Biflavones from *Ginkgo biloba* as inhibitors of human thrombin

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**ABSTRACT**

*Ginkgo Biloba* leaf extract has been widely used for the prevention and treatment of thrombosis and cardiovascular disease in both eastern and western countries, but the bioactive constituents and the underlying mechanism of anti-thrombosis have not been fully characterized. The purpose of this study was to investigate the inhibitory effects of major constituents in *Ginkgo biloba* on human thrombin, a key serine protease regulating the blood coagulation cascade and the processes of thrombosis. To this end, a fluorescence-based biochemical assay was used to assay the inhibitory effects of sixteen major constituents from *Ginkgo biloba* on human thrombin. Among all tested natural compounds, four biflavones (ginkgetin, isoginkgetin, bilobetin and amentoflavone), and five flavonoids (luteolin, apigenin, quercetin, kaempferol andisorhamnetin) were found with thrombin inhibition activity, with the IC\(_{50}\) values ranging from 8.05μM to 82.08μM. Inhibition kinetic analyses demonstrated that four biflavones were mixed inhibitors against thrombin-mediated Z-GGRAMC acetate hydrolysis, with the \( K_i \) values ranging from 4.12μM to 11.01μM. Molecular docking method showed that the four biflavones could occupy the active cavity with strong interactions of salt bridges and hydrogen bonds. In addition, mass spectrometry-based lysine labeling reactivity assay suggested that the biflavones could bind on human thrombin at exosite I rather than exosite II. All these findings suggested that the biflavones in *Ginkgo biloba* were naturally occurring inhibitors of human thrombin, and these compounds could be used as lead compounds for the development of novel thrombin inhibitors with improved efficacy and high safety profiles.

1. Introduction

It is well-known that cardiovascular disease (CVD) is the leading cause of morbidity and mortality in most countries [1,2]. Myocardial infarction, stroke and venous thromboembolism, three major clinical forms of CVD, are the top causes of CVD-associated death in the world [3]. *Ginkgo Biloba* leaf extract, one of the most commonly consumed non-prescription food supplements, has been registered as a prescription medication in many countries. EGB 761, the standardized leaf extract of *Ginkgo Biloba*, contains approximately 24% ginkgo flavonoids and 6% terpene lactones [4,5], and has been widely used for the prevention and treatment of thrombosis and cardiovascular disease. Siegel et al. found that oral administration of EGB761 (240 mg daily for 2 months) could significantly decrease atherosclerotic nanoplaque formation by 11.9 ± 2.5% (p < 0.0078) and size by 24.4 ± 8.1% (p < 0.0234) in cardiovascular high-risk patients [6]. Recently, a systematic review (2529 patients) demonstrated that combination therapy with *Ginkgo biloba* in angina showed a better therapeutic effect when compared with the routine western medicine alone [7]. In a clinical study for the treatment of acute ischemic stroke, *Ginkgo biloba* leaf extract can significantly decrease The National Institutes of Health Stroke Scale (NIHSS) score compared to a placebo group [8].

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findings clearly demonstrated that *Ginkgo biloba* leaf extract can bring multiple beneficial effects for patients with cardiovascular disease or patients at high CVD risk by promoting blood circulation, decreasing blood stasis, lowering blood lipids and improving microcirculation. However, the bioactive compounds in *Ginkgo biloba* and their underlying molecular mechanisms for the prevention and treatment of CVD diseases have not been fully characterized.

Thrombin, a crucial serine protease responsible for the proteolytic hydrolysis of many endogenous peptides and proteins, has been recognized as a key target for regulation of the blood coagulation cascade and the processes of thrombosis, as well as platelet activation and aggregation [9]. As the key enzyme catalyzing many coagulation-related reactions, thrombin has become an important clotting promoter in mammals [10]. Thrombin inhibitor therapy has been recognized as one of the most effective therapeutic options for the prevention and treatment of CVD. Direct thrombin inhibitors (DTIs), such like dabigatran, bivalirudin, argatroban, desirudin and lepirudin [11,12], display promising preventive and therapeutic effects for stroke, venous thromboembolism and acute coronary syndrome. Currently, several direct thrombin inhibitors have been approved by the Food and Drug Administration (FDA) for the prevention and treatment of CVD, such as argatroban, bivalirudin, dabigatran etexilate, etc. Although these DTIs display strong anti-thrombin activity, these drugs can cause severe side effects, such as hemorrhage, pain and cardiac arrest [13]. Therefore, it is necessary to find more promising thrombin inhibitors with strong inhibitory capability and a good safety profile.

Over the past half-century, there are increasing interest in discovering natural compounds from herbal medicines and edible plants as drug lead compounds, by considering the records of safe use of these herbal medicines during long-term clinical applications [14,15]. In fact, some natural flavonoids and polyphenols from herbs have been identified as thrombin inhibitors [16]. However, the inhibitory effects of constituents from *Ginkgo biloba* leaf extract on human thrombin have not been fully investigated. Recent studies have found that some biflavone and terpene lactones from *Ginkgo biloba* displayed strong inhibition against a panel of serine hydrolases, such as pancreatic lipase, phosphodiesterases and proteases [17–19]. Considering that the catalytic triad (Ser, His, and Glu) is highly conserved in all serine hydrolases [20], as well as the highly overlapped inhibitor spectra of mammalian serine hydrolases, *Ginkgo biloba* constituents may also serve as thrombin inhibitors and thereby regulate the blood coagulation cascade via thrombin inhibition.

In this study the inhibitory effects of major constituents in *Ginkgo biloba* on human thrombin were investigated systematically. Firstly, a fluorescence-based biochemical assay was used for highly efficient screening and characterization of the inhibition potentials of each constituent. Preliminary screening demonstrated that four major biflavones from *Ginkgo biloba* including ginkgetin (1), isoginkgetin (2), bilobetin (3), and amentoflavone (4), displayed strong inhibitory effects on human thrombin, with IC_{50} values ranging from 8.05 μM to 17.83 μM. Then, the inhibition kinetics of the four biflavones were characterized to explore their inhibitory mechanism on human thrombin. The results showed that all the four biflavones were mixed-type inhibitors against thrombin-mediated Z-GGRAMC acetate hydrolysis, with the K_{i} values of 6.32 μM, 11.01 μM, 4.12 μM, and 8.06 μM, respectively. Finally, molecular docking method was conducted to gain the microscopic insights into the interactions between these biflavones and human thrombin. The result showed that all the four biflavones could occupy the active cavity with strong interactions with Arg-221A and (or) Lys-60F via salt bridges. Further, a newly developed mass spectrometry-based lysine labeling reactivity method was used to identify the ligand-binding sites of ginkgetin on thrombin. The results showed that ginkgetin could bind to the lysine residues (Lys-107, Lys-109 or Lys-110) of exosite I while the binding to the lysine residues (Lys-236 or Lys-240) of exosite II were not observed, suggesting that the biflavones could bind to exosite I rather than exosite II. Collectively, our results demonstrated that the biflavones in *Ginkgo biloba* were naturally occurring inhibitors of human thrombin, and these compounds could be used as lead compounds for the development of novel biflavone-type thrombin inhibitors.

2. Material and methods

2.1. Chemicals and reagents

Human thrombin (Lot No. F1700752P2) was purchased from Hyphen BioMed. (France). Z-Gly-Gly-Arg-AMC acetate (Z-GGRAMC acetate) was purchased from Med Chem Express CO., Ltd. (Shanghai, China). 7-Amino-4-methylcoumarin was purchased from GL Biochem, Ltd (Shanghai, China). Dabigatran (a synthetic direct thrombin inhibitor) was purchased from Medchem Express (Shanghai, China). The flavonoids including apigenin, luteolin, genkwanin, querce tin, kaempferol, myricetin, isorhamnetin, the biflavones including ginkgetin, isoginkgetin, bilobetin, amentoflavone, sciadopitysin, and the terpene lactones including bilobalide, ginkgolide A, ginkgolide B, ginkgolide C from *Ginkgo biloba* were purchased from Chengdu Fufei De Biotech Co., Ltd. (Chengdu, China). Formaldehyde (HCHO), formaldehyde-D2 (DCDO), sodium cyanoborohydride (NaBH3CN), formic acid (FA), di-thiothreitol (DTT), iodoacetamide (IAA) chymotrypsin and other chemical reagents were purchased from Sigma (St. Louis, MO, USA). The purities of all tested natural compounds were higher than 98%. The chemical structures of sixteen major constituents from *Ginkgo biloba* and positive controls were shown in Fig. 1. The stock solution of each compound (100 mM) were prepared in DMSO and stored at 4 °C. Stock solution of Z-GGRAMC acetate (5 mM) and human thrombin (10 NIH/ ml) were prepared in DMSO and stored at ~ 20 °C. Tris buffer (50 mM, pH 8.3) was prepared using Millipore water and stored at 4 °C for further use. Millipore water (Millipore, Bedford, USA), LC grade DMSO (Tedia, USA) were used throughout.

2.2. Enzyme inhibition assays

Enzyme inhibition assays were conducted according to a previously reported method with minor modification, using Z-GGRAMC acetate as the probe substrate [19]. In brief, a typical incubation mixture with a total volume of 0.2 ml consisted of human thrombin (0.1 NIH/ml, final concentration), Tris buffer (50 mM, pH 8.3), NaCl (100 mM), BSA (2 mg/ml), and each inhibitor. Following pre-incubation at 37 °C for 10 min, the reaction was started by the addition of Z-GGRAMC acetate (50 μM, final concentration), with the final concentration of DMSO at 1% (v/v), a concentration that had no effect on catalytic activity. Z-GGRAMC acetate hydrolysis with or without inhibitor (DMSO only) was performed and the fluorescence signals of its hydrolytic metabolite (7-amino-4-methylcoumarin, AMC) were recorded by a multi-mode microplate reader (SpectraMax iD3, Molecular Devices, Austria), with an interval of 60 s. Baicalein and dabigatran were used as the positive controls [16]. The excitation wavelength of AMC was set at 380 nm, while the emission wavelength was 440 nm. The standard curve of 7-amino-4-methylcoumarin fluorescence is shown in Fig. S1. The residual activity of human thrombin was calculated by the following formula: the residual activity (%) = (the fluorescence intensity of AMC in the presence of inhibitor)/the fluorescence intensity of AMC in negative control (DMSO only) × 100%.

2.3. Inhibition kinetic analyses

The inhibition kinetic modes and the corresponding inhibition constant (K_{i}) values of ginkgetin (1), isoginkgetin (2), bilobetin (3), and amentoflavone (4), against human thrombin were investigated by performing a set of analyses in which the concentrations of both the fluorescent substrate and the inhibitor were varied. To determine the inhibition kinetic types (competitive inhibition, non-competitive inhibition, etc.) using the following formula: the residual activity (%) = (the fluorescence intensity of AMC in the presence of inhibitor)/the fluorescence intensity of AMC in negative control (DMSO only) × 100%.
inhibition, or mixed inhibition), multiple inhibitor concentrations and various concentrations of substrates were used to determine the corresponding reaction rates. The second plot of the slopes from the Lineweaver-Burk plots as a function of inhibitor was used to calculate the corresponding inhibition constant \( (K_i) \) value. The following equations for competitive inhibition Eq. (1), noncompetitive inhibition Eq. (2), or mixed inhibition Eq. (3) were used to fit all inhibition kinetic data and to calculate the \( K_i \) values [21],

\[
V = \frac{(V_{max} \cdot S)}{[K_m + (1/K_i) + S]}
\]  

(1)
\[ V = \frac{V_{\text{max}} S}{(K_m + S)(1 + I/K_i)} \]  
(2)  
\[ V = \frac{V_{\text{max}} S}{(K_m + S)(1 + I/k)} \]  
(3)

where \( V \) is the velocity of the reaction; \( V_{\text{max}} \) is the maximum velocity; \( S \) and \( I \) are the substrate and inhibitor concentrations, respectively; \( K_m \) is the Michaelis constant (substrate concentration at 0.5 \( V_{\text{max}} \)); \( K_i \) is the inhibition constant describing the affinity of the inhibitor towards the target enzyme (human thrombin).

2.4. Molecular docking simulations

The molecular docking simulation was performed using Discovery Studio (BIOVIA Discovery Studio 2016, Dassault Systèmes, San Diego, USA). The protein structure of thrombin was obtained from the Protein Data Bank (PDB ID: 4UFD) [22]. The procedure for molecular docking simulations has been described previously [21]. The active cavity was defined in a sphere centered at Ser-195. The exosite I was defined in a sphere containing key residues Lys-36, Arg-67, Arg-73, Arg-75, Try-76, Arg-77A, Lys-107, Lys-109 and Lys-110. The compounds ginkgetin (1), isoginkgetin (2), bilobetin (3), amentoflavone (4) were docked into the active site and exosite I of thrombin. The protein-ligand complexes with low binding energy and high LibDock score were taken for further analysis.

2.5. Lysine reactivity profiling by LC-MS/MS

An aliquot of 20 μg thrombin was dissolved in 200 μL labeling buffer containing 20 mM HEPES and 200 mM NaCl (pH 7.4). DMSO (4 μL) as the control group or ginkgetin (1.15 mg/μL, final concentration) as the experimental group was added and incubated with thrombin at 37 °C for 30 min. Then, the lysine reactivity profiling of thrombin was conducted as previous reported [23,24]. Briefly, 2 μL 0.6 M NaBH₃CN and 2 μL 4% CH₂O were added to the thrombin solution in sequence and kept at 37 °C for 20 min. The reaction was quenched by adding 2 μL 5 M NH₄AC. The labeled thrombin was alkylated by DTT and IAA, then subjected to digestion with chymotrypsin at 37 °C overnight. Finally, the generated peptides were desalted and lyophilized for LC-MS/MS analysis.

The peptides were analyzed by Thermo Fusion Lumos coupled with Vanquish UHPLC Systems. 0.1 μg peptides were loading onto a 200 μm × 3 cm trap column (C18, 5 μm, 130 Å) with a flowrate of 5 μL/min and separated by a 75 μm × 15 cm column (C18, 3 μm, 130 Å) with a flow rate of 300 μL/min. The gradient was set from 5 to 35% ACN in 30 min. The mass spectrometry was operated in positive ionization mode. The data were collected in a data-dependent acquisition manner using a top-speed approach with a cycle time of 3 s. The full scan was conducted by orbitrap with a resolution of 60 K and a scan range of m/z 375 to m/z 1800. The precursor ions with a charge state of 2–5 were isolated by quadrupole with an isolation window of 1.6 Da and fragmented by HCD with a normalized energy of 30%. The produced fragment ions were analyzed by orbitrap with a resolution of 15 K. Dynamic exclusion was enabled with a time of 60 s and a mass tolerance of 10 ppm.

The MS data was proceeded using MaxQuant (version 1.6.7) against the thrombin sequence with default settings unless specified. The dimethyl and methyl of lysine, oxidation of methionine and acetylation of protein N-termini were set as variable modifications and carbamidomethylation of cysteine was set as the fix modification. The digestion specification was set as chymotrypsin (LFWMY) with a max missed cleavage of 5. The labeling ratio was calculated by label-free quantification (spectral counting) as following:

\[ \text{labeling ratio} = \frac{\text{spectal counts of labeled peptides}}{\text{spectal counts of labeled and unlabeled peptides}} \]

2.6. Statistical analysis

All experiments were carried out in triplicate and the data are shown as mean ± SD. The IC₅₀ values (the concentration of inhibitor that reduces enzyme activity by 50%) were calculated by nonlinear regression using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, USA).

Fig. 2. Inhibitory effects of major constituents in G. Biloba on human thrombin at different concentrations (0 μM, 1 μM, 10 μM, 100 μM).
3. Results and discussion

3.1. The inhibitory effects of natural constituents in Ginkgo biloba against thrombin

Firstly, the inhibitory potentials of major constituents in Ginkgo biloba on human thrombin were assayed using three different inhibitors concentrations (1 μM, 10 μM and 100 μM). As shown in Fig. 2, nine Ginkgo biloba constituents inhibited thrombin-mediated Z-GGRAMC acetate hydrolysis by 50% or more at the high concentration (100 μM). The dose-dependent inhibition curves of these nine constituents on thrombin were generated using different inhibitor concentrations (Fig. 3), while baicalein (a natural flavonoid thrombin inhibitor reported previously [16]) and dabigatran (the synthetic direct thrombin inhibitor) were used as positive controls. The IC50 values of these nine constituents were evaluated and are listed in Table 1. Among all tested Ginkgo biloba constituents, four natural biflavones, including ginkgetin (1), isoginkgetin (2), bilobetin (3) and amentoflavone (4) displayed strong inhibitory effects on human thrombin, while five flavonoids displayed moderate inhibitory effects on thrombin, while the terpene lactones showed negligible inhibitory activity (Table 1, Fig. 3). The IC50 values of ginkgetin (1), isoginkgetin (2), bilobetin (3), and amentoflavone (4), were determined as 8.05 ± 1.50 μM, 13.97 ± 0.92 μM, 9.39 ± 0.51 μM, 17.83 ± 1.11 μM respectively, while the IC50 value of Baicalein (the positive control) was 36.11 ± 4.68 μM. These results suggested that the biflavones from Ginkgo biloba were strong naturally occurring thrombin inhibitors and encouraged us to further investigate the inhibition modes and constants (K) of these four biflavones on human thrombin.

Table 1
The IC50 values of the major constituents in G. Biloba on human thrombin.

<table>
<thead>
<tr>
<th>Class</th>
<th>No.</th>
<th>Constituents</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavones</td>
<td>1</td>
<td>Luteolin</td>
<td>23.90 ± 2.06</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Apigenin</td>
<td>26.80 ± 1.74</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Genkwanin</td>
<td>&gt; 100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Quercetin</td>
<td>67.73 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Myricetin</td>
<td>&gt; 100</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Kaempferol</td>
<td>31.56 ± 3.08</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Isorhamnetin</td>
<td>82.08 ± 18.05</td>
</tr>
<tr>
<td>Biflavones</td>
<td>8</td>
<td>Ginkgetin</td>
<td>8.05 ± 1.50</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Isoginkgetin</td>
<td>13.97 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Bilobetin</td>
<td>9.39 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Amentoflavone</td>
<td>17.83 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Sciadopitysin</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Terpene lactones</td>
<td>13</td>
<td>Bilobalide</td>
<td>&gt; 100</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Ginkgolide A</td>
<td>&gt; 100</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Ginkgolide B</td>
<td>&gt; 100</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Ginkgolide C</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Positive inhibitors</td>
<td>17</td>
<td>Baicalein</td>
<td>36.11 ± 4.68</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Dabigatran</td>
<td>0.016 ± 0.001</td>
</tr>
</tbody>
</table>

1 Baicalein and dabigatran were used as the positive inhibitors in this study [16].
3.2. The inhibitory behaviors of four biflavones against thrombin

Prior to inhibition kinetic analyses, time-dependent inhibition assays were performed to investigate the inhibition types of these biflavones on the proteolytic activity of thrombin. As shown in Fig. S2, the four tested biflavones with or without pre-incubation displayed similar inhibitory tendencies and IC_{50} values against thrombin-mediated Z-GGRAMC acetate hydrolysis, suggesting that these biflavones were reversible inhibitors against thrombin [21,25]. The inhibitory behavior of the four identified biflavones against thrombin-mediated Z-GGRAMC acetate hydrolysis were carefully characterized by performing a set of analyses with varying concentrations of both the fluorescent substrate and the inhibitor. As depicted in Fig. 4, Lineweaver-Burk plots demonstrated that ginkgetin (1), isoginkgetin (2), bilobetin (3), and amentoflavone (4) against thrombin-mediated Z-GGRAMC acetate hydrolysis. Right: The secondary plots from the Lineweaver-Burk plot for thrombin inhibition by ginkgetin (E), isoginkgetin (F), bilobetin (G) and amentoflavone (H). All data are shown as mean ± SD.

Fig. 4. Inhibition kinetics of four biflavones on human thrombin. Left: The Lineweaver-Burk plots of ginkgetin (A), isoginkgetin (B), bilobetin (C), and amentoflavone (D) against thrombin-mediated Z-GGRAMC acetate hydrolysis. Right: The secondary plots from the Lineweaver-Burk plot for thrombin inhibition by ginkgetin (E), isoginkgetin (F), bilobetin (G) and amentoflavone (H). All data are shown as mean ± SD.
amentoflavone (4) were mixed-type inhibitors against thrombin-mediated Z-GRGMC acetate hydrolysis. These results suggested that most of biflavones in *Ginkgo biloba* leaves, isoginkgetin (2), bilobetin (3), and amentoflavone (4), were evaluated against thrombin-mediated Z-GGRAMC acetate hydrolysis. The *K*ᵢ values of ginkgetin (1), isoginkgetin (2), bilobetin (3), and amentoflavone (4), were 6.32 μM, 11.01 μM, 4.12 μM, and 8.06 μM, respectively (Table 2).

These biflavones could strongly interact with Gly-219, Gly-216, and Glu-146 via hydrogen bonding, while isoginkgetin (2) could form hydrogen bonds with Gly-219 and Gly-193 in the active site of this enzyme. These interactions suggested that ginkgetin (1), isoginkgetin (2), bilobetin (3), and amentoflavone (4) could strongly interact with the key amino acids in the active site mainly via salt bridges and hydrogen bonding.

### 3.3. Identification of ligand-binding sites of biflavones on thrombin

It is well-known that human thrombin has multiple ligand-binding sites, including the substrate-binding site (catalytic cavity), exosite I (also called fibrinogen-binding exosite) and exosite II (also known as the heparin-binding exosite) [26]. In this study, molecular docking simulations and mass spectrometry-based lysine labeling reactivity method were performed to explore the ligand-binding sites of these biflavones on human thrombin. Firstly, a newly developed mass spectrometry-based lysine labeling reactivity method was used to identify whether or not these ligands could bind on exosite I or exosite II, by considering that exosite I and exosite II contain several lysines on the surface of this protein which could be easily labeled in water environment [27]. Once bound with the ligand, the label ratio of the lysines would decline compared to the control group. In this study, ginkgetin was used as a representative compound of the four biflavones-type thrombin inhibitors. The label ratios of the key lysines in the active site, exosite I and exosite II were shown in Table 4. As shown in Table 4, the labeling ratio of the three key lysines (Lys-107, Lys-109, and Lys-110) of exosite I declined from 80% to 0% when ginkgetin was co-incubated with human thrombin, suggesting that this region was occupied by ginkgetin. By contrast, the labeling ratios of the key lysines of exosite II (Lys-236 and Lys-240) with or without ginkgetin were similar. These findings clearly demonstrated that the biflavones could bind to exosite I rather than exosite II. Meanwhile, the docking simulations of ginkgetin, isoginkgetin, bilobetin and amentoflavone on exosite I were also conducted and the results were depicted in Fig. 6 and Figs. S3–S5. All these four biflavones could create strong interactions with Lys-110 via salt bridges, fully supported the above mentioned results.

It is worth noting that the lysine (Lys-60F) in the active site of thrombin could not be labeled under the same conditions of both the control group and the lysine labeling group, owing to that the lysine was buried deeply in the catalytic cavity. In this case, molecular docking simulations were performed to gain microscopic insight into the interactions between these biflavones-type inhibitors and the catalytic cavity of human thrombin. As shown in Fig. 5, ginkgetin (1), isoginkgetin (2), bilobetin (3) and amentoflavone (4) could be well- docked into the catalytic cavity of human thrombin. The key interactions between these four biflavones and the amino acids surrounding on the catalytic cavity of human thrombin were analyzed. As depicted in Fig. 5 and Table 3, the C-7 or C-4’’ hydroxyl group of ginkgetin (1), isoginkgetin (2), bilobetin (3) or amentoflavone (4) could strongly interact with Arg-221A or Lys-60F via a salt bridge in the active site of human thrombin. Furthermore, ginkgetin (1), bilobetin (3) and amentoflavone (4) could strongly interact with Gly-219, Gly-216, and Glu-146 via hydrogen bonding, while isoginkgetin (2) could form hydrogen bonds with Gly-219 and Gly-193 in the active site of this enzyme. These interactions clearly demonstrated that biflavones (such as bilobetin and ginkgetin) were mixed-type inhibitors against thrombin-mediated Z-GRGMC acetate hydrolysis. The *K*ᵢ values of ginkgetin (1), isoginkgetin (2), bilobetin (3), and amentoflavone (4) were 4.12 μM, 6.32 μM and 8.06 μM, implying that these biflavones display high affinity towards human thrombin. Besides the strong anti-thrombin effects of biflavones, some natural flavonoids in *Ginkgo biloba* leaves, such as luteolin, apigenin and kaempferol display moderate inhibitory effects on human thrombin, with *IC*₅₀ values ranging from 23.90 μM to 31.56 μM. Terpene lactones display negligible effects on the proteolytic activity of human thrombin. These findings clearly demonstrate that the biflavones and some natural flavonoids in *Ginkgo biloba* leaves may regulate the blood coagulation cascade via inhibition of human thrombin.

It should be noted that the ginkgolides (such as ginkgolide B and ginkgolide C) are known antagonists of the platelet-activating factor (PAF) receptor, and these compounds can strongly block platelet-activating factor (PAF)-induced platelet activation and aggregation, with the *K*ᵢ values less than 1.0 μM [29–32]. Considering that the biflavones and ginkgolides are two leading constituents in *Ginkgo biloba* leaves, these two classes may regulate the blood coagulation cascade via thrombin inhibition, while the ginkgolides can promote blood circulation via targeting PAF. In addition, *Ginkgo biloba* extract and its constituents can also bring other beneficial effects to CVD patients by lowering blood lipids or reducing blood viscosity [5, 28, 33]. Therefore, the pharmacodynamic effects of *Ginkgo biloba* constituents on the prevention and treatment of CVD should be carefully investigated from the perspective of the synergistic effects of various constituents on multiple targets.

Although the biflavones in *Ginkgo biloba* leaves may be easily modified by medicinal chemists. Notably, the biflavones can also be obtained via total synthesis [36, 37], which strongly facilitates the ability of medicinal chemists to design novel biflavone derivatives as potent thrombin inhibitors. In this study, molecular docking simulations demonstrated that the hydroxyl group at the C-7 or C-4’’ site is essential for the formation of a salt bridge
Fig. 5. A stereo view of human thrombin docked with ginkgetin (A), isoginkgetin (B), bilobetin (C), and amentoflavone (D) on the active site and 2D representation of the key interactions in the active site of human thrombin.
group within the catalytic cavity of human thrombin. Thus, to design the next generation of biflavone-type thrombin inhibitors, this key interaction between the biflavones and human thrombin should be fully considered by medicinal chemists. Moreover, considering that most natural biflavones display poor oral bioavailability and poor metabolic stability, it is necessary to optimize the pharmacokinetic properties of biflavones via chemical or formulation approaches. In the future some novel strategies should be used to improve the dissolution and/or oral bioavailability of these biflavones, which will be very helpful for enhancing the anti-thrombin effects of these biflavones in vivo.

Currently, long-term oral administration of warfarin is highly recommended for the prevention and treatment of cardiovascular disorders [38,39]. As one of the most frequently used herbs in clinic, Ginkgo biloba extract has been widely used for the prevention and adjuvant treatment of thrombosis and CVD [5,28]. Thus, the likelihood of co-administration of Ginkgo biloba extract and warfarin or other anticoagulant agents is very high, especially for the patients with cardiovascular disorders. In these cases, the beneficial or undesirable effects of the potential herb-drug interactions, as well as the consequences of Ginkgo biloba combination therapy on the circulatory system should be carefully monitored. It has been reported that some constituents in Ginkgo biloba are strong inhibitors of human P450 enzymes (such as CYP2C9, CYP3A4) [40–42], which may enhance warfarin’s effects by reducing the metabolic clearance of this anticoagulant agent. Meanwhile, the biflavones and the ginkgolides from Ginkgo biloba can also exhibit anticoagulant or antiplatelet activity via targeting thrombin and platelet-activating factor (PAF), respectively. Thus, it is easily conceivable that the use of warfarin combined with Ginkgo biloba extract may display more potent inhibitory effects on blood coagulation. Indeed, a case report has demonstrated that a 76-year-old patient experienced intracerebral bleeding, which returned to normal with the cessation of Ginkgo biloba extract intake [43], and a review summarized fifteen published case reports about Ginkgo biloba-associated bleeding effects [44]. Therefore, more attention should be paid to the possible herb-drug interactions (HDI) when Ginkgo biloba and warfarin are combined in the clinic.

4. Conclusion

In summary, this study investigated the inhibitory effects of sixteen major constituents in Ginkgo biloba on human thrombin, a crucial target regulating the blood coagulation cascade and the processes of

Table 3
The binding energy of ginkgetin (1), isoginkgetin (2), bilobetin (3) and amentoflavone (4) on different ligand binding sites of human thrombin, as well as the key interactions between each inhibitor and the amino acids surrounding the active site (H-bonds and salt bridge).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Binding energy (Kcal/mol)</th>
<th>Active site</th>
<th>Exosite I</th>
<th>H-bond</th>
<th>Salt bridge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Active site</td>
<td>Exosite I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ginkgetin</td>
<td>−234.07</td>
<td>−355.97</td>
<td>Gly-219, Gly-216, Gly146</td>
<td>Lys-60F</td>
<td></td>
</tr>
<tr>
<td>Iso(ginkgetin)</td>
<td>−293.68</td>
<td>−422.15</td>
<td>Gly-219, Gly-193</td>
<td>Arg-221A</td>
<td></td>
</tr>
<tr>
<td>Bilobetin</td>
<td>−293.58</td>
<td>−425.42</td>
<td>Gly-219, Gly-216, Gly146</td>
<td>Arg-221A, Lys-60F</td>
<td></td>
</tr>
<tr>
<td>Amentoflavone</td>
<td>−290.34</td>
<td>−533.28</td>
<td>Gly-219, Gly-216, Gly146</td>
<td>Arg-221A, Lys-60F</td>
<td></td>
</tr>
</tbody>
</table>

* Other labeling ratios of lysine residues are provided in Table S1.
inhibitory effects on human thrombin, with the IC50 values ranging from 8.05 μM to 17.83 μM. Inhibition kinetics analyses showed that these four biflavones were mixed inhibitors against thrombin-mediated Z-GRAMC acetate hydrolysis, with Ki values ranging from 4.12 μM to 11.01 μM. Docking simulations suggested that the hydroxyl groups at the C-7 and the C-4’ play key roles in thrombin inhibition by forming salt bridges. Mass spectrometry-based lysine labeling reactivity assay showed that ginkgetin could bind on human thrombin at exosite I rather than exosite II. All these findings provide new evidence for explaining the beneficial effects of *Ginkgo biloba* extract on CVD, also suggest that the biflavones could be used lead compounds for the development of biflavone-type thrombin inhibitors.

**Declaration of Competing Interest**

The authors state that no conflicts of interest exist.

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