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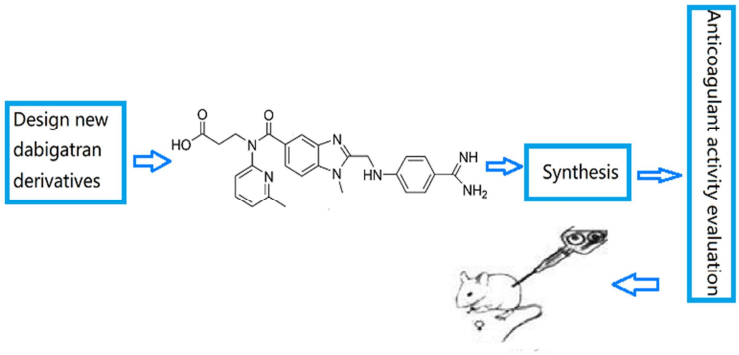

Design, Synthesis and Biological Activity Evaluation of Novel Methyl Substituted Benzimidazole Derivatives

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Abstract

Ten new dabigatran derivatives (7a–j) with high docking scoring were designed, synthesised and biologically evaluated. The inhibitory in vitro activity of these compounds on thrombin was evaluated on the basis of preliminary activity screening results. The IC_{50} values of compounds 7a, 7d and 7j were 1.92, 2.17 and 1.54 nM, respectively, and are equivalent to the dabigatran (IC_{50} = 1.20 nM). Therefore, the most active compound, 7j, was selected to further investigate the anticoagulant activity in rats. Compound 7j presented excellent in vivo inhibitory effects on arteriovenous thrombosis, and the inhibition rate was (84.19±1.14) %. The anticoagulant activity of compound 7k synthesised in the previous work was evaluated in vivo, and its inhibition rate was (85.58±2.89) %. This rate was nearly equivalent to that of dabigatran (85.07 ± 0.61) %. Results indicated that compounds 7a, 7d, 7j and 7k can be further studied as novel antithrombin drug candidates.

Keywords: Design； Synthesis； Methyl Substituted Benzimidazole； biological eva-luation，Molecular docking

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide. The World Health Organisation reported that 17.5 million people die of CVDs annually, accounting for approximately 31% of the world's death population [1]. Therefore, scientists have exerted considerable effort into researching different kinds of drugs to treat CVDs. Arterial and venous thrombotic diseases represent a major pathogenic factor of CVDs and can be fatal if improperly managed. Excessive stimulation of the coagulation cascade is closely linked to thrombotic diseases [2]. Thrombin is a multifunctional specific trypsin-like serine proteinase that is crucial in the coagulation cascade. Thrombin also plays an important role in anticoagulation processes by converting soluble fibrinogen to insoluble fibrin. Therefore, thrombin is an attractive target for CVD therapy. Antithrombotic drugs have been recognised as the best for the treatment of CVDs and their complications [3-5]. Antithrombotic drugs are mainly classified into indirect thrombin inhibitors and direct thrombin inhibitors (DTIs) [6]. Thrombin is crucial in the blood coagulation process in these inhibitors and facilitates coagulation by specifically hydrolysing fibrinogen to fibrin [7]. At present, indirect thrombin inhibitors mainly involve unfractionated heparin. However, unfractionated heparin would interfere with platelet function and raise the risk of increased bleeding due to its large molecular weight [8], These conditions would result in the gradual withdrawal of unfractionated heparin from the market. Direct thrombin inhibitors

(DTIs) (**Fig. 1**), which can directly bind to thrombin, are the most promising new anticoagulants [9], mainly include argatroban [10, 11], ximelagatran [12] and dabigatran etexilate [13]. These DTIs have been extensively studied due to their unique anticoagulant properties [14]. DTIs are a group of small molecules that can directly inhibit thrombin activity without cofactors, which can effectively inhibit free and bound thrombins to blood clots. Moreover, DTIs do not bind to plasma proteins, thereby resulting in predictable pharmacodynamic and pharmacokinetic characteristics [15]. However, the therapeutic effect of argatroban is limited because of its low oral bioavailability. Meanwhile, ximelagatran has been withdrawn from clinical development due to drug-induced severe liver damage and low oral bioavailability [16, 17]. Therefore, dabigatran etexilate has become the only oral, safe and effective DTI available on the market [18-20]. Dabigatran was initially approved in Europe and later in 2010 by the United States Food and Drug Administration (FDA) for reducing the risk of stroke in non-valvular atrial fibrillation. (AF) [21-24]. Dabigatran is orally administered as dabigatran etexilate and converted into its active form by dabigatran etexilate [25]. Monoclonal anti-idarucizumab has been recently found to reverse the anticoagulant activity induced by dabigatran by rapidly binding to the latter. This reversal can effectively resolve dyspepsia, liver damage and bleeding events [26-28]. Therefore, dabigatran etexilate currently remains a safe and effective drug for the treatment of CVDs. Over 75% of CVD deaths occurred in low and middle-income countries [1], but the high price of dabigatran etexilate imposes a heavy financial burden on patients and families. However, dabigatran etexilate efficacy is limited by poor bioavailability and the bleeding risk at high does [29]. Its bioavailability is only 6.5% [30,31]. Therefore, the structure of dabigatran could be modified to identify active candidate compounds with comparable or superior antithrombin activity and bioavailability. Finding additional dabigatran analogues may be vital to alleviate the economic burden of patients, improve bioavailability, reduce the rise of bleeding.

Hauel [32] reported that the benzamidine ring of dabigatran interacted with residues, such as Leu99, Ile174 and Trp215, in the thrombin active site cleft and formed a bidentate salt bridge with the carboxylate of residue Asp189; the pyridine ring of dabigatran is not essential for bioactivity. N-methyl is highly suitable for the P-pocket located in the active site of the enzyme, and benzamidine is docked to the D-pocket via hydrophobic interactions [33]. Guo-qiang Lin [34] obtained a novel non-peptide thrombin inhibitor by replacing the pyridine ring in dabigatran etexilate with a benzene ring. Johnson et al. [35] established that the introduction of a single methyl group with a lipophilic efficiency can effectively increase the efficacy of a compound commonly known as ‘magic methyl’. The introduced methyl group optimally occupies the lipophilic pocket on the target protein, and the van der Waals force between the ligand and the protein increases, thereby effectively increasing the binding efficiency [36-39]. Therefore, given the above research conclusions, the pyridine ring of dabigatran was replaced with a benzene ring and a methyl group was simultaneously introduced at different positions to explore a remarkably effective thrombin inhibitor as a candidate compound (**Fig. 2**).

This study employed a molecular model and a scoring technology to avoid blindness and save resources during the synthesis and predict the bioactivity of the designed compounds. Targeted compounds with a total score higher than the positive control dabigatran were selected for synthesis (Table 1). The synthesised compounds were screened by their thrombin inhibitory activities in vitro. Most of the compounds exhibit promising inhibitory activities within the range of the reference drug dabigatran. On this basis, the most active compound in vitro was further selected to complete the antithrombotic activity test in vivo. The results of the present study will lay the foundation for the design, synthesis and biological evaluation of novel dabigatran derivatives and further provide candidate compounds for new anticoagulant drugs.

2. Results and discussions

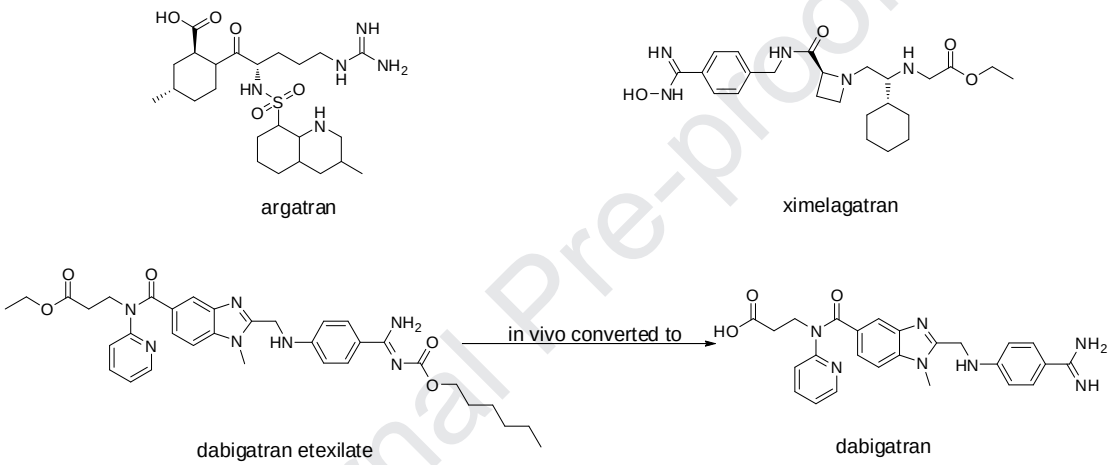


Fig.1. Chemical structures of direct thrombin inhibitors.

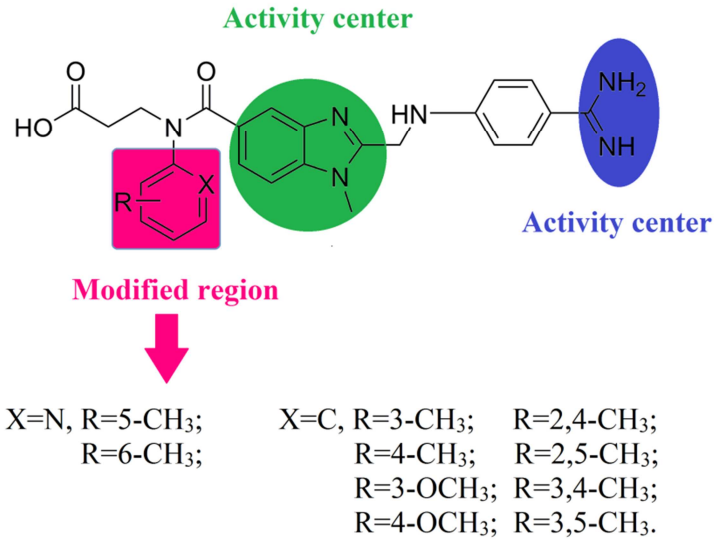


Fig. 2. Design strategy of dabigatran derivatives.

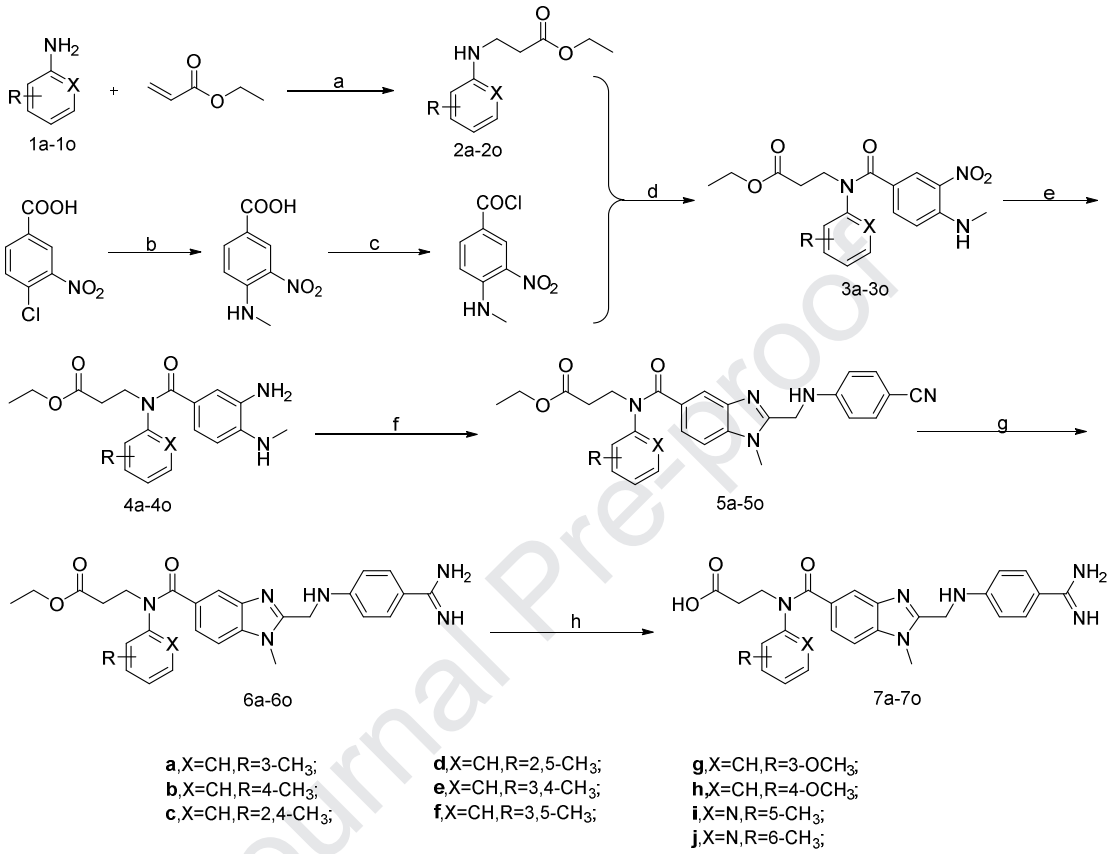
Table 1 The results obtained from docking studies of compounds **7a-j**.

Compound	Total-score	Key Residues
7a	12.3874	Asp189; Gly219; Gly216; Thr172
7b	11.2158	Asp189(2); Gly219
7c	11.7354	Asp189(2); Gly216; Thr172(2)
7d	12.8474	Asp189; Gly219; Gly216
7e	12.8667	Asp189; Gly219
7f	11.8716	Asp189; Gly219; Glu97A
7g	10.8182	Asp189(2); Gly216; Thr172
7h	10.9033	Asp189(2); Gly219; Gly216; Asn98
7i	12.4723	Asp189;Gly219; Glu217; Thr172
7j	12.5338	Asp189(2); Gly219; Gly216; Thr172
Dabigatran	10.3345	Asp189(2); Gly219; Gly216

2.1 Chemistry

The synthesis of compounds **7a–j** using a convenient synthetic route is shown in **Scheme 1**. Ten target compounds with different substituents **2a–j** were synthesised using ethyl acrylate and corresponding amines **1a–j** with trifluoromethanesulfonic acid as a catalyst at 100 °C by refluxing for 16 h. Amongst these compounds, **2i**–**j** were white solid with low yield (44%–50%), whilst **2g**–**h** were brown-red oil with high yield (77%–89%). Electron-donating methoxy-substituted aniline may be beneficial for the Michael addition reaction. Compounds **2a–j** reacted with 4-(methylamino)-3-nitrobenzoyl chloride to generate important intermediates **3a–j**. Hauel [32] used 10% Pd/C and H_2 in methanol/dichloromethane at room temperature. Meanwhile, the reductant of zinc and ammonium chloride in tetrahydrofuran with water at 80 °C was previously explored for 5 h under N_2 to reach the reduction reactions of compounds **3a–j** to **4a–j**. Zinc in acetic acid was used with water as a solvent at room temperature only for 1 h to prepare compounds **4a–j**. This method was simple and did not require any gas piping. Interestingly, the reductant can be easily recycled, and the yields were all more than 90%. This conventional reduction method of the nitro could be applied for the technical study of dabigatran. Compounds **5a–j** were prepared by treating **4a–j** with 2-[(4-cyanophenyl)-amino]-acetic acid in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 1-hydroxybenzotriazole. Cyano compounds **5a–j** with hydroxylamine hydrochloride and triethylamine under ethanol provided the corresponding hydroxamic acids by

refluxing at 80 °C for 3 h, followed by 10% Pd/C reduction under an N_2 atmosphere to obtain the amidino compounds **6a–j**. These amidino compounds were hydrolysed in the presence of sodium hydroxide in ethanol with water at room temperature for 1 h to obtain the target compounds **7a–j.** These target compounds were then characterised by ^{1}H-NMR, ^{13}C-NMR and HR-MS.



Scheme 1 Synthesis of dabigatran derivatives. Reagents and conditions: (a) TfOH, N_2, 100 °C, reflux, 12h; (b) CH_3NH_2, 75 °C, reflux, 5h; (c) DCM, $SOCl_2$, DMF, 45 °C, reflux,1h; (d) DCM, Et_3N, rt, 4h; (e) Zn, AcOH/H_2O, rt, 1h; (f) ① EDCI, HOBt, 4-cyano-2-substituted-phenylamino)-acetic acid, THF/DMF, rt, 5h; ②AcOH, 120 °C, reflux, 2.5h; (g) ① $NH_2OH\cdot HCl$, Et_3N, EtOH, 80 °C, reflux, 3h; ② $HCOONH_4$, Pd/C, AcOH, 120 °C, reflux, 5h; (h) NaOH, EtOH/H_2O, rt, 1h.

2.2 Antithrombin activity in vitro

The thrombin inhibitory activities of the synthesised dabigatran analogues **7a–j** were measured with the chromogenic assay in vitro, with dabigatran as the positive control. All these compounds exhibited more than 50% inhibition effects after preliminarily screening for inhibitory activity against thrombin at 1 μg/mL concentration (**Fig. 3**). The IC_{50} values of these compounds for thrombin inhibition are shown in **Table 2**. Most of the compounds exhibit excellent activity against thrombin in vitro, except for compounds **7g–h**. The activity data were consistent with the results predicted by molecular docking simulation.

Amongst the synthesised compounds, **7a**, **7d**, **7i** and **7j** showed the most potent thrombin inhibition with respective IC_{50} values of 1.92, 2.17, 2.58 and 1.54 nM.

These values are comparable to those of dabigatran (1.20 nM). Table 2 indicates that the positions of the electron-donating methyl group on the ring may affect the antithrombin activity. Compound **7a** with the methyl group at the *para* position exhibited lower activities relative to **7b**. Compounds **7i**–7**j** with a methyl group on the pyridine ring exhibited inhibitory activities in the following order: **7i** (5-CH_3) < **7j** (6-CH_3). Therefore, the methyl group at a position farther from the nitrogen atom in the pyridine ring may weaken antithrombin activities. When two methyl groups were placed on the ring and one methyl group was at the *para* position, the thrombin inhibitory activity of compounds **7c** and **7e** decreased to 4.62 and 4.37 nM, respectively, regardless of another methyl position. Overall, the modification of the *para* position may be detrimental to thrombin inhibitory activities. The introduction of a methoxy group to the rings of **7g** and **7h** was more detrimental than that observed in the ring of **7a**. Therefore, a strong electron-donating effect on the ring could negatively influence the antithrombin activity.

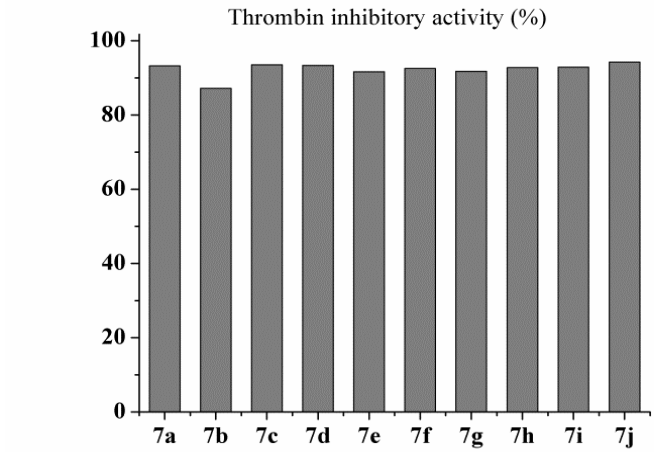


Fig. 3 Thrombin inhibitory activity of synthesized compounds at 1 μg/mL concentration

Table 2 Thrombin inhibitory activities of twelve compounds.

Compound	R	X	IC_{50}(nM)[a,b]
7a	3-CH_3	CH	1.92±0.25
7b	4-CH_3	CH	4.44±0.60
7c	2,4-CH_3	CH	4.62±0.24
7d	2,5-CH_3	CH	2.17±0.24
7e	3,4-CH_3	CH	4.37±0.48
7f	3,5-CH_3	CH	5.90±1.28
7g	3-OCH3	CH	-
7h	4-OCH3	CH	-
7i	5-CH_3	N	2.58±0.41
7j	6-CH_3	N	1.54±0.12
7k	2-CH_3	CH	2.74±0.20
Dabigatran			1.20±0.09

a) The concentrations that causes a 50% inhibition of thrombin;

b) Results were expressed as means±SD of six independent experiments.

2.3 Inhibitory effects on rat arteriovenous thrombosis in vivo

An arteriovenous thrombosis model was used to evaluate the antithrombotic activities of intravenous administration of compounds **7j** and known compound **7k**. Dabigatran served as a reference drug. Compounds **7j** and **7k** were given as a pre-treatment and exerted significant effects on rat arteriovenous thrombosis with inhibition rates of (84.19 ± 1.14) % and (85.58 ± 2.89) %, respectively. Such outcomes are comparable to those of dabigatran (85.07 ± 0.61) %. The results are shown in **Table 3**.

Table 3 Antithrombotic activities of intravenous administration of synthesized compounds

Compounds	Concentrations	Thromboembolic weight(mg)	The inhibition rate (%)[a]
CMC-Na	-	42.04±5.09	0.00±12.11
Dabigatran	0.5 mg/mL	6.28±0.26**	85.07±0.61**
7j	0.5 mg/mL	6.65±0.48**	84.19±1.14**
7k	0.5 mg/mL	6.06±1.22**	85.58±2.89**

**）$p<0.01$ versus the CMC-Na group;

a) Results were expressed as means±SD of eight independent experiments.

2.4 Computer-aided simulation

2.4.1 Activity predicted by Computer simulation

By introduction of giving-electronics substituent into the benzene ring to replace the pyridine ring of dabigatran, ten new dabigatran derivatives were designed. Molecular docking and scoring technologies were applied to discover drugs and predict the activities of these new derivatives. Targeted compounds with a total score higher than dabigatran (10.3345) were selected for synthesis. Except for compounds 7g and 7h, other compounds showed considerable antithrombin activities. It is noteworthy that the R group of compounds 7g and 7h linked to a methoxyl, which may be one of the main reasons for no activities.

2.4.2 Molecular docking analysis

To further validate the rationality of our designed compounds, the molecular docking study was performed using the Surflex-docking module in SYBYL-X 2.0. The cognate ligand (Dabigatran) and compound **7j** with good antithrombotic activity were docked into the active site of the thrombin receptor (PDB code: 1KTS), as shown in **Fig. 4**. It was found that the compound 7j we synthesized formed two significant hydrogen bond interactions with the active pocket from **Fig. 4D**. The distance of hydrogen bonds observed were 2.99Å (Glu97=O···H-N-) and 2.91 Å (Trp215-OH···O=). Compound 7j was tightly fixed around the receptor through these two hydrogen bonds. Moreover, residue His57 and Ile174 formed T-shaped π-π stacking and π- Alkyl interactions, respectively, which illustrated that the multi-interactions enhanced the degree of binding pattern. Docking results and

interactions map of Dabigatran were shown in **Fig. 4A and 4C**. Four important hydrogen bond interactions between Dabigatran and pocket were formed. The distances were 2.91 Å (Phe227=O···H-N-), 3.10Å (Phe227=O•••H-N-), 2.96Å (Cys220-S•••H-N-) and 2.82 Å (Gly219=O···H-N-), respective. Furthermore, Pi-Pi stacked, carbon hydrogen bond and Pi-Alkyl interactions also enhanced the pesticide effect of Dabigatran. Different weak interactions make the binding conformations of **7j-1KTS** and Dabigatran-**1KTS** different. Meanwhile, compounds 7g and 7k were docked into **1KTS** pocket and the results were illustrated in **Fig. S1**.

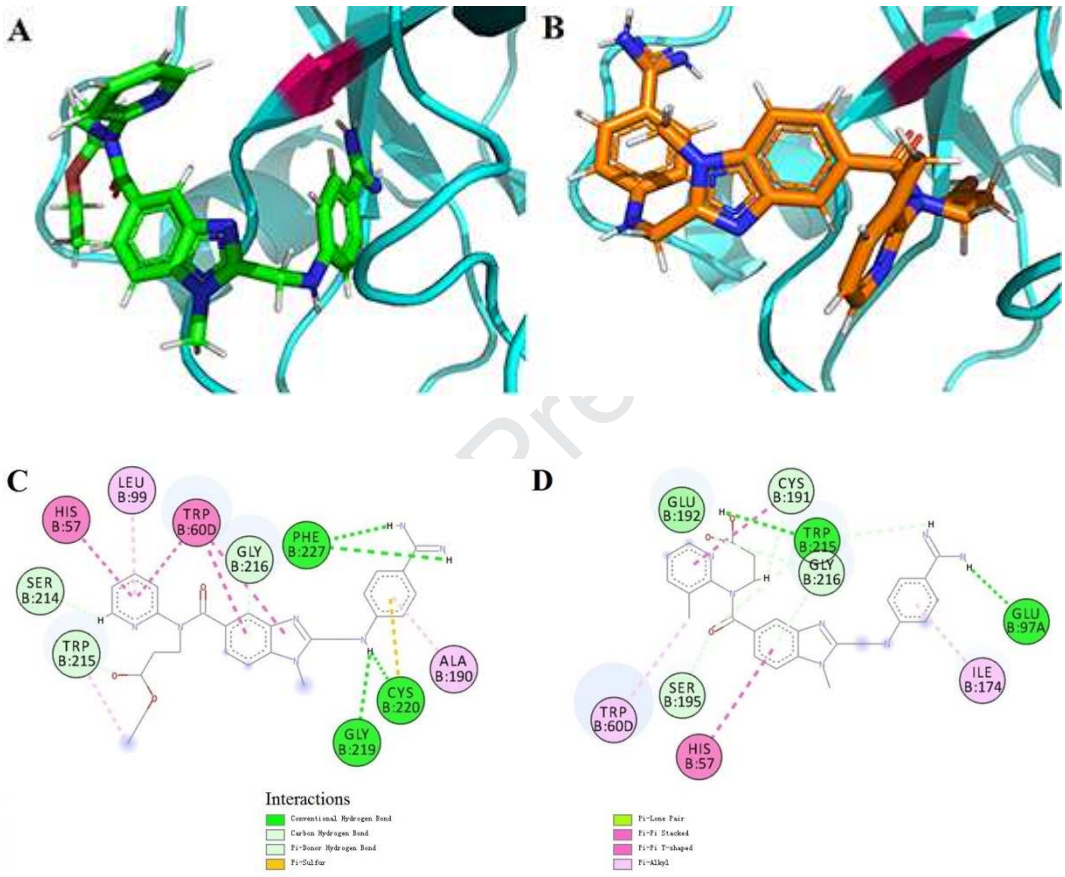


Fig. 4 Docking results and 2D interaction maps of Dabigatran (A and C) and selected compound 7j (B and D) in the binding site of thrombin receptor (PDB code: 1KTS).

Conclusion

Ten dabigatran derivatives (7a–7j) were synthesised in this paper by introducing methyl and methoxy groups at different positions in dabigatran. The thrombin inhibitory activities in vitro were then evaluated. In addition to compounds 7g and 7h, other compounds showed effective inhibitory activity against thrombin during the initial screening in vitro. The preliminary relationship between the structure and activity of these compounds revealed that the introduction of methyl groups on the benzene ring can enhance the anticoagulant activity. Moreover, the methyl group can effectively activate the docking pocket and increase the lipophilic efficiency. Comparative studies indicated that the introduction of methoxy at these positions

reduces inhibitory potency. The order of thrombin inhibition activities in vitro is as follows: compounds 7j > 7a. In particular, the IC_{50} values of compounds 7a, 7d and 7j were 1.92, 2.17 and 1.54 nM, respectively. These outcomes are comparable to the anticoagulant activity of the reference drug dabigatran. The anticoagulant activities in rats of compounds 7j and 7k were further studied. Both activities demonstrated effective in vivo activities in inhibiting arteriovenous thrombosis. The inhibition rates of compounds 7j and 7k were (84.19 ± 1.14) % and (85.58 ± 2.89) %, respectively, and these values are nearly equivalent to those of dabigatran (85.07 ± 0.61) %. Molecular docking studies were conducted to generate valuable insights into the binding of active compounds to receptors and help clarify their inhibitory mechanisms. The in vitro results were consistent with those from molecular docking. Hence, compounds 7a, 7d, 7j and 7k can be further studied as antithrombin drug candidates.

4 Experimental

4.1. Materials and methods

Unless otherwise noted, all the chemicals and solvents were purchased from Darui and Titan Corporation, and used without further purification. Reaction course was routinely monitored by thin-layer chromatography on silica gel under UV light (254 nm). The products were purified by column chromatography (200–300 mesh) with designated solvents. The melting points were measured using a WPS-2A digital melting point apparatus and were uncorrected. High-resolution mass spectra (HRMS) were obtained using a SolariX-70FT-MS Bruker spectrometer equipped with electrospray ionisation (ESI) equipment. 1H and 13C NMR spectra were obtained in $CDCl_3$ or DMSO-d_6 solution using a Bruker Avance 400 magnetic resonance spectrometer at 400 MHz, respectively, with tetramethylsilane (TMS) as the internal reference.

4.2. General procedure for preparation of compounds 2a-2j

Compounds **1a-1j**(50mmol) and ethyl acrylate **2** (6.0g, 60mmol) were mixed in a 100 mL dry round bottom flask. TfOH (5mmol) as catalyst was added. The mixture was stirred for 16h at reflux temperature in a nitrogen atmosphere. The reaction was detected by TLC. The unreacted ethyl acrylate was concentrated under reduced pressure. The residue was purified by silica gel chromatography (PE/ EtOAc = 6:1-1:1) to afford pure compounds **2a-2j** as brown red oil or white solid in 44-89% yield.

4.3. General procedure for preparation of compounds 3a-3j

4-Chloro-3-nitrobenzoic acid (9.0 g, 45 mmol) and aqueous methylamine (865 mmol) were mixed in 250 mL dry round bottom flask. The mixture was heated to reflux for 6h at 75 °C. Upon completion, PH value of the mixture was adjusted with acetic acid to 4~5. The solids were filtrated and washed carefully with water and methanol, and dried to afford yellow 4-(methylamino)-3-nitrobenzoic acid. To a DCM (50 mL) solution of 4-(methylamino)-3-nitrobenzoic acid (6.8 g, 35 mmol), five drops of DMF were added. $SOCl_2$ (20 mL, 0.23 mol) was added slowly via addition funnel. The mixture was stirred at room temperature (30°C) for 5h. The reaction was

detected by TLC. After completion of the reaction, the mixture was concentrated under reduced pressure to obtain bright yellow solid 4-methylamino-3-nitrobenzoyl chloride. To a DCM (50 mL) solution of compound **2a-2j** (35 mmol) containing triethylamine (TEA) (35 mmol), a DCM (40 mL) solution of the yellow solid chloride was slowly added at room temperature and then the mixture was stirred for 5h until completion of the reaction. Upon completion, the reaction mixture was washed with water and extracted with DCM in the separatory funnel, dried by anhydrous sodium sulfate (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel chromatography (PE/ EtOAc =3:1-1:1) to afford pure yellow solid compounds **3a-3j** in 69-82% yield.

4.4. General procedure for preparation of compounds 4a-4j

To a solution of compounds **3a-3j** (21mmol) in 100 mL acetic acid and 50 mL water was added Zn (6.6g, 105mmol). This mixture was stirred for 1h at room temperature. After completion of the reaction, the Zn was filtered, and the mixture was extracted with DCM (3×40 mL). The DCM layer was dried by anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (PE/ EtOAc =1:1) to obtain white solid compounds **4a-4j**.

4.4.1.

acid ethyl ester (4a). Yield: 94.0%; mp: 138.9-139.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 (t, *J* = 7.7 Hz, 1H, Ar-H), 6.92 (d, *J* = 7.6 Hz, 1H, Ar-H), 6.89 (s, 1H, Ar-H), 6.84 (s, 1H, Ar-H), 6.79 (d, *J* = 7.9 Hz, 1H, Ar-H), 6.70 (d, *J* = 8.3 Hz, 1H, Ar-H), 6.25 (d, *J* = 8.3 Hz, 1H, Ar-H), 4.13 (t, *J* = 7.4 Hz, 2H, -CH_2-), 4.03 (q, *J* = 7.2 Hz, 2H, -CH_2-), 2.74 (s, 3H, -NCH_3), 2.66 (t, *J* = 7.4 Hz, 2H, -CH_2-), 2.24 (s, 3H, Ar-CH_3), 1.17 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.4.2.

3-[(3-Amino-4-methylamino-benzoyl)-(4-methyl-phenyl)-amino]-propionic acid ethyl ester (4b). Yield: 92.8%; mp: 134.4-135.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.00 (d, *J* = 8.1 Hz, 2H, Ar-H), 6.91 (d, *J* = 8.2 Hz, 2H, Ar-H), 6.82 (s, 1H, Ar-H), 6.71 (d, *J* = 8.2 Hz, 1H, Ar-H), 6.26 (d, *J* = 8.3 Hz, 1H, Ar-H), 4.12 (t, *J* = 7.4 Hz, 2H, -CH_2-), 4.03 (q, *J* = 7.1 Hz, 2H, -CH_2-), 2.75 (s, 3H, -NCH_3), 2.65 (t, *J* = 7.4 Hz, 2H, -CH_2-), 2.25 (s, 3H, Ar-CH_3), 1.17 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.4.3.

3-[(3-Amino-4-methylamino-benzoyl)-(2,4-dimethyl-phenyl)-amino]-propionic acid ethyl ester (4c). Yield: 94.9%; mp: 121.7-122.8 °C. 1H NMR (400 MHz, $CDCl_3$) δ 6.92 (s, 1H, Ar-H), 6.89 (m, 2H, Ar-H), 6.83 (s, 1H, Ar-H), 6.66 (d, J = 7.7 Hz, 1H, Ar-H), 6.24 (d, J = 8.1 Hz, 1H, Ar-H), 4.28 (m, 1H, -CH2-), 4.03 (q, J = 7.2 Hz, 2H, -CH2-), 3.75 (m, 1H, -CH2-), 2.75 (s, 3H, -NCH3), 2.68 (d, J = 15.0, 6.9 Hz, 2H, -CH2-), 2.24 (s, 3H, Ar-CH3), 2.11 (s, 3H, Ar-CH3), 1.18 (t, J = 7.1 Hz, 3H, -CH3).

4.4.4.

3-[(3-Amino-4-methylamino-benzoyl)-(2,5-dimethyl-phenyl)-amino]-propionic acid ethyl ester (4d). Yield: 97.0%; mp: 125.6-126.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (d, *J* = 7.6 Hz, 1H, Ar-H), 6.91 (d, *J* = 7.9 Hz, 1H, Ar-H), 6.86 (d, *J* = 7.0 Hz, 2H, Ar-H), 6.65 (d, *J* = 7.7 Hz, 1H, Ar-H), 6.23 (d, *J* = 8.0 Hz, 1H, Ar-H), 4.23 (d, *J* = 13.7, 7.0 Hz, 1H, -CH_2-), 4.03 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.82(m, 1H, -CH_2-), 2.75 (s, 3H, -NCH_3), 2.69 (t, *J* = 7.3 Hz, 2H, -CH_2-), 2.23 (s, 3H, Ar-CH_3), 2.07 (s, 3H, Ar-CH_3), 1.18 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.4.5.

3-[(3-Amino-4-methylamino-benzoyl)-(3,4-dimethyl-phenyl)-amino]-propionic acid ethyl ester (4e). Yield: 96.3%; mp: 126.9-128.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.93 (d, *J* = 8.0 Hz, 1H, Ar-H), 6.85 (s, 2H, Ar-H), 6.76 – 6.72 (m, 1H, Ar-H), 6.71 (d, *J* = 2.1 Hz, 1H, Ar-H), 6.27 (d, *J* = 8.3 Hz, 1H, Ar-H), 4.10 (t, *J* = 7.5 Hz, 2H, -CH_2-), 4.03 (q, *J* = 7.1 Hz, 2H, -CH_2-), 2.75 (s, 3H, -NCH_3), 2.65 (t, *J* = 7.5 Hz, 2H, -CH_2-), 2.16 (s, 3H, Ar-CH_3), 2.15 (s, 3H, Ar-CH_3), 1.18 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.4.6.

3-[(3-Amino-4-methylamino-benzoyl)-(3,5-dimethyl-phenyl)-amino]-propionic acid ethyl ester (4f). Yield: 91.7%; mp: 134.6-135.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.87 (s, 1H, Ar-H), 6.75 (s, 1H, Ar-H), 6.72 (d, *J* = 8.2 Hz, 1H, Ar-H), 6.65 (s, 2H, Ar-H), 6.27 (d, *J* = 8.3 Hz, 1H, Ar-H), 4.10 (t, *J* = 7.5 Hz, 2H, -CH_2-), 4.04 (q, *J* = 7.1 Hz, 2H, -CH_2-), 2.76 (s, 3H, -NCH_3), 2.65 (t, *J* = 7.5 Hz, 2H, -CH_2-), 2.19 (s, 6H, Ar-CH_3), 1.18 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.4.7.

3-[(3-Amino-4-methylamino-benzoyl)-(3-methoxy-phenyl)-amino]-propionic acid ethyl ester (4g). Yield: 92.8%; mp: 144.8-145.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (t, J = 8.3 Hz, 1H, Ar-H), 6.83 (s, 1H, Ar-H), 6.75 (d, J = 6.4 Hz, 1H, Ar-H), 6.66 (d, J = 8.7 Hz, 1H, Ar-H), 6.61 (d, J = 7.0 Hz, 2H, Ar-H), 6.28 (d, J = 8.3 Hz, 1H, Ar-H), 4.14 (t, J = 7.4 Hz, 2H, -CH_2-), 4.04 (q, J = 7.1 Hz, 2H, -CH2-), 3.68 (s, 3H, -OCH_3), 2.76 (s, 3H, -NCH_3), 2.67 (t, J = 7.4 Hz, 2H, -CH_2-), 1.18 (t, J = 7.1 Hz, 3H, -CH_3).

4.4.8.

3-[(3-Amino-4-methylamino-benzoyl)-(4-methoxy-phenyl)-amino]-propionic acid ethyl ester (4h). Yield: 97.1%; mp: 112.9-114.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.93 (d, *J* = 8.7 Hz, 2H, Ar-H), 6.79 (s, 1H, Ar-H), 6.71 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.65 (d, *J* = 6.8 Hz, 1H, Ar-H), 6.22 (d, *J* = 8.3 Hz, 1H, Ar-H), 4.07 (t, *J* = 7.3 Hz, 2H, -CH_2-), 4.01 (d, *J* = 14.8, 7.5 Hz, 2H, -CH_2-), 3.70 (s, 3H, -OCH_3), 2.70 (s, 3H, -NCH_3), 2.61 (t, *J* = 7.4 Hz, 2H, -CH_2-), 1.15 (t, *J* = 7.0 Hz, 3H, -CH_3).

4.4.9.

3-[(3-Amino-4-methylamino-benzoyl)-(5-methyl-pyridin-2-yl)-amino]-propionic acid ethyl ester (4i). Yield: 96.0%; mp: 114.5-115.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (s, 1H, Py-H), 7.17 (d, *J* = 8.2 Hz, 1H, Py-H), 6.80 (s, 1H, Ar-H), 6.70 (d, *J* = 9.4 Hz, 1H, Ar-H), 6.59 (d, *J* = 8.2 Hz, 1H, Py-H), 6.28 (d, *J*

= 8.2 Hz, 1H, Ar-H), 4.30 (t, *J* = 7.4 Hz, 2H, -CH_2-), 4.02 (q, *J* = 7.1 Hz, 2H, -CH_2-), 2.77 (s, 3H, -NCH_3), 2.70 (t, *J* = 7.3 Hz, 2H, -CH_2-), 2.23 (s, 3H, Py-CH_3), 1.17 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.4.10.

3-[(3-Amino-4-methylamino-benzoyl)-(6-methyl-pyridin-2-yl)-amino]-propionic acid ethyl ester (4j). Yield: 97.4%; mp: 119.6-120.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 (t, *J* = 7.7 Hz, 1H, Py-H), 6.78 (d, *J* = 7.5 Hz, 1H, Py-H), 6.75 (s, 1H, , Ar-H), 6.62 (d, *J* = 8.2 Hz, 1H, Ar-H), 6.39 (d, *J* = 7.9 Hz, 1H, Py-H), 6.20 (d, *J* = 8.3 Hz, 1H, Ar-H), 4.28 (t, *J* = 7.3 Hz, 2H, -CH2-), 3.97 (q, *J* = 7.1 Hz, 2H, -CH2-), 2.68 (s, 3H, -NCH_3), 2.65 (d, *J* = 7.4 Hz, 2H, -CH2-), 2.44 (s, 3H, Py-CH_3), 1.12 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.4.11.

3-[(3-Amino-4-methylamino-benzoyl)-(2-methyl-phenyl)-amino]-propionic acid ethyl ester (4k). Yield: 92.2%; mp: 138.3-139.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (d, J = 8.2 Hz, 3H, Ar-H), 7.00 (s, 1H, Ar-H), 6.75 (s, 1H, Ar-H), 6.58 (d, J = 7.5 Hz, 1H, Ar-H), 6.14 (d, J = 7.7 Hz, 1H, Ar-H), 3.98 (q, J = 7.1 Hz, 2H, -CH_2-), 2.64 (s, 3H, -NCH_3), 2.11 (s, 3H, Ar-CH_3), 1.13 (t, J = 7.1 Hz, 3H, -CH_3).

4.5. General procedure for preparation of compounds 5a-5j

To a solution of (4-cyano-2-substitutedphenylamino)-acetic acid (3.3g, 19 mmol), HOBt (2.5g, 19 mmol) and EDCI (3.6g, 19 mmol) in 70 mL tetrahydrofuran (THF) and 10 mL DMF was stirred under the ice baths. After 30 min, the compounds 4a-4o (16mmol) were dissolved in 40 mL tetrahydrofuran (THF), and slowly added into this system. The mixture was stirred at room temperature for 5h. The solvent was concentrated and extracted with DCM (3×40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. To solution of the residue, 40 mL acetic acid was added. The mixture was heated to reflux for 2.5h at 120 °C. After cooled to room temperature, the acetic acid was removed under reduced pressure, and the residue was neutralized to PH=7-8 with concentrated aqueous ammonia ($NH_3 \cdot H_2O$). Then the mixture was extracted with DCM (3×40 mL). The DCM layer was dried by anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (DCM/ MeOH=100:1) to obtain white solid compounds 5a-5j.

4.5.1.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3-methyl-phenyl)-amino)-propionic acid ethyl ester (5a). Yield: 61.8%; mp: 206.7-207.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H, Ar-H), 7.42 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.26 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.04 (s, 1H, Ar-H), 7.00 (m, 1H, Ar-H), 6.88 (d, *J* = 6.9 Hz, 1H, Ar-H, s, *J* = 6.9 Hz, 1H, Ar-H), 6.77 (d, *J* = 7.8 Hz, 1H, Ar-H), 6.68 (d, *J* = 8.6 Hz, 2H, Ar-H), 4.43 (d, *J* = 4.5 Hz, 2H, -CH_2-), 4.19 (t, *J* = 7.4 Hz, 2H, -CH_2-), 4.05 (q, *J* = 7.1 Hz, 2H,

-CH_2-), 3.64 (s, 3H, -NCH_3), 2.69 (t, *J* = 7.4 Hz, 2H, -CH_2-), 2.20 (s, 3H, Ar-CH_3), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.2.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(4-methyl-phenyl)-amino)-propionic acid ethyl ester (5b). Yield: 61.2%; mp: 199.8-200.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (s, 1H, Ar-H), 7.42 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.25 (d, *J* = 10.0 Hz, 2H, Ar-H), 7.04 (d, *J* = 8.5 Hz, 1H, Ar-H), 6.95 (d, *J* = 8.2 Hz, 2H, Ar-H), 6.90 (d, *J* = 8.2 Hz, 2H, Ar-H), 6.69 (d, *J* = 8.5 Hz, 2H, Ar-H), 4.44 (d, *J* = 4.5 Hz, 2H, -CH_2-), 4.18 (t, *J* = 7.3 Hz, 2H, -CH_2-), 4.05 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.64 (s, 3H, -NCH_3), 2.68 (t, *J* = 7.3 Hz, 2H, -CH_2-), 2.19 (s, 3H, Ar-CH_3), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.3.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2, 4-dimethyl-phenyl)-amino)-propionic acid ethyl ester (5c). Yield: 79.3%; mp: 199.8-201.2 °C; 1H NMR (400 MHz, $CDCl_3$) δ 7.59 (s, 1H, Ar-H), 7.42 (d, J = 8.5 Hz, 2H, Ar-H), 7.26 (d, J = 8.4 Hz, 1H, Ar-H), 7.03 (d, J = 8.4 Hz, 1H, Ar-H), 6.89 (d, J = 7.8 Hz, 1H, Ar-H), 6.86 (s, 1H, Ar-H), 6.82 (d, J = 7.8 Hz, 1H), 6.68 (d, J = 8.5 Hz, 2H, Ar-H), 4.42 (d, J = 3.9 Hz, 2H, -CH2-), 4.05 (q, J = 7.0 Hz, 2H, -CH2-), 3.76 (d, J = 15.8, 8.5 Hz, 2H, -CH2-), 3.63 (s, 3H, -NCH3), 2.71 (dd, J = 15.9, 9.0 Hz, 2H, -CH2-), 2.17 (s, 3H, Ar-CH3), 2.14 (s, 3H, Ar-CH3), 1.19 (t, J = 7.1 Hz, 3H, -CH3).

4.5.4.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2, 5-dimethyl-phenyl)-amino)-propionic acid ethyl ester (5d). Yield: 51.7%; mp: 201.7-202.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 1H, Ar-H), 7.42 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.27 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.02 (d, *J* = 8.5 Hz, 1H, Ar-H), 6.93 (d, *J* = 7.9 Hz, 1H, Ar-H), 6.86 (d, *J* = 6.9 Hz, 2H), 6.68 (d, *J* = 8.5 Hz, 1H, Ar-H), 4.43 (d, *J* = 4.3 Hz, 2H, -CH_2-), 4.34 (m, 1H, -CH_2-), 4.05 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.85 (m, 1H, -CH2-), 3.63 (s, 3H, -NCH_3), 2.73 (t, *J* = 7.0 Hz, 2H, -CH_2-), 2.17 (s, 3H, Ar-CH_3), 2.10 (s, 3H, Ar-CH_3), 1.20 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.5.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3, 4-dimethyl-phenyl)-amino)-propionic acid ethyl ester (5e). Yield: 63.8%; mp: 193.7-194.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (s, 1H, Ar-H), 7.42 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.28 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.04 (d, *J* = 8.5 Hz, 1H, Ar-H), 6.86 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.69 (d, *J* = 8.5 Hz, 3H, Ar-H), 4.44 (d, *J* = 4.5 Hz, 2H, -CH_2-), 4.17 (t, *J* = 7.5 Hz, 2H, -CH_2-), 4.05 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.65 (s, 3H, -NCH_3), 2.68 (t, *J* = 7.4 Hz, 2H, -CH_2-), 2.10 (s, 6H, Ar-CH_3), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.6.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3, 5-dimethyl-phenyl)-amino)-propionic acid ethyl ester (5f). Yield: 43.7%; mp: 188.9-190.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (s, 1H, Ar-H),

7.42 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.28 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.04 (d, *J* = 8.5 Hz, 1H, Ar-H), 6.86 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.85 (s, 1H, Ar-H), 6.69 (d, *J* = 8.4 Hz, 2H, Ar-H), 4.44 (d, *J* = 4.4 Hz, 2H, -CH_2-), 4.17 (t, *J* = 7.4 Hz, 2H, -CH_2-), 4.05 (q, *J* = 7.2 Hz, 2H, -CH_2-), 3.65 (s, 3H, -NCH_3), 2.68 (t, *J* = 7.4 Hz, 2H, -CH_2-), 2.10 (s, 6H, Ar-CH_3), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.7.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3-methoxy-phenyl)-amino)-propionic acid ethyl ester (5g). Yield: 80.9%; mp: 206.7-207.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (s, 1H, Ar-H), 7.42 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.28 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.04 (m, 2H, Ar-H), 6.68 (d, *J* = 8.3 Hz, 2H), 6.60 (m, 3H, Ar-H, Ar-H, Ar-H), 4.43 (d, *J* = 4.4 Hz, 2H, -CH_2-), 4.20 (t, *J* = 7.3 Hz, 2H, -CH_2-), 4.06 (q, *J* = 7.2 Hz, 2H, -CH_2-), 3.64 (s, 6H, -OCH_3, -NCH_3), 2.70 (t, *J* = 7.3 Hz, 2H, -CH_2-), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.8.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(4-methoxy-phenyl)-amino)-propionic acid ethyl ester (5h). Yield: 77.4%; mp: 198.6-199.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (s, 1H), 7.40 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.22 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.01 (d, *J* = 8.5 Hz, 1H, Ar-H), 6.93 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.67 (t, *J* = 7.8 Hz, 4H, Ar-H, Ar-H), 4.42 (d, *J* = 4.5 Hz, 2H, -CH_2-), 4.16 (t, *J* = 7.3 Hz, 2H, -CH_2-), 4.05 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.66 (s, 3H, -OCH_3), 3.62 (s, 3H, -NCH_3), 2.67 (t, *J* = 7.3 Hz, 2H, -CH_2-), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.9.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(5-methyl-pyridin-2-yl)-propionic acid ethyl ester (5i). Yield: 66.6%; mp: 188.6-189.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (s, 1H, Py-H), 7.66 (s, 1H), 7.41 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.23 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.11 (d, *J* = 6.4 Hz, 1H, Ar-H), 7.04 (d, *J* = 8.4 Hz, 1H, Py-H), 6.69 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.57 (d, *J* = 8.1 Hz, 1H, Py-H), 4.46 (d, *J* = 4.5 Hz, 2H, -CH2-), 4.35 (t, *J* = 7.3 Hz, 2H, -CH_2-), 4.04 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.66 (s, 3H, -NCH_3), 2.74 (t, *J* = 7.2 Hz, 2H, -CH_2-), 2.19 (s, 3H, Py-CH_3), 1.18 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.10.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(6-methyl-pyridin-2-yl)-propionic acid ethyl ester (5j). Yield: 62.5%; mp: 199.9-201.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H), 7.41 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.23 (d, *J* = 5.8 Hz, 1H, Ar-H), 7.15 (t, *J* = 7.8 Hz, 1H, Py-H), 7.04 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.81 (d, *J* = 7.5 Hz, 1H, Py-H), 6.69 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.40 (d, *J* = 7.9 Hz, 1H, Py-H), 4.46 (d, *J* = 4.5 Hz, 2H, -CH_2-), 4.39 (t, *J* = 7.3 Hz, 2H, -CH_2-), 4.04 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.67 (s, 3H, -NCH_3), 2.76 (t, *J* = 7.3 Hz, 2H, -CH_2-), 2.47 (s, 3H, Py-CH_3), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.5.11.

3-({2-[(4-Cyano-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2-methyl-phenyl)-amino)-propionic acid ethyl ester (5k). Yield: 45.0%; mp: 202.4-203.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H, Ar-H), 7.42 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.26 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.24 (t, 1H, Ar-H), 7.03 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.00(t, 1H, Ar-H), 6.88 (d, *J* = 6.9 Hz, 2H, Ar-H), 6.77 (d, *J* = 7.8 Hz, 1H, Ar-H) , 6.68 (d, *J* = 8.6 Hz, 2H, Ar-H), 4.43 (d, *J* = 4.5 Hz, 2H, -CH_2-), 4.19 (t, *J* = 7.4 Hz, 2H, -CH_2-), 4.05 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.64 (s, 3H, -NCH_3), 2.69 (t, *J* = 7.4 Hz, 2H, -CH_2-), 2.20 (s, 3H, Ar-CH_3), 1.19 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6. General procedure for preparation of compounds 6a-6j

Compounds **5a-5j** (10mmol), hydroxylamine hydrochloride (1.4g, 20mmol) and trimethylamine (Et_3N) (20 mmol) were mixed in 20mL ethanol. The mixture was refluxed for 3h at 80 °C. Ethanol was then evaporated under reduced pressure. To a acetic acid (HOAC) (20 mL) solution of the residue, ammonium formate (1.9g, 30mmol) and 10% Pd/C (1.9g) were added with stirring. The mixture was heated to reflux for 5h at 120 °C under a nitrogen atmosphere. After completion of the reaction, the contents were cooled. The solution was evaporated under vacuum, then the residue was purified by silica gel chromatography (DCM/ MeOH=7:1), recrystallized from ethyl acetate to obtain white solid compounds **6a-6j**.

4.6.1.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3-methyl-phenyl)-amino]-propionic acid ethyl ester (6a). Yield: 78.3%; mp: 188.9-190.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.58 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.45 (s, 1H, Ar-H), 7.33 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.28 (t, *J* = 5.5 Hz, 1H, Ar-H), 7.16 (d, *J* = 8.6 Hz, 1H, Ar-H), 7.05 (s, 1H, Ar-H), 7.03 (d, *J* = 7.9 Hz, 1H, Ar-H), 6.91 (d, *J* = 7.7 Hz, 1H, Ar-H), 6.81 (d, *J* = 8.7 Hz, 2H, Ar-H), 4.58 (d, *J* = 5.4 Hz, 2H, -CH_2-), 4.02 (t, *J* = 7.2 Hz, 2H, -CH_2-), 3.95 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.71 (s, 3H, -NCH_3), 2.55 (t, *J* = 7.1 Hz, 2H, -CH_2-), 2.16 (s, 3H, Ar-CH_3), 1.10 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6.2.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(4-methyl-phenyl)-amino]-propionic acid ethyl ester (6b). Yield: 72.1%; mp: 197.8-198.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.59 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.44 (s, 1H, Ar-H), 7.32 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.14 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.00 (s, 4H, Ar-H), 6.81 (d, *J* = 8.9 Hz, 2H, Ar-H), 4.58 (d, *J* = 5.4 Hz, 2H,- CH_2-), 4.01 (t, *J* = 7.2 Hz, 2H,-CH_2-), 3.95 (q, *J* = 7.1 Hz, 2H,-CH_2-), 3.71 (s, 3H, -NCH_3), 2.54 (t, *J* = 7.2 Hz, 2H, -CH_2-), 2.14 (s, 3H, Ar-CH_3), 1.67 (s, 2H, -NH_2), 1.10 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6.3.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2, 4-dimethyl-phenyl)-amino]-propionic acid ethyl ester (6c). Yield: 84.5%; mp: 155.5-156.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.62

(d, *J* = 8.6 Hz, 2H, Ar-H), 7.45 (s, 1H, Ar-H), 7.33 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.16 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.05 (s, 1H, Ar-H), 7.03 (d, *J* = 7.9 Hz, 1H, Ar-H), 6.90 (d, *J* = 7.5 Hz, 1H, Ar-H), 6.82 (d, *J* = 8.6 Hz, 2H, Ar-H), 4.59 (d, *J* = 5.2 Hz, 2H, -CH_2-), 4.02 (t, *J* = 7.1 Hz, 2H, -CH_2-), 3.95 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.72 (s, 3H, -NCH_3), 3.32 (s, 3H, Ar-CH_3), 2.56 (t, *J* = 7.1 Hz, 2H, -CH_2-), 2.16 (s, 3H, Ar-CH_3), 1.10 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6.4.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2, 5-dimethyl-phenyl)-amino]-propionic acid ethyl ester (6d). Yield: 78.9%; mp: 165.5-166.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.60 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.39 (s, 1H, Ar-H), 7.32 (d, *J* = 7.6 Hz, 1H, Ar-H), 7.18 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.07 (s, 1H), 6.94 (d, *J* = 7.7 Hz, 1H, Ar-H), 6.88 (d, *J* = 7.5 Hz, 1H, Ar-H), 6.80 (d, *J* = 8.4 Hz, 2H, Ar-H), 4.57 (d, *J* = 4.6 Hz, 2H, -CH_2-), 3.95 (d, *J* = 13.9, 7.0 Hz, 2H, -CH_2-), 3.83 – 3.50 (m, 5H, -NCH3, -CH_2-), 2.60 (t, 2H, -CH_2-), 2.15 (s, 3H, Ar-CH_3), 1.99 (s, 3H, Ar-CH_3), 1.10 (t, *J* = 6.9 Hz, 3H, -CH_3).

4.6.5.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3, 4-dimethyl-phenyl)-amino]-propionic acid ethyl ester (6e). Yield: 77.4%; mp: 226.5-227.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.57 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.46 (s, 1H, Ar-H), 7.33 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.16 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.01 (s, 1H, Ar-H), 6.91 (d, *J* = 8.0 Hz, 1H, Ar-H), 6.80 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.75 (d, *J* = 7.5 Hz, 1H, Ar-H), 4.57 (d, *J* = 5.3 Hz, 2H, -CH_2-), 4.00 (t, *J* = 5.3 Hz, 2H, -CH2-), 3.95 (q, J = 12.5, 5.4 Hz, 2H, -CH2-), 3.71 (s, 3H, -NCH3), 2.54 (t, J = 7.2 Hz, 2H, -CH2-), 2.07 (s, 3H, Ar-CH3), 2.05 (s, 3H, Ar-CH3), 1.10 (t, J = 7.1 Hz, 3H, -CH3).

4.6.6.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3, 5-dimethyl-phenyl)-amino]-propionic acid ethyl ester (6f). Yield: 79.5%; mp: 215.7-216.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.63 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.47 (s, 1H, Ar-H), 7.34 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.18 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.82 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.76 (s, 2H, Ar-H), 6.74 (s, 1H, Ar-H), 4.59 (d, *J* = 5.3 Hz, 2H, -CH2-), 3.96 (dt, *J* = 14.5, 7.2 Hz, 4H, -CH_2-), 3.72 (s, 3H, -NCH_3), 2.55 (t, *J* = 7.1 Hz, 2H, -CH_2-), 2.08 (s, 6H, Ar-CH_3), 1.10 (t, *J* = 7.0 Hz, 3H, -CH_3).

4.6.7.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3-methoxy-phenyl)-amino]-propionic acid ethyl ester (6g). Yield: 77.9%; mp: 231.5-232.1 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.57 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.47 (s, 1H, Ar-H), 7.34 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.18 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.07 (t, *J* = 8.1 Hz, 1H, Ar-H), 6.81 (s, 1H, Ar-H), 6.78 (d, *J* = 4.5 Hz, 2H, Ar-H), 6.64 (d, *J* = 8.8 Hz, 1H, Ar-H), 4.57 (d, *J* = 5.4 Hz, 2H, -CH_2-), 4.05 (t, *J* = 7.1 Hz, 2H, -CH_2-), 3.96 (q, *J* = 7.1 Hz, 2H, -CH_2-),

3.71 (s, 3H, -OCH_3), 2.56 (t, *J* = 7.1 Hz, 2H, -CH_2-), 1.67 (s, 3H, -NCH_3), 1.10 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6.8.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(4-methoxy-phenyl)-amino]-propionic acid ethyl ester (6h). Yield: 80.0%; mp: 229.6-230.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.60 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.44 (s, 1H, Ar-H), 7.32 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.13 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.05 (d, *J* = 8.8 Hz, 2H, Ar-H), 6.81 (d, *J* = 8.8 Hz, 2H, Ar-H), 6.74 (d, *J* = 8.8 Hz, 2H, Ar-H), 4.58 (d, *J* = 5.4 Hz, 2H, -CH_2-), 4.00 (t, *J* = 5.7 Hz, 2H, -CH_2-), 3.96 (q, 2H, -CH_2-), 3.71 (s, 3H, -OCH_3), 3.61 (s, 3H, -NCH_3), 2.54 (t, *J* = 7.1 Hz, 2H, -CH_2-), 1.10 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6.9.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(5-methyl-pyridin-2-yl)-amino]-propionic acid ethyl ester (6i). Yield: 71.5%; mp: 200.5-201.4 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.93 (s, 1H, Py-H), 12.32 (d, *J* = 8.8 Hz, 2H, Ar-H), 12.19 (s, 1H, Ar-H), 12.09 (dd, *J* = 11.5, 5.1 Hz, 2H, Ar-H), 11.86 (d, *J* = 8.4 Hz, 1H, Py-H), 11.55 (d, *J* = 8.8 Hz, 2H, Ar-H), 11.51 (d, *J* = 8.1 Hz, 1H, Py-H), 9.34 (d, *J* = 5.4 Hz, 2H, -CH_2-), 8.89 (t, *J* = 7.1 Hz, 2H, -CH_2-), 8.69 (q, *J* = 7.1 Hz, 2H, -CH2-), 8.48 (s, 3H, -NCH_3), 7.37 (t, *J* = 7.1 Hz, 2H, -CH_2-), 6.40 (s, 3H, Py-CH_3), 5.84 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6.10.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(6-methyl-pyridin-2-yl)-amino]-propionic acid ethyl ester (6j). Yield: 75.8%; mp: 197.7-198.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.59 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.44 (s, 1H, Ar-H), 7.34 (d, *J* = 8.8 Hz, 1H, Ar-H), 7.26 (t, *J* = 5.4 Hz, 1H, Py-H), 7.11 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.94 (d, *J* = 7.6 Hz, 1H, Py-H), 6.81 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.55 (d, *J* = 7.9 Hz, 1H, Py-H), 4.60 (d, *J* = 5.3 Hz, 2H, -CH_2-), 4.17 (t, *J* = 7.0 Hz, 2H, -CH_2-), 3.94 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.73 (s, 3H, -NCH_3), 2.63 (t, *J* = 7.0 Hz, 2H, -CH_2-), 2.37 (s, 3H, Py-CH_3), 1.08 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.6.11.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2-methyl-phenyl)-amino]-propionic acid ethyl ester (6k). Yield: 71.4%; mp: 191.9-192.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.58 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.45 (s, 1H, Ar-H), 7.33 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.28 (t, *J* = 5.5 Hz, 1H, Ar-H), 7.16 (d, *J* = 8.6 Hz, 1H, Ar-H), 7.05 (s, 1H, Ar-H), 7.03 (d, *J* = 7.9 Hz, 1H, Ar-H), 6.91 (d, *J* = 7.7 Hz, 1H, Ar-H), 6.81 (d, *J* = 8.7 Hz, 2H, Ar-H), 4.58 (d, *J* = 5.4 Hz, 2H, -CH_2-), 4.02 (t, *J* = 7.2 Hz, 2H, -CH_2-), 3.95 (q, *J* = 7.1 Hz, 2H, -CH_2-), 3.71 (s, 3H, -NCH_3), 2.55 (t, *J* = 7.1 Hz, 2H, -CH_2-), 2.16 (s, 3H, Ar-CH_3), 1.10 (t, *J* = 7.1 Hz, 3H, -CH_3).

4.7. General procedure for preparation of compounds 7a-7j

The compounds **6a-6j** (1.0 mmol) were dissolved in 5 mL ethanol, then a solution of sodium hydroxide(160mg, 4mmol) in 2 mL H_2O was added into this

system. The mixture was stirred at room temperature for 1h. After completion of the reaction, the reaction solution was neutralized to PH=6-7 with diluted acetic acid. The solids were filtrated and washed carefully with water and methanol, and dried to afford compounds **7a-7j** as white solid. Purities of these target compounds have been tested.

4.7.1.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3-methyl-phenyl)-amino]-propionic acid (7a). Yield: 77.5%; mp: 246.5-247.1 °C; Purity: 96.8% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.91 (s, 1H, -OH), 8.66 (s, 1H, =NH), 7.66 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.51 (s, 1H, Ar-H), 7.39 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.22 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.11 (s, 1H, Ar-H), 7.07 (d, *J* = 7.7 Hz, 1H, Ar-H), 6.95 (d, *J* = 7.5 Hz, 1H, Ar-H), 6.87 (d, *J* = 8.7 Hz, 3H, Ar-H), 4.65 (s, 2H, -CH_2-), 4.02 (t, *J* = 7.2 Hz, 2H, -CH_2-), 3.79 (d, *J* = 14.6 Hz, 3H, -NCH_3), 2.55 (t, *J* = 7.5 Hz, 2H, -CH_2-), 2.20 (s, 3H, Ar-CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 172.56, 169.84, 164.33, 164.25, 164.18, 164.10, 153.07, 152.94, 143.25, 138.62, 136.40, 129.98, 129.62, 128.81, 127.99, 127.28, 125.27, 123.10, 118.94, 111.71, 109.46, 46.39, 32.11, 30.02, 20.79. ESI-HRMS: calcd for $C_{27}H_{28}N_6O_3$ $[M+H]^+$, 485.2256, found 485.2300.

4.7.2.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(4-methyl-phenyl)-amino]-propionic acid (7b). Yield: 85.8%; mp: 253.0-254.5 °C; Purity: 97.0% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (s, 1H, -OH), 8.65 (s, 1H, =NH), 7.67 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.52 (s, 1H, Ar-H), 7.43 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.22 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.04 (d, *J* = 8.8 Hz, 4H, Ar-H), 6.88 (d, *J* = 8.3 Hz, 2H, Ar-H), 4.69 (s, 2H, -CH_2-), 4.02 (t, *J* = 7.1 Hz, 2H, -CH_2-), 3.79 (s, 3H, -NCH_3), 2.54 (d, *J* = 7.2 Hz, 2H, -CH_2-), 2.18 (s, 3H, Ar-CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 172.53, 169.29, 164.33, 164.24, 164.17, 164.09, 153.20, 152.63, 140.34, 136.02, 135.09, 131.78, 129.74, 129.66, 127.77, 113.83, 111.93, 110.52, 46.23, 32.04, 30.61, 20.40, 18.44. ESI-HRMS: calcd for $C_{27}H_{28}N_6O_3$ $[M+H]^+$, 485.2256, found 485.2325.

4.7.3.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2, 4-dimethyl-phenyl)-amino]-propionic acid (7c). Yield: 83.6%; mp: 239.9-241.1 °C; Purity: 98.8% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (s, 1H, -OH), 8.75 (s, 1H, =NH), 7.70 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.50 (s, 2H, Ar-H), 7.28 (d, *J* = 8.4 Hz, 1H, Ar-H), 7.13 (d, *J* = 7.7 Hz, 1H, Ar-H), 6.94 (s, 3H, Ar-H), 6.89 (d, *J* = 8.5 Hz, 2H, Ar-H), 4.75 (s, 2H, -CH_2-), 4.22(m, 2H, -CH_2-), 3.82 (s, 3H, -NCH_3), 2.57 (dd, *J* = 13.4, 6.7 Hz, 2H, -CH_2-), 2.15 (s, 3H, Ar-CH_3), 2.10 (s, 3H, Ar-CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 172.51, 168.65, 164.15, 164.08, 164.00, 153.39, 152.13, 138.17, 137.32, 134.38, 134.07, 133.29, 131.81, 129.73, 129.37, 128.58, 127.55, 125.36,

114.55, 113.60, 112.29, 112.22, 112.14, 45.22, 31.69, 31.55, 20.37, 17.32. ESI-HRMS: calcd for $C_{28}H_{30}N_6O_3$ $[M+H]^+$, 499.2412, found 499.2447.

4.7.4.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2, 5-dimethyl-phenyl)-amino]-propionic acid (7d). Yield: 81.3%; mp: 247.9-249.0 °C; Purity: 98.4% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.07 (s, 1H, -OH), 8.82 (s, 1H, =NH), 7.73 (d, *J* = 7.7 Hz, 3H, Ar-H), 7.60 (s, 1H, Ar-H), 7.42 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.15 (s, 1H, Ar-H), 7.00 (d, *J* = 7.6 Hz, 1H, Ar-H), 6.93 (t, *J* = 8.4 Hz, 3H, Ar-H), 4.92 (s, 2H, -CH_2-), 4.20(m, 2H, -CH_2-), 3.91 (s, 3H, -NCH_3), 2.60 (t, *J* = 7.3 Hz, 2H, -CH_2-), 2.18 (s, 3H, Ar-CH_3), 2.06 (s, 3H, Ar-CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 173.10, 169.69, 164.77, 164.69, 164.62, 153.77, 153.00, 141.54, 136.74, 135.34, 134.37, 132.44, 131.94, 131.49, 130.18, 129.12, 124.74, 116.56, 114.43, 113.43, 112.54, 111.24, 56.51, 46.09, 32.35, 31.31, 20.82, 17.51. ESI-HRMS: calcd for $C_{28}H_{30}N_6O_3$ $[M+H]^+$, 499.2412, found 499.2457.

4.7.5.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3, 4-dimethyl-phenyl)-amino]-propionic acid (7e). Yield: 94.2%; mp: 255.7-256.2 °C; Purity: 96.5%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 1H, -OH), 8.67 (s, 1H, =NH), 7.68 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.55 (s, 1H, Ar-H), 7.45 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.25 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.08 (s, 1H, Ar-H), 6.96 (d, *J* = 7.8 Hz, 1H, Ar-H), 6.88 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.81 (d, *J* = 7.7 Hz, 1H, Ar-H), 4.70 (s, 2H, -CH_2-), 4.00 (t, *J* = 7.0 Hz, 2H, -CH_2-), 3.80 (s, 3H, -NCH_3), 2.55 (d, *J* = 7.4 Hz, 2H, -CH_2-), 2.11 (s, 3H, Ar-CH_3), 2.09 (s, 3H, Ar-CH_3). ^{13}C NMR (101 MHz, DMSO) δ 173.07, 169.02, 164.78, 164.70, 164.63, 153.77, 152.58, 140.33, 138.03, 135.85, 134.82, 133.70, 130.61, 130.23, 129.33, 128.94, 126.22, 126.08, 114.57, 112.82, 112.65, 56.51, 46.78, 32.45, 32.05, 19.78, 19.24, 18.71. ESI-HRMS: calcd for $C_{28}H_{30}N_6O_3$ $[M+H]^+$, 499.2412, found 499.2474.

4.7.6.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(3, 5-dimethyl-phenyl)-amino]-propionic acid (7f). Yield: 87.9%; mp: 259.5-260.2 °C; Purity: 96.2% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (s, 1H, -OH), 8.68 (s, 1H, =NH), 7.67 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.55 (s, 1H, Ar-H), 7.45 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.27 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.88 (d, *J* = 8.2 Hz, 2H, Ar-H), 6.82 (s, 2H, Ar-H), 6.79 (s, 1H, Ar-H), 4.69 (s, 2H, -CH_2-), 3.99 (t, *J* = 7.0 Hz, 2H, -CH_2-), 3.80 (s, 3H, -NCH_3), 2.55 (d, *J* = 7.2 Hz, 2H, -CH_2-), 2.13 (s, 6H, Ar-CH_3). ^{13}C NMR (101 MHz, DMSO) δ 173.03, 168.94, 164.69, 164.61, 164.53, 153.73, 152.57, 142.51, 139.04, 134.73, 133.68, 130.23, 129.24, 129.19, 126.10, 115.06, 114.49, 112.81, 112.68, 56.49, 46.74, 32.47, 32.08, 21.11. ESI-HRMS: calcd for $C_{28}H_{30}N_6O_3$ $[M+H]^+$, 499.2412, found 499.2474.

4.7.7.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidaz

ole-5-carbonyl}-(3-methoxy-phenyl)-amino]-propionic acid (7g). Yield:84.6%; mp: 248.7-249.9 °C; Purity: 98.5% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ8.97 (s, 1H, -OH), 8.73 (s, 1H, =NH), 7.69 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.58 (s, 1H, Ar-H), 7.53 (d, *J* = 7.3 Hz, 1H, Ar-H), 7.31 (d, *J* = 8.2 Hz, 1H, Ar-H), 7.11 (t, *J* = 8.0 Hz, 1H, Ar-H), 6.90 (s, 1H, Ar-H), 6.87 (d, *J* = 5.8 Hz, 2H, Ar-H), 6.71 (t, *J* = 6.9 Hz, 2H, Ar-H), 4.75 (s, 2H, -CH_2-), 4.05 (t, *J* = 7.3 Hz, 2H, -CH_2-), 3.83 (s, 3H, -OCH3), 3.65 (s, 3H, -NCH_3), 2.56 (t, *J* = 7.2 Hz, 2H, -CH_2-). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 172.60, 169.49, 164.25, 164.17, 164.10, 159.55, 153.20, 152.70, 144.18, 135.50, 129.84, 129.66, 123.76, 120.31, 117.51, 113.70, 112.21, 111.86, 110.23, 99.49, 55.14, 46.21, 32.11, 30.45. 18.41.ESI-HRMS: calcd for $C_{27}H_{28}N_6O_4$ $[M+H]^+$, 501.2205, found 501.2260.

4.7.8.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidaz ole-5-carbonyl}-(4-methoxy-phenyl)-amino]-propionic acid (7h). Yield: 83.5%; mp: 244.2-245.1 °C; Purity: 98.4% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (s, 1H, -OH), 8.67 (s, 1H, =NH), 7.67 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.51 (s, 1H, Ar-H), 7.41 (d, *J* = 7.6 Hz, 1H, Ar-H), 7.21 (d, *J* = 8.2 Hz, 1H, Ar-H), 7.11 (d, *J* = 8.2 Hz, 2H, Ar-H), 6.88 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.79 (d, *J* = 8.2 Hz, 2H, Ar-H), 4.67 (s, 2H, -CH_2-), 4.00 (t, *J* = 7.1 Hz, 2H, -CH_2-), 3.78 (s, 3H, -OCH3), 3.65 (s, 3H, -NCH_3), 2.54 (d, *J* = 7.3 Hz, 2H, -CH_2-).^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm)173.16, 170.38, 164.85, 164.77, 164.70, 164.63, 157.97, 153.57, 153.32, 138.90, 136.41, 131.08, 130.14, 129.72, 123.85, 118.84, 114.78, 113.92, 113.85, 112.32, 110.21, 56.51, 55.63, 46.83, 32.57, 30.68, 18.86. ESI-HRMS: calcd for $C_{27}H_{28}N_6O_4$ $[M+H]^+$, 501.2205, found 501.2272.

4.7.9.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidaz ole-5-carbonyl}-(5-methyl-pyridin-2-yl)-amino]-propionic acid (7i). Yield: 80.4%; mp: 254.9-255.5 °C; Purity: 95.9% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 1H, -OH), 8.71 (s, 1H, =NH), 8.21 (d, *J* = 2.3 Hz, 1H, Py-H), 7.68 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.52 (s, 1H, Ar-H), 7.46 (d, *J* = 8.4 Hz, 1H), 7.41 (dd, *J* = 8.3, 2.1 Hz, 1H, Ar-H), 7.18 (d, *J* = 8.4 Hz, 1H, Py-H), 6.89 (d, *J* = 7.3 Hz, 2H, Ar-H), 6.87 (s, 1H, Py-H), 4.70 (s, 2H, -CH_2-), 4.14 (t, *J* = 7.5 Hz, 2H, -CH_2-), 3.81 (s, 3H, -NCH_3), 2.60 (t, *J* = 7.5 Hz, 2H, -CH_2-), 2.17 (d, *J* = 9.0 Hz, 3H, Py-CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 177.66, 174.38, 169.54, 169.47, 169.39, 169.31, 158.75, 158.34, 157.70, 153.97, 144.00, 139.98, 137.81, 136.37, 134.94, 129.92, 126.77, 121.27, 119.37, 117.32, 49.76, 37.79, 36.33, 22.46. ESI-HRMS: calcd for $C_{26}H_{27}N_7O_3$ $[M+H]^+$, 486.2208, found 486.2260.

4.7.10.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidaz ole-5-carbonyl}-(6-methyl-pyridin-2-yl)-amino]-propionic acid (7j). Yield: 85.0%; mp: 246.3-247.2 °C; Purity: 97.1%; ^{1}H NMR (400 MHz, DMSO-d_6) δ

8.94 (s, 1H), 8.69 (s, 1H), 7.68 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.50 (s, 1H, Ar-H), 7.43 (dd, *J* = 15.8, 8.0 Hz, 2H, Ar-H, Py-H), 7.18 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.99 (d, *J* = 7.6 Hz, 1H, Py-H), 6.88 (d, *J* = 8.9 Hz, 2H, Ar-H), 6.67 (d, 1H, Py-H), 4.69 (s, 2H, -CH_2-), 4.17 (t, *J* = 7.5 Hz, 2H, -CH_2-), 3.80 (s, 3H, -NCH_3), 2.62 (t, *J* = 7.5 Hz, 2H, -CH_2-), 2.40 (s, 3H, Py-CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 172.97, 169.14, 164.75, 164.68, 164.61, 164.54, 157.68, 154.16, 153.71, 152.65, 140.56, 134.25, 134.16, 130.23 129.61, 126.06, 122.67, 120.42, 115.15, 114.84, 112.92, 46.48, 45.22, 32.96, 32.09, 23.34. ESI-HRMS: calcd for $C_{26}H_{27}N_7O_3$ $[M+H]^+$, 486.2208, found 486.2259.

4.7.11.

3-[{2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1H-benzoimidazole-5-carbonyl}-(2-methyl-phenyl)-amino]-propionic acid (7k). Yield: 80.3%; mp: 249.5-250.3 °C; Purity: 95.3% ; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 1H, -OH), 8.71 (s, 1H, =NH), 7.68 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.44 (d, *J* = 11.6 Hz, 2H, Ar-H), 7.25 (d, *J* = 6.6 Hz, 2H, Ar-H), 7.12 (s, 3H, Ar-H), 6.87 (d, *J* = 8.0 Hz, 2H, Ar-H), 4.68 (s, 2H, -CH_2-), 3.78 (s, 3H, -NCH_3), 3.67(m, 2H, -CH_2-), 2.59 (s, 2H, -CH_2-), 2.11 (d, *J* = 19.5 Hz, 3H, Ar-CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm) 173.02, 170.30, 164.86, 164.79, 164.71, 164.64, 153.61, 153.39, 142.21, 136.73, 135.23, 131.67, 130.13, 130.00, 128.11, 127.33, 123.44, 113.27, 112.26, 110.00, 45.92, 32.41, 30.59, 21.50, 18.00. ESI-HRMS: calcd for $C_{27}H_{28}N_6O_3$ $[M+H]^+$, 485.2256, found 485.2323.

4.2 Anticoagulant assay

The thrombin inhibitory effects (IC_{50}) of new compounds **7a-j** were determined with a commercially available chromogenic assay against human thrombin. Lyophilized human thrombin (national standard), which was purified from human blood, substrate and the test compounds were dissolved and diluted to needed concentrations. Dabigatran was as the reference. Firstly, lyophilized human thrombin (5.4μg/mL) and tested compounds with different concentrations were preincubated for 10 minutes at 37 °C in the 96-well plate. Nextly the specificity substrate, Ac-FVR-AMC (5μM), was added into the preincubation mixture. Then the dynamic changes offluorescence intensity was measured in a microplate detector Envision (PerkinElmer) at room temperature within 10 min. The slope of the linear enzyme dynamics curve during the initial stage of the reaction was referred to as the initial velocity of enzyme reaction. The inhibition rates were calculated from the ultraviolet absorption values of inhibited wells and positive control wells. Finally, the IC_{50} values were gained using a regression analysis method between the concentration and inhibition rate. The inhibitory rate and the concentration that induced a 50% inhibition of thrombin (IC_{50}) were calculated by the following formulas:

Inhibition rate (%) = $[V_{DMSO}-V_{sample}] / V_{DMSO}\times100\%$

V_{sample}: initial velocity of compound group

V_{DMSO}: initial velocity of blank group, treated with DMSO alone

Inhibition rate (%) =$100 / [1+10^{(LogIC50-X)h}]$, h is the Hill coefficient.

4.3 Inhibitory effects on rat arteriovenous thrombosis in vivo

32 SD rats (Shanghai Sippr Bk Laboratory Animals L.T.D.;220-260 g) were divided into blank control (CMC-Na), positive drug (dabigatran), and two test substances (7j and 7k) groups, equally with eight rats in each group. The positive drug and test substances were dissolved with 0.9% CMC-Na to adjust to the concentration of 0.5mg/mL. SD rats were administrated for 3 days(2mg/kg/day). On the fourth day, the rats were anesthetized by intraperitoneal injection of 12% chloral hydrate and fixed in supine position. The neck skin was incised to separate the left carotid artery and right external jugular vein, connecting by a bypass pipe with a No. 4 surgical thread inside. After the tested compounds were injected by intravenous administration, making blood flow for 15 minutes, then the thread was removed and weighed, subtracting the thread of its own weight, that is, wet weight of thrombus. The average value and standard deviation between the experimental groups of thrombus wet weights were calculated.

Inhibition rate of rat arteriovenous thrombosis $_{sample}$ (%) =

[Control group (thromboembolism weight)-sample group (thromboembolism weight)]/control group (thromboembolism weight) × 100%.

Control group: treated with CMC-Na only.

Sample group: treated with CMC-Na and compounds.

4.4 Molecular docking

The structures of the newly synthesized compounds were constructed in the sketch process in SYBYL-X 2.0 package running on windows workstation. The structure was optimized using Gasteiger–Huckel method and the Tripos force field, following by 10000 iterations and the energy gradient limit is set to 0.005 kcal/ (mol*Å). For the protein preparation, the ligand and the water were removed from the receptor protein (PDB entry code: 1KTS). Remaining protein was optimized using Gasteiger–Huckel charges, following by 20 iterations and the energy gradient limit was set to 0.05 kcal/ (mol*Å).

Conflicts of interest

There are no conflicts to declare.

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References

[1] Dahri K.2019. The Pharmacist Guide to Implementing Pharmaceutical Care. vol. 72. Springer International Publishing, 29:353–367.

[2] Weitz J I. 2001. New Anticoagulant Drugs [J]. Journal of Thrombosis and Thrombolysis, 12(1):7-17.

[3] Crawley J T B, Zanardelli S , Chion C K N K , et al 2007. The central role of thrombin in hemostasis [J]. Journal of Thrombosis and Haemostasis, 5(s1):95-101.

[4] Mosesson M W. 2005. Fibrinogen and fibrin structure and functions [J]. Journal of thrombosis and haemostasis: JTH, 3(8):1894-1904.

[5] Bode W . 2005. The structure of thrombin, a chameleon-like proteinase [J]. Journal of Thrombosis and Haemostasis, 3(11):2379-2388.

[6] Kong Y, Chen H, Wang Y-Q, et al. 2014. Direct thrombin inhibitors: Patents 2002-2012 (Review). Mol Med Rep,9(5):1504–1514.

[7] Candia E. 2011.Mechanisms of platelet activation by thrombin: A short history. Thromb Res,129(3):250–256.

[8] Siddique A, Shantsila E, YH Lip G. 2008. The search for new anticoagulants: Dabigatran etexilate. Therapy,5(6):793–796.

[9] Dabbous M, Sakr F, N Malaeb D. 2014.Anticoagulant therapy in pediatrics. J Basic Clin Pharm,5(2):27–33.

[10] Treichl B, Bachler M, Lorenz I, et al. 2015. Efficacy of Argatroban in Critically Ill Patients with Heparin Resistance: A Retrospective Analysis. Semin Thromb Hemost,41(01):61–67.

[11] Jeske W, Fareed J, Hoppensteadt D, et al. 2010. Pharmacology of argatroban. Expert Rev Hematol,3(5):527–539.

[12] Sarich T, Wolzt M, Eriksson U, et al. 2003. Effects of ximelagatran, an oral direct thrombin inhibitor, r-hirudin and enoxaparin on thrombin generation and platelet activation in healthy male subjects. J Am Coll Cardiol,41(4):557–564.

[13] Stangier J, Stähle H, Rathgen K, et al. 2008. Pharmacokinetics and Pharmacodynamics of Dabigatran Etexilate, an Oral Direct Thrombin Inhibitor, Are Not Affected by Moderate Hepatic Impairment. J Clin Pharmacol,48(12):1411–1419.

[14] Bauer K. 2011. Recent progress in anticoagulant therapy: Oral direct inhibitors of thrombin and factor Xa. J Thromb Haemost,9(s1):12–19.

[15] Günther A, Ruppert C. 2006. Coagulation Cascade | Fibrinogen and Fibrin III. Encycl Respir Med, 25(4):503–509.

[16] Testa L, Bhindi R, Agostoni P, et al. 2007. The direct thrombin inhibitor ximelagatran/melagatran: A systematic review on clinical applications and an evidence based assessment of risk benefit profile. Expert Opin Drug Saf,6(4):397–406.

[17] Yip V, Alfirevic A, Pirmohamed M.2014. Genetics of Immune-Mediated Adverse Drug Reactions: a Comprehensive and Clinical Review. Clin Rev Allergy Immunol ,48(2-3):165–175.

[18] Huber K, J Connolly S, Kher A, et al.2013. Practical use of dabigatran etexilate for stroke prevention in atrial fibrillation. Int J Clin Pract ,67(6):516–526.

[19] Kate M, Gioia L, Buck B, et al.2015. Dabigatran Therapy in Acute Ischemic Stroke Patients Without Atrial Fibrillation. Stroke ,46(9):2685–2687.

[20] Ageno W, Spyropoulos A, Turpie A.2012. Role of new anticoagulants for the prevention of venous thromboembolism after major orthopaedic surgery and in hospitalised acutely ill medical patients. Thromb Haemost ,107(6):1027–1034.

[21] Ganetsky M, Babu K, Salhanick S. et al.2011.Dabigatran: Review of Pharmacology and Management of Bleeding Complications of This Novel Oral Anticoagulant. J Med Toxicol ,7(4):281–287.

[22] Armbruster A, Tellor K, H Min S, et al.2014. Evaluation of Dabigatran for Appropriateness of Use and Bleeding Events in a Community Hospital Setting. Am Heal Drug Benefits ,7(7):376–384.

[23] D Bendel S, Bona R, Baker W.2011. Dabigatran: An oral direct thrombin inhibitor for use in atrial fibrillation. Adv Ther ,28(6):460–472.

[24] Ahmed S, Levin V, Malacoff R, et al.2012. Dabigatran - A New Chapter in Anticoagulation. Cardiovasc Hematol Agents Med Chem ,10(2):116–123.

[25] Albert T, Rigault C, Eickhoff J, et al.2014. Characterization of molecular and cellular functions of the cyclin-dependent kinase CDK9 using a novel specific inhibitor. Br J Pharmacol ,171(1):55–68.

[26] Kanuri S, Kreutz R. et al.2019. Pharmacogenomics of Novel Direct Oral Anticoagulants: Newly Identified Genes and Genetic Variants. J Pers Med ,9(1):7 –24.

[27] E. Dager W, Banares L.2017. Reversing the anticoagulation effects of dabigatran. Hosp Pract ,45(2):29–38.

[28] Listed N.2012. Dabigatran and atrial fibrillation: the alternative to warfarin for selected patients. Prescrire International, 21(124):33-36.

[29] Fries, D., Giurea, A., Gütl, M., et al 2013. Management of dabigatran-induced bleeding: expert statement. Wiener Klinische Wochenschrift, 125(21-22), 721–729.

[30] Haas S . 2008. New oral Xa and IIa inhibitors: updates on clinical trial results[J]. Journal of Thrombosis and Thrombolysis, 25(1):52-60.

[31] Stangier J., Rathgen, K., Stähle, H., Gansser, D., & Roth, W. 2007. The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects. British Journal of Clinical Pharmacology, 64(3), 292–303.

[32] H Hauel N, Nar H, Priepke H, et al.2002. Structure-Based Design of Novel Potent Nonpeptide Thrombin Inhibitors. J Med Chem ,45(9):1757–1766.

[33] Wang F, Ren YJ, Dong MH.2016. Molecular design, synthesis and anticoagulant activity evaluation of fluorinated dabigatran analogues. Bioorganic Med Chem ,24(12):2739–2753.

[34] Lin, G. Q.; Ma, J. Y.; Xu, L.; Sun, X.; Sun, Z. H.; Wang, J. Y. CN 1,861,596A, 2006–05–18.

[35] Johnson T, A. Gallego R, Edwards M.2018. Lipophilic Efficiency as an Important Metric in Drug Design. J Med Chem ,61(15):6401–6420.

[36] Schönherr H, Cernak T.2014. ChemInform Abstract: Profound Methyl Effects in Drug Discovery and a Call for New C-H Methylation Reactions. Angew Chem Int Ed Engl ,45(5):12256–12267.

[37] Backes A, Zech B, Felber B, et al.2008. Small-molecule inhibitors binding to protein kinases. Part I: Exceptions from the traditional pharmacophore approach of type I inhibition. Expert Opin Drug Discov ,3(12):1409–1425.

[38] Barreiro E, Kummerle A, Fraga C.2011. The Methylation Effect in Medicinal Chemistry. Chem Rev ,111(9):5215–5246.

[39] S Leung C, Leung S, Tirado-Rives J, et al.2012 Methyl Effects on Protein-Ligand Binding. J Med Chem ,55(9):4489–4500.

1. Ten novel methyl substituted benzimidazole derivatives as direct thrombin inhibitors for the treatment of cardiovascular diseases were designed, synthesised and biologically evaluated.

2. At nanomolar levels, eight compounds presented excellent in vitro inhibitory effects on arteriovenous thrombosis.

3. Compounds 7j and 7k exhibited a potent inhibition of venous thrombosis in rats compared with dabigatran.

Conflicts of interest

There are no conflicts to declare.