approximately 20% in men and 8% in women [3]. Although most HUA patients do not have any symptoms of HUA, it affects their quality of life since it will lead to other disruptive conditions, for example, gout, arthritis [4], kidney diseases [5], and is also a risk of cardiovascular diseases [6]. SMP is a classic Chinese herbal formula developed during the Qing dynasty (1904 C.E.) and recorded in *Convenient Reader of Established Prescriptions* [7]. It has been used for the management of HUA-related symptoms for more than 100 years and it continues to be used in clinical practice in China today [8]. In our recently published study, we found that nitric oxide synthase 3 (NOS3) is one of the most significant target proteins of SMP for HUA [1]. NOS3 is a kind of enzyme that produces nitric oxide (NO) and is involved in the regulation of vascular functions physiologically [9]. In the disordered purine metabolism, the superoxide anion formed, accompanied by uric acid synthesis, will decrease the bioavailability of NO and promote the conversion of NOS3 from a NO-synthesizing enzyme to a superoxide-generating enzyme, thus inducing vascular dysfunction [10, 11]. Therefore, searching for suitable ligands for NOS3 to maintain the normal function of vascular endothelium may be a novel approach for treating HUA-associated cardiovascular diseases. In our previous study, we also identified that limonin (HB106), which can also be found in the metabolic profiling study mentioned in our published paper, is a potential inhibitor of NOS3 [1]. Concurrently, we also published a paper regarding a new method to identify compounds which may act in synergy and that may lead to the development of novel combination drugs based on a Chinese herbal formula using a structure-based multi-ligand molecular modeling approach [12]. Therefore, we used this new method to identify potent synergistic ligands in the formula for NOS3.

We retrieved the docking results of limonin against NOS3 from our previous work and set limonin as the initial inhibitor of NOS3 (Figures 1a and 1b). Then we utilized MGLTools 1.5.6 to convert the limonin-NOS3 complex’s PDB file to the “receptor.PDBQT” files [13]. We performed multiple ligand molecular docking between all 403 compounds identified from SMP and the limonin-NOS3 complex, in order to identify compounds that may appear to exert potential synergistic effects, for example, by docking close to the limonin site. A total of 403 docking results were obtained. Docking was performed using AutoDock Vina (v1.2.3) and PyRx GUI (v0.8) on the Sunway TaihuLight supercomputer (12-cores Chinese-designed SW26010 manycore 64-bit RISC processors) [14, 15]. The binding affinities of these 403 docking results ranged from −2.818 to −12.143 kcal/mol (average affinity −8.297 kcal/mol) (Figure 1c, Supplementary Table S1). In our previous work, we found that compounds at the same binding pocket as the initial inhibitor may produce synergistic effects with the initial inhibitor [12]. Interestingly, we found that 387 compounds (96% of all compounds) were predicted to bind to the same binding pockets of limonin. The binding affinity of the 387 compounds ranged from −2.988 to −12.143 kcal/mol with an average binding affinity of −8.427 kcal/mol. We also found that 277 compounds (72% of all compounds) in the binding sites had a high binding affinity (<−7 kcal/mol). We selected the top 10 compounds with the highest binding affinity that were located at the same binding pocket of limonin. The top 10 compounds included NX133 ((−12.143 kcal/mol), NX089 ((−12.138 kcal/mol), YR032 ((−11.975 kcal/mol), NX114 ((−11.811 kcal/mol), YR021 ((−11.39 kcal/mol), HB032 ((−11.389 kcal/mol), HB041 ((−11.389 kcal/mol), HB069 ((−11.387 kcal/mol), NX138 ((−11.323 kcal/mol), and HB011 ((−11.309 kcal/mol).

To improve the success rate of developing a medication, we conducted a virtual pharmacokinetics and toxicity prediction study via several online platforms. We first used SwissADME (http://www.swissadme.ch/) to filter out pan-assay interference compounds (PAINS) as those compounds may lead to false positives in biological screening assays [16, 17]. We found that except for NX133, NX089 and NX114, all compounds passed the PAINS screening. Then the absorption, distribution, metabolism and excretion properties were further predicted via the platform. Among the 10 compounds, six compounds have low gastrointestinal absorption, including NX133, NX089, YR032, NX114, YR021 and HB069. These compounds may require further modification if they were used orally, or may require a different administration method, for example, intravenous drips. For the blood-brain barrier, only HB041 was predicted to be permeable. In addition, YR021, HB069 and NX138 received a poor solubility result. Arguably the most important set of predictions from the server is medicinal chemical properties prediction, also known as drug-likeness. The server provides a stricter criterion for drug-likeness prediction, called Leadlikeness. Among the 10 candidates, none of them passed the screening. Five compounds including NX133, NX089, NX114, HB032 and HB011 failed the test due to their molecular weight (larger than 350 g/mol). However, it should be noted that newly approved drugs with large molecular weight can be found in recent years, for example, posulma (flutolufastat F 18) and qalsoby (toferzen) [18]. Further, we used Pro Tox-II (http://tox.charite.de) to evaluate their toxicity properties. All compounds passed the hepatotoxicity screening. In terms of toxicity class prediction, except for HB032, which received a Class 2 result, indicating it may be fatal if swallowed, the nine compounds passed the toxicity test. Based on the screening and toxicity properties, we selected HB011 (obacunone) for subsequent analyses, as it passed all tests. Detailed information regarding the virtual pharmacokinetics and toxicity predictions can be found in Supplementary Table S2.

We further analyzed the docking complex of limonin-obacunone-NOS3 (Figures 1d and 1e). Our previous study reported that limonin is bound to two active site residues via hydrogen bonds, including Trp536 (3.09 Å) and Trp447 (3.12 Å). Trp366 was also identified as a key residue of NOS3. Multiple-ligand docking indicated that an H-bond was formed between obacunone and His461 with a distance of 3.01 Å. Concurrently, a total of nine different types of bonds were found between obacunone and NOS3, including pi-pi stacked (Trp447), alkyl (Val104), pi-alkyl (Trp74, Phe105 and Tyr475), pi-sigma (Trp74) and carbon-hydrogen bond (Ala446). Based on the docking result, we assumed that these 10 chemical bonds form a strong connection between obacunone and NOS3. It is interesting to find that, although there is no direct connection between obacunone and limonin, both of them were bound to the same active site residue Trp447 through different types of bonds (pi-pi stacked and H-bond, respectively). Additionally, it can be observed that the H-bond formed by obacunone was connected to chain A of NOS3 and the pi-pi stacked bond connected to chain B of NOS3. These two bonds may assist in stabilizing both chain A and chain B of the protein, which means it may assist in maintaining the position of limonin within NOS3. Therefore, considering that these ligands stay close to each other and are bound to the same residues of the protein, based on the concepts and assumptions discussed in our previous work, it is reasonable to predict that synergistic effects may occur between these two ligands for NOS3.

To see whether one limonin or obacunone species binds to NOS3 and affects the binding efficiency of the other ingredient, we conducted additional docking. Docking results indicated that obacunone binding alone to NOS3 resulted in a binding affinity of −10.3 kcal/mol; and when one limonin first binds to NOS3, the subsequent binding affinity of obacunone changes to −11.309 kcal/mol. Vice versa, when limonin binds to NOS3, its binding affinity is −10.7 kcal/mol; and when obacunone first binds to NOS3, the subsequent binding affinity of limonin changes to −10.033 kcal/mol. Limonin (when bound within the active site) causes a substantial increase in affinity (more negative) for obacunone, where the latter binds tightly to an adjacent site that further reinforces the stability of the complex. In contrast, obacunone (when docked to the active site) causes a slight decrease in the affinity (slightly less negative) of limonin. It is, therefore, more likely that obacunone acts as an effective synergistic ligand “partner” to limonin, as we have proposed,
rather than the other way around.

To further validate the synergistic effects between limonin and obacunone against NOS3, we performed an all-atom molecular dynamics simulation for the limonin-obacunone-NOS3 complex, limonin-NOS3 complex and NOS3 apo, via the SiBioLead online molecular dynamics simulation platform (https://sibiolead.com/) (GPU-based high-performance cluster system, running on Ubuntu OS, NVIDIA GeForce RTX3050 GPUs) [19]. We used all default settings recommended by the platform to perform our simulations. We analyzed the conformational stability and feasibility of root mean square deviation of backbone Cα atoms (RMSD) and root mean square fluctuation (RMSF) in a 50 ns simulation (Figure 2, Supplementary Video S1). All three systems reached equilibration at around 10 ns. For RMSD in Figure 2a, all three systems did not have major displacements beyond 0.3 nm, and only small fluctuations were observed after 10 ns, which means all three complexes were likely equilibrated. The average RMSD of NOS3 apo was 0.2 nm, whereas the NOS3-HB106-HB011 complex was 0.205 nm and the NOS3-HB106 complex was 0.178 nm. Although the NOS3-HB106 complex exhibited the lowest average RMSD, the average RMSD of the NOS3-HB106-HB011 complex was closer to NOS3 apo, which means that the NOS3-HB106-HB011 complex appeared to be more stable than the NOS3-HB106 complex. In terms of the RMSF and changes in RMSF (Figures 2b and 2c), we focused on the active binding pocket in chain B around residues Ala350 to Pro450. We observed that in these areas, the peaks of the three systems were similar to each other. Compared to the apo system, the RMSF and changes of RMSF of the NOS3-HB106-HB011 complex and NOS3-HB106 complex were lower, which means that the ligand-bound systems were more stable than the apo at the active binding pocket. Additionally, most of the peaks of the NOS3-HB106 complex were slightly higher than the NOS3-HB106-HB011 complex, which means that the synergistic system may be more stable than the single-ligand bound complex. Concurrently, for the average RMSF for all residues, the NOS3-HB106-HB011 complex is 0.119 nm, compared to the NOS3-HB106 complex (0.12 nm) and the apo system (0.113 nm), which also supported that synergistic effects may exist.

Figure 1 The binding patterns of limonin and obacunone from Simiao pill against nitric oxide synthase 3. (a & b) Binding poses of limonin and NOS3; (c) overview of Simiao pill compounds binding to limonin-NOS3 complex; (d & e) the binding poses of limonin and obacunone against NOS3. The green dash lines represent hydrogen bonds and the purple dash lines in (e) represent pi-pi stacking interactions. NOS3, nitric oxide synthase 3.
Figure 2 Structural analyses of the NOS3 apo-, NOS3-HB106-HB011 and NOS3-HB106. (a) protein RMSD; (b) RMSF; (c) Changes in RMSF. NOS3, nitric oxide synthase 3; RMSD, root mean square deviation of backbone Cα atoms; RMSF, root mean square fluctuation.

It is significant to note that even though obacunone has more binding sites and various types of chemical bonds, it did not bind to the key active site residues, including Trp366 (356), Met368 (358) and Glu 371 (361), as we mentioned in our previous published paper [1]. Also, from Supplementary Video S1 we provided, we did not observe physical displacements of the main inhibitor, limonin, due to interference from obacunone, confirming that limonin still interacts more strongly with the active site. However, there are more chemical bonds between obacunone and NOS3, which means that the biological role of obacunone may be a strong complementary ligand that provides additional stability to help limonin bind to the key residues of NOS3.

To our knowledge, synergy is a key component in traditional medicine theory since herbal practitioners, most of the time, prescribe more than one herb to patients, and therefore, synergy will occur in the complex herbal mixture [12]. This current study, on the other hand, provides additional evidence to support the synergistic effects of herbal compounds in a formula, for a specific condition, at the molecular level. Furthermore, NOS3 is an endothelial cell-derived enzyme involved in the production of nitric oxide to manipulate...
vascular smooth muscle relaxation [9]. In addition, NOS3 has been shown to affect VEGF-induced coronary angiogenesis and platelet activation, thereby simulating blood clotting [9]. Evidence has shown that uric acid increases the development of cardiovascular disorders, which may be due to NOS3 damage and endothelial dysfunction [11, 20, 21]. Notably, both limonin and obacunone are limonoids identified from Huangbai (Phellodendri Chinensis Cortex) in SMP and have been found to affect the regulation of vascular function related to NO bioavailability [22, 23]. In our research, the synergy of these two limonoids demonstrated more bioactivity with NOS3 compared to their separate actions, which partly accounts for the integrative effect of traditional herbal prescriptions. Therefore, it is essential to note that the combination of limonin and obacunone may play a role in the maintenance of NOS3’s normal function under high uric acid levels and then relieve endothelial deterioration in the human body.

In conclusion, based on the docking and dynamics simulation calculations, we propose that limonin and obacunone from SMP may have synergistic effects against NOS3. Further studies, such as cell and animal studies, are still required, to validate our in silico findings and contribute more evidence of the concurrent synergistic theory in Chinese herbal formulation.

References