Original Article

Functional and binding studies of gallic acid showing platelet aggregation inhibitory effect as a thrombin inhibitor

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Abstract

Objective: This study was devoted to identifying natural thrombin inhibitors from traditional Chinese medicine (TCM) and evaluating its biological activity in vitro and binding characteristics.

Methods: A combination strategy containing molecular docking, thrombin inhibition assay, surface plasmon resonance (SPR) and molecular dynamics simulation were applied to verify the study result.

Results: Gallic acid was confirmed as a direct thrombin inhibitor with IC50 of 9.07 μmol/L and showed a significant inhibitory effect on thrombin induced platelet aggregation. SPR-based binding studies demonstrated that gallic acid interacted with thrombin with a KD value of 8.29 μmol/L. Molecular dynamics and binding free energy analysis revealed that thrombin-gallic acid system attained equilibrium rapidly with very low fluctuations, the calculated binding free energies was –14.61 kcal/mol. Ala230, Glu232, Ser235, Gly258 and Gly260 were the main amino acid residues responsible for thrombin inhibition by gallic acid, providing a mechanistic basis for further optimization.

Conclusion: This study proved that gallic acid is a direct thrombin inhibitor with platelet aggregation inhibitory effect, which could provide a basis for the follow-up research and development for novel thrombin inhibitors.

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1. Introduction

Thrombin has become a crucial target of multiple cardiocerebrovascular diseases therapy because of its central role in coagulation cascade. Thrombin pervades in the vascular system widespread and contributes to multiple pathological processes such as stroke, thrombosis-fibrinolysis, blood clotting, cancer invasion and neurodegenerative diseases (Posma et al., 2016). To date, several thrombin inhibitors are available on the market, including argatroban, bivalirudin, lepirudin and dabigatran etexilate. However, the side effects of part of thrombin inhibitors on the market have been reported, such as hypotension, hemorrhagic events, nausea and dyspnea (Wert et al., 2018). Argatroban (Bambrah et al., 2013), ximelagatran (Hirsh, O'Donnell, & Eikelboom, 2007) and dabigatranetexilate (Thorne et al., 2014) have varying degree side effects on the liver, kidney or gastrointestinal tract. Argatroban has been suggested to reduce its dosage in patients with hepatic dysfunction (Brand, Egberts, Scholz, Weiler, & Bein, 2008). There are 7.9 % ximelagatran-taking patients whose level of transaminase is three times higher than that in normal people (Mohapatra, Tran, Gore, & Spencer, 2005). Dabigatranetexilate was reported to have side effects including gastrointestinal bleeding. Therefore, discovery of novel thrombin inhibitor, especially sourced from natural products, remains a promising research for the significant roles of thrombin in the regulation of many pathological processes.

Traditional Chinese medicines (TCM) provide a large pool of bioactive compounds, such as ginkgo acid (Yang et al., 2017), safflower yellower (Bai et al., 2020), ligustrazine (Gao et al., 2015), matrine (Zhang et al., 2019) and berberine (Feng et al., 2019), which have made significant contributions to prevent cardiovasculare and cerebrovascular diseases. This study aimed to discover direct thrombin inhibitors from TCM sourced ingredients and evaluate their biological activity and binding characteristics with the target protein using the computational and experimental method. Finally, gallic acid was identified as a direct thrombin inhibitor showing platelet aggregation inhibitory effects from a self-built reference substance solution library containing 315 TCM compounds. Gallic acid, an organic small-molecule, was well known
in TCM for the various biological activities including antimicrobial, anticancer, anti-inflammatory and antioxidant activities. According to Kang et al., gallic acid showed therapeutic effect on cardiovascular disease owing to the potent vasorelaxant and antihypertensive effect (Kang et al., 2015). Patel et al. suggest gallic acid as a favorable molecule for the treatment of myocardial damage (Patel & Goyal, 2011). In addition, gallic acid is widely distributed in various medicinal plants such as Carthamus tinctorius L., one of the main representative medicines promoting blood circulation and removing blood stasis. The relative content of gallic acid was measured in the range of 1.24–3.37 mg/g in C. tinctorius (Wu et al., 2019). Treatment with prescription is one of the most important characteristics in the clinic of TCM. The content of gallic acid sourced from Taohong Siwu Decoction, which showed potent efficacy on activating blood and dissolving stasis, was determined as 187.5–344.4 μg/g according to a recent study (Cheng et al., 2020). The content of gallic acid sourced from Yuanhu Zhitong Soft Capsule, which have good pharmacological actions such as antiplatelet aggregation and anti-inflammation, have been measured in the range of 1.24–1.26 mg/g (Li, Niu, & Dou, 2015; Du and Liu, 2014). The wide distribution and biological activities of gallic acid in medicinal plants presents a possible avenue for future inquiry on mechanism of related TCM herbs. This study provides a potential bioactive compound to be a natural thrombin inhibitor. Moreover, thrombin-gallic acid binding characteristic was investigated using SPR-based binding kinetics and molecular dynamics simulation.

2. Materials and methods

2.1. Molecular docking

In order to facilitate the subsequent experimental verification, a self-built reference substance solution library containing 315 compounds derived from traditional Chinese medicine was selected to perform virtual screening. These molecules were downloaded and converted to mol2 format in SYBYL-X 1.2 software. After being added with hydrogen atoms, the electrostatic charge of these molecules was calculated using Gasteiger-Hückel method in SYBYL X-1.2 software package. Then, each molecule was energy optimized according to the 0.05 kcal/mol Å convergence criterion for energy gradient to carry out molecular docking as initial conformations.

The B chain of thrombin crystal structure (PDB ID: 4BAH) was used as a target protein for docking (Winquist et al., 2013). After deleting the crystallographic water molecules and adding hydrogen atoms, the crystal structure of thrombin was energy minimized with AMBER7 F99 force field following default parameter settings of SYBYL-X 1.2 software. The thrombin active site within the binding pocket was defined as the inside space within 0.5 Å of the known inhibitor melagatan (MEL, CAS: 159776-70-2) (Winquist et al., 2013). In order to verify the rationality and validity of the docking program, MEL was extracted from the co-crystal structure and re-docked into the binding pocket of thrombin using the preset surfex docking program. According to the following formula (1), the difference between the co-crystallized and docked conformations of all heavy atoms in MEL could be quantified by root mean square deviation (RMSD) (X. Wang et al., 2017). The distance among N pairs of equivalent atoms other than hydrogen atoms is denoted by d. A lower RMSD value was indicative of a greater degree of overlap between co-crystalline and docked conformation of MEL.

\[
\text{RMSD} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} d_i^2}
\]  

2.2. Thrombin inhibition assay

A thrombin activity assay kit was used to test the thrombin inhibitory activity for the hit compound through virtual screening. Briefly, 40 μL of diluted standard thrombin solution (0.33 μg/mL) and 10 μL of sample (300 μmol/L, 5% DMSO) were incubated in a black, non-binding, flat-bottom 96-well plate for 10 min at 37 °C. Then add 50 μL of diluted substrate and incubated for 1 h at 37 °C. Then, fluorescence intensity was determined (excitation/emission = 490/520 nm) using EnVision Multilabel Reader (2104 PerkinElmer). Argatroban (600 μmol/L, Sigma-Aldrich) was served as positive control, while the negative control was 5% DMSO (Skelley, Kyle, & Roberts, 2016). In order to avoid false positives, the autofluorescence for each test compound (300 μmol/L) was detected, whose purity was over 98%. The biochemical 50% inhibitory concentration (IC₅₀) was calculated using GraphPad Prism V5 software.

2.3. Thrombin induced platelet aggregation assay

The antithrombin activity of the hit was evaluated by assessing its platelet aggregation capacities using a turbidimetric method. The research method has obtained approval from Ethics Committee in Capital Medical University (AEEI-2019-157). Male Sprague-Dawley rats (weighing 480–520 g) were anesthetized with 20% urethane dissolved in physiological saline solution. The blood collected by abdominal aortic method containing 109 mmol/L sodium citrate (citrate/blood = 1/9, volume percent) was collected by twice centrifugation at 800 rpm with 15 min to get platelet-rich plasma (PRP). PRP were then centrifuged for 10 min at 3000 rpm to get platelet-poor plasma. The platelets were then resuspended in Ca²⁺ free Tyrode’s buffer (214 mg/L MgCl₂·6H₂O, 200 mg/L KCl, 1000 mg/L NaHCO₃, 8000 mg/L NaCl, 65 mg/L NaH₂PO₄, 1000 mg/L D-glucose) and incubated at 37 °C for 20 min. This wash-step was repeated twice. Washed platelets (WP) were then resuspended in Tyrode’s buffer including 200 mg/L CaCl₂ at a density of 200 × 10⁶ platelets per milliliter. Briefly, 200 μL WP was co-incubated with gallic acid (12.50, 25.00 and 50.00 μmol/L), argatroban (0.33 nmol/L) or ultrapure water (blank control) at assay tube. Add 25 μL of thrombin (1U/mL) to initiate thrombin aggregation and run for 15 min at 37 °C. Four-channel platelet aggregation remote analyzer module (AggRam™, Helena Laboratories, USA) was applied in this assay. Platelet aggregation rate (%) and maximum platelet aggregation rate (Max%) were computed automatically in the system following the built-in formulas:

\[
\text{Platelet aggregation rate(%) =} \frac{\text{PointValue} - \text{InitialMax}}{\text{InitialMin} - \text{InitialMax}} \times 100\% 
\]  

\[
\text{Max(%) =} \frac{\text{MaxPCPoint} - \text{InitialMax}}{\text{InitialMin} - \text{InitialMax}} \times 100\% 
\]  

2.4. SPR-based binding assay

The BIAcore T200 system was used to detect the binding capacity of thrombin and gallic acid. Ten millimole thrombin (Sigma, pH 5.0) was immobilized on the surface of CMS sensor chip following standard amine coupling protocol. Different concentrations of gallic acid were diluted in 5% DMSO contained PBS-P (0.05% P20, pH 7.4) and flow through the chip surface at a rate of 30 μL/min at 25 °C. In this assay, amine coupled chip without immobilized thrombin was set as the blank control. The association and dissociation kinetic processes of gallic acid on thrombin were continuously monitored for 4 and 10 min, respectively. Glycine-HCl
A mixture of thrombin protein, gallic acid, ions, and water were used to construct a simulation system. To perform MD simulation, the required initial atomic coordinates of the thrombin-gallic acid complex were acquired using the SurfleX-Dock approach. The box of the simulation was generated for the simulation of the MD of gallic acid interacting with thrombin. The geometry of each ligand was optimized at the B3LYP/6-31G* level of theory and the formal charges was derived from HF/6-31G* computations using Gaussian 09 program (Frisch et al., 2016). The partial charges were generated by fitting the electrostatic potentials derived by the Gaussian program through the restrained electrostatic potential (RESP) fitting technique in Amber20 package (Case et al., 2020). To build the water box, the TIP3P water model (Zheng et al., 1997) was used for the system, and to neutralize the system, the aqueous atomic coordinates were added before performing the MD simulation. The FF4SB force field to thrombin protein and general Amber force field (GAFF) to the ligand were added using the LEaP module in the Amber20 package (Maier et al., 2015; J. Wang, Wolf, Caldwell, Kollman, & Case, 2004).

Energy minimization for the box of the simulation was carried out to achieve a starting conformation with low-energy for successive MD simulations. First of all, four-thousand steepest descent method were employed followed by six-thousand steps of the conjugate gradient method. The whole system of simulation (protein, ligand, ions, and water) was minimized, and after that, minimization of the solutes (protein and ligand) was performed. To heat the system, the Langevin thermostat was used under canonical ensemble from 0 to 310 K for 300 ps, with the force constant for the harmonic restraint was set at 10.0 kcal mol⁻¹ Å⁻². Then the system was tried to equilibrate for 10 ns under an isothermal-isobaric condition with a constant pressure of 1.0 bar. For the barostat bath, the time of relaxation was set at 2.0 ps. Consequently, the production simulation was carried out for 100 ns provided with an environment of having isothermal-isobaric conditions with periodic boundary conditions. The time step was set at 2 fs and the SHAKE algorithm was used to constrain the bonds connected with hydrogen atoms. The long-range electrostatic interactions were handled by us using a method of the particle-mesh Ewald (Darden, York, & Pedersen, 1993). For short-range interactions, the cut-off value was set at 10.0 Å.

The free energy of binding, achieved while thrombin binds to gallic acid, was measured by using molecular mechanics energies and methods such as generalized born and surface area continuum solvation (Y. Wang et al., 2015; Y. Wang et al., 2013). To explore the most important residues involved in the binding thrombin to gallic acid, the total free energy of binding was decomposed according to contributions played by each residue (i = 1, 2, . . . , 253): where ΔGbind represents the per-residue contributions, and that of ΔGbind were the contributions of the pairwise interaction of residue. The calculations were provided by the MMPBSA.py. MPI module of AMBER20 (Miller et al., 2012).

\[
\Delta G_{\text{bind}} = \sum_{i=1}^{253} \Delta G_{\text{bind}}^i = \sum_{i=1}^{253} \sum_{j=1}^{253} \Delta G_{\text{bind}}^{ij} \quad (4)
\]

2.6. Statistical method

Statistical analysis and graphical presentation were performed using GraphPad Prism V5 software. Statistical comparisons were done by one-way ANOVA followed by Dunnett’s multiple-comparison test. For all analyses, values of ***P < 0.001 were considered significant.

3. Results

3.1. Primary screening result in 53 hits from 315 natural compounds

To validate the docking program, MEL was extracted from the co-crystal structure and re-docked into the active site of thrombin. As shown in Fig. 1A, the total score of thrombin-MEL was calculated as 13.00 and the RMSD between co-crystallized and docked conformation of MEL was 1.03 Å. The result showed that the docked conformation was almost in the same position with the co-crystallized conformation, which indicated a high reliability of the docking program in reproducing the experimentally observed binding mode. His79, Asp229, Ala230, Gly258 and Gly260 were key amino acids that inhibit thrombin activity in MEL binding site. Then, a total of 315 natural compounds were screened for thrombin inhibitors with docking procedures and 53 compounds with docking scores above 6.00 were acquired (Table S1). These 53 hits were further assessed for their in vitro inhibition activity against thrombin.

3.2. In vitro evaluation of thrombin inhibitory activity

SensoLyte 520 Thrombin Activity Assay Kit was used to determine the thrombin inhibitory activity of 53 potential hits from molecular docking. In primary screening, only gallic acid reached 50% inhibition (Table S1) compared with the positive control (0.60 μmol/L argatroban). Further dose–response relationship showed that the IC₅₀ value of gallic acid in thrombin inhibition was determined to be 9.07 μmol/L, while argatroban had an IC₅₀ value of 14.07 nmol/L in a parallel test (Fig. 1D) (X. Wang et al., 2017).

3.3. Interaction analysis based molecular docking

To investigate the binding sites between thrombin and ligands, molecular docking was performed and the results showed that both argatroban (Fig. 1B) and gallic acid (Fig. 1C) had stable binding modes with thrombin. Gallic acid had four hydrogen bond interactions with the side chain of Glu232, Ser235, Gly258 and Gly260, and an σ-h interaction with the side chain of Ala230. Argatroban could form five hydrogen bond interactions with the side chain of Asp229, Ala230, Ser235, Gly258 and Gly260. Gallic acid and argatroban could both interact with thrombin through several common binding sites, Ala230, Gly258 and Gly260, which may be key residues for thrombin inhibitory activity. Compared to argatroban, gallic acid could bind to Glu232 and Ser235 in the form of intermolecular hydrogen bonds, which may be key factors affecting its binding to thrombin.

3.4. Effects on thrombin-induced platelet aggregation

Platelet aggregation assays indicated that gallic acid groups (50.00 μmol/L and 25.00 μmol/L) showed significant inhibitory effect on thrombin in vitro. Gallic acid (50.00 μmol/L) reduced about 35% aggregation effects compared with thrombin stimulated platelet group (**P < 0.001), while argatroban (0.33 nmol/L) reduced 40% rate (Fig. 2A). Aggregation curves of 1 U/mL thrombin
(blue curve) triggered washed platelet aggregation quickly, while the maximum of aggregation rate significantly reduced (Fig. 2B) in the presence of gallic acid (orange curve) and argatroban (green curve).

3.5. Binding analysis based on surface plasmon resonance biosensor

To ascertain whether the newfound thrombin inhibitor has satisfactory binding properties, the binding kinetics of thrombin and gallic acid was analyzed using a BIAcore T200 system with a CM5 chip immobilized with thrombin. Compared with argatroban \( (K_D = 53.78 \, \mu \text{mol/L}) \) reported in our previous work (Wang et al., 2017), gallic acid has a tighter bound to thrombin \( (K_D = 8.29 \, \mu \text{mol/L}) \) in a parallel test (Fig. 3).

3.6. Molecular dynamics simulations

Molecular dynamics simulation was carried out based on the initial conformations of thrombin-inhibitor complexes, which were acquired from the docking program. The molecular dynamics simulations (100 ns) of the thrombin-gallic acid system was performed through the Particle Mesh Ewald Molecular Dynamics (PMEMD) (Götz et al., 2012) module in Amber 20 software package. To explore the conformation dynamics of the system, the RMSD of the backbone atoms of thrombin was calculated with the reference of the initial structure (Fig. 4A). The results showed that the RMSD values of the system reached equilibrium rapidly (within 40 ns) with very low fluctuations (below 1 Å), indicating that gallic acid could bind to thrombin protein and stabilize the protein conformation rapidly. Considering that the system reached...
equilibrium after 40 ns, the binding free energy and free energy decomposition were calculated on the basis of the last 20-ns trajectories data. An approach of MM/GBSA was applied to calculate the binding affinities, and the calculated binding free energies ($DG_{\text{bind}}$) for the thrombin-gallic acid system was found to be $-14.61$ kcal/mol (Table 1). For the thrombin-gallic acid system, the van der Waals interactions ($DE_{\text{vdw}}$) constitutes the major component of the $DG_{\text{bind}}$, suggesting that van der Waals interactions played a vital role in the interaction among thrombin and gallic acid. Furthermore, the root mean square fluctuation (RMSF) of each amino acid residue of thrombin protein was determined to explore the binding stability of gallic acid with the thrombin (Fig. 4B). Overall, the RMSF values of key common amino acid residues (i.e., Ala230, Glu232, Ser235, Gly258 and Gly260) were found to be significantly lower than those in other regions of thrombin. MM/GBSA decomposition results of the total binding free energies per residue analysis showed that Ala230, Glu232, Ser235, Gly258 and Gly260 played critical roles in gallic acid binding to thrombin (Fig. 4C). Therefore, the above statement indicates that these amino acid residues had a strong binding force with gallic acid.

4. Discussion

The results obtained by the different experimental methods used in this study could support each other. The half inhibitory concentration (IC$_{50}$ of 9.07 µmol/L) of gallic acid in the thrombin inhibition assay was close to the dissociation rate constant value (KD of 8.29 µmol/L) in the SPR assay. In addition, the inhibition rate of 25.00 µmol/L gallic acid on thrombin activity and on platelet aggregation was 12% and 23%, respectively. The slight difference might due to the different reaction environment. Another reason is that the enzyme activity assay was detected at a single time point, while the platelet aggregation test recorded the changes over a period of time. The RMSD results of molecular dynamics

**Table 1**

| Energy components | Average energy/ (kcal mol$^{-1}$) | Standard deviation | Standard error of mean |
|-------------------|-----------------------------------|--------------------|------------------------|
| $\Delta E_{\text{vdw}}$ | $-21.55$                          | $2.65$             | $0.08$                 |
| $\Delta E_{\text{ele}}$ | $-16.06$                          | $11.62$            | $0.37$                 |
| $\Delta E_{\text{GB}}$ | $26.01$                          | $9.53$             | $0.30$                 |
| $\Delta E_{\text{SURF}}$ | $-3.01$                          | $0.26$             | $0.01$                 |
| $\Delta G_{\text{total}}$ | $23.00$                          | $9.40$             | $0.30$                 |
| $\Delta G_{\text{bind}}$ | $-14.61$                          | $3.73$             | $0.12$                 |

$E_{\text{vdw}}$ and $E_{\text{ele}}$ are the van der waals and coulomb energies. $E_{\text{GB}}$ and $E_{\text{SURF}}$ are the polar and non-polar solvation contribution calculated by solving the GB equations. $G_{\text{total}}$ is the total solvation free energy. $G_{\text{bind}}$ is the binding energy between the thrombin protein and ligands. $\Delta G_{\text{bind}} = G_{\text{bind}} - (G_{\text{pro}} + G_{\text{lig}}) = \Delta E_{\text{vdw}} + \Delta E_{\text{ele}} + \Delta G_{\text{GB}}$. 

**Fig. 3.** SPR-based binding analysis between gallic acid and thrombin. (A) Sensorgram of gallic acid and thrombin. The concentrations of gallic acid were 37.50, 18.75, 9.38, 4.69, 2.34 and 1.17 µmol/L (from top to bottom). (B) Fit curve of thrombin with different concentrations of gallic acid generated by the ‘Affinity’ module in Biacore T200 evaluation software.

**Fig. 4.** (A) RMSD of the backbone atoms of thrombin-gallic acid system. (B) RMSF values of amino acid residues in thrombin-gallic acid system. Key amino acid residues that bind to gallic acid are labelled with dashed lines. (C) MM/GBSA decomposition results of total binding free energies per residue for thrombin-gallic acid system.
simulation showed that gallic acid and thrombin reached equilibrium after 50 ns, which was consistent with the 'quick association' characteristic determined in the SPR-based binding study. The binding free energy results indicated the amino acid residues with lower RMSF values, indicating that they were more stable in the binding model between gallic acid and thrombin. Moreover, previous study has been reported that only high concentration of gallic acid could inhibit platelet aggregation induced by collagen, thrombin receptor activator peptide 6, ADP or U46619 (Chang et al., 2012; Gaspar et al., 2020). While this work showed that gallic acid could significantly inhibit thrombin-induced washed platelet aggregation at a concentration of 25.00 μmol/L. This inconsistency might due to the difference in the inducer and sample size. Our study provides a valuable supplement for investigating the activity of gallic acid in inhibiting platelet aggregation.

In this study, gallic acid was identified as a direct inhibitor of thrombin from a library containing 315 natural compounds sourced from various herbal medicine. Some herbal medicine such as *Ligusticum chuanxiong* Hort., *Carthamus tinctorius* L., *Salvia miltiorrhiza* Bunge have the effect of promoting blood circulation. All compounds in the screening database were purchased from commercial companies, and each compound has a purity over 95%. Virtual screening based on molecular docking was first used to identify potential thrombin inhibitors. A total of 53 hits with docking total score above 6.0 were further verified for biological activity (Table S1). The hit compounds are mainly sourced from *Ginkgo biloba* Linn., *Curcuma longa* Linn., *Panax ginseng* and so on. However, only *Paeonia lactiflora* Pall and *Curcuma longa* Linn are typical herbal medicine showing efficacy of promoting blood circulation and removing blood stasis. As described previously, gallic acid has a higher content in *P. lactiflora*, which indicated that the efficacy of herbal medicine is consistent with the pharmacological activity of the main chemical composition to some extent. In addition, the application of the molecular docking model has greatly improved the hit ratio and increased screening efficiency. Moreover, it is a general consensus that the effect of TCM is contributed by the integration of multiple components and multiple targets. The identification of active components and their targets could provide a basis to explain the integrated action of TCM. This study confirmed that gallic acid is a direct thrombin inhibitor with anti-platelet aggregation effect, and its binding model and dynamics characteristics were further described. The study results could help reveal the pharmacodynamic material basis of TCM that gallic acid is one of the major component.

5. Conclusion

In this work, gallic acid was identified as a direct thrombin inhibitor from TCM herbs using a systematic strategy based on the combination of computational simulation, biological verification and biophysical studies. Multiple methods were used to evaluate the antithrombin activity of gallic acid from different perspectives. Enzyme activity assay focuses on assessing the thrombin inhibition activity of gallic acid at the molecular level by calculating its IC₅₀ value. Thrombin induced platelet aggregation confirm the in vitro thrombin inhibitory effect of gallic acid at cellular level. SPR-based binding assay revealed the intermolecular binding kinetic characteristic between thrombin and gallic acid according to the calculated Kₜ value. Molecular docking was carried out to characterize the binding sites between thrombin and gallic acid in a visualized way. MD simulation and MM/GBSA calculation were carried out to estimate the free energy of the binding of gallic acid to thrombin and identify the hotspots of the protein–ligand binding. All the above studies confirmed that gallic acid is a thrombin inhibitor with platelet aggregation inhibitory effect. In addition, the binding characteristics between gallic acid and thrombin provide a valuable reference for structural optimization to improve its efficacy and selectivity in the follow-up study.

In summary, gallic acid was identified as a direct thrombin inhibitor from multiperspective point of view, which provides a basis for the development of agents targeting thrombin.

Author contributions

Y.J.Q., X.W. and Z.R.P. conceived and designed the experiments. Y.X.Z. wrote the manuscript and performed experiments. X.W. analyzed the data. Y.B.G., B.N.L and Y.L.Z. contributed to discussion and revised the manuscript. Y.T.L., H.J.N. and L.F. involved in performing experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2021.09.001.

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