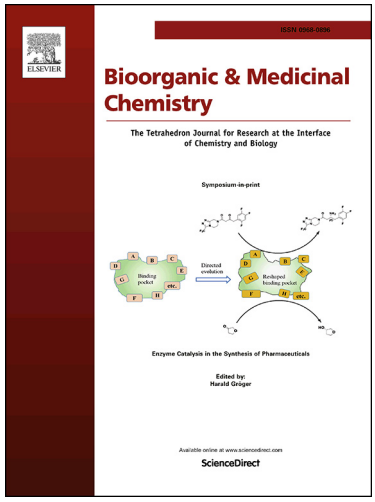
Accepted Manuscript



Design, synthesis and biological evaluation of anthranilamide derivatives as potential factor Xa (fXa) inhibitors

Junhao Xing, Lingyun Yang, Jinpei Zhou, Huibin Zhang

PII: S0968-0896(18)30840-X
DOI: https://doi.org/10.1016/j.bmc.2018.09.012
Reference: BMC 14537

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 1 May 2018
Revised Date: 27 August 2018
Accepted Date: 10 September 2018

Please cite this article as: Xing, J., Yang, L., Zhou, J., Zhang, H., Design, synthesis and biological evaluation of anthranilamide derivatives as potential factor Xa (fXa) inhibitors, *Bioorganic & Medicinal Chemistry* (2018), doi: https://doi.org/10.1016/j.bmc.2018.09.012

Design, synthesis and biological evaluation of anthranilamide derivatives as potential factor Xa (fXa) inhibitors

*Junhao Xing[a], Lingyun Yang[b], Jinpei Zhou[c], Huibin, Zhang[b, d, *]*

[a]*Department of Organic Chemistry, School of Science, China Pharmaceutical University, 639 Longmian Avenue, Nanjing 211198, PR China.*

[b]*Center of Drug Discovery, State Key Laboratory of Natural Medicines, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, PR China.*

[c]*Department of Medicinal Chemistry, China Pharmaceutical University, TongjiaXiang 24, 210009 Nanjing, PR China*

[d]*Jiangsu Key Laboratory of Drug Discovery for Metabolic Disease, China Pharmaceutical University, Nanjing 210009, PR China*

Abstract

Factor Xa (fXa) is a crucial player in various thromboembolic disorders. Inhibition of fXa can provide safe and effective antithrombotic effects. In this study, a series of anthranilamide compounds were designed by utilizing structure-based design strategies. Optimization at P1 and P4 groups led to the discovery of compound **16g**: a highly potent, selective fXa inhibitor with pronounced *in vitro* anticoagulant activity. Moreover, **16g** also displayed excellent *in vivo* antithrombotic activity in the rat venous thrombosis (VT) and arteriovenous shunt (AV-SHUNT) models. The bleeding risk evaluation showed that **16g** had a safer profile than that of betrixaban at 1 mg/kg and 5 mg/kg dose. Additionally, **16g** also exhibited satisfactory PK profiles. Eventually, **16g** was selected to investigate its effect on hypoxia-reoxygenation-induced H9C2 cell viability. MTT results showed that H9C2 cell viability can be remarkably alleviated by **16g**.

Keywords factor Xa; venous thrombosis; arteriovenous shunt; bleeding risk; H9C2 cell.

1. Introduction

Factor Xa (fXa), a trypsin-like serine protease, plays a key role in the blood coagulation cascade[1]. It can form pro-thrombinase complex with fVa, phospholipids, and Ca^{2+} to produce thrombin by activating prothrombin during blood clotting in intrinsic and extrinsic coagulation pathways[2, 3]. fXa is upstream from thrombin in the coagulation cascade. Inhibition of fXa instead of thrombin can be more effective in diminishing the coagulation cascade. Moreover, Inhibition of fXa has lower risk of bleeding due to its ability to reduce the further generation of thrombin without affecting the existing level of thrombin which should be sufficient to ensure

* Corresponding authors. Center of Drug Discovery, State Key Laboratory of Natural Medicines, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China. Tel: +86-25-83271302; Fax: +86-25-83271480.
E-mail addresses: hb_zhang@hotmail.com (H. Zhang) or zhanghb80@163.com, xingjhcpu@163.com (J. Xing)

primary hemostasis[4]. Therefore, fXa is a particularly promising target for the development of novel anticoagulants and has attracted great interest in the past decades.

Currently, four oral, direct and selective fXa inhibitors have been approved by the U.S. Food and Drug Administration (FDA): rivaroxaban[5, 6], apixaban[7], edoxaban[8] and betrixaban[9]. Compared with traditional anticoagulants, these novel fXa inhibitors exhibited good oral bioavailability, high specificity and minor drug and food interactions[10, 11]. However, they still faced with challenging scenarios due to lack of required monitoring and specific reversal agents for significant bleeding[12]. In addition, they have narrow clinical indications and should not be used in patients with severe renal and hepatic impairment[13, 14], patients with mechanical heart valves[15]. Therefore, it is also necessary to further develop novel and safer fXa inhibitors to promote their use in clinic.

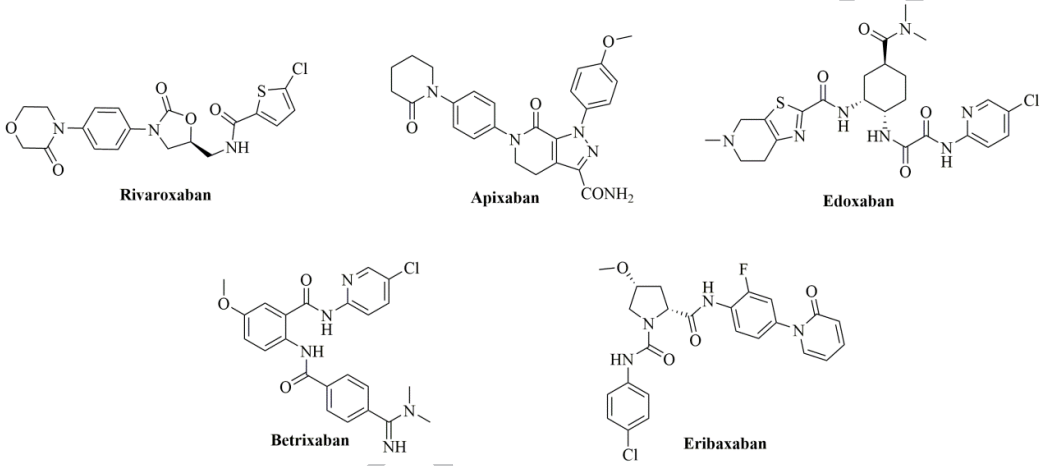


Fig. 1. The structures of rivaroxaban, apixaban, edoxaban, betrixaban and eribaxaban.

In our previous studies, we have reported a series of anthranilamide and 2, 3-dihydroquinazolin- 4(1H)-one derivatives as fXa inhibitors[16, 17]. As a part of our ongoing research program to develop safer and more effective fXa inhibitors, guided by X-ray crystallography and structure-based design strategies, a series of novel anthranilamide fXa inhibitors were designed and synthesized. The following optimization resulted in the discovery of compound **16g**, which was identified as a highly potent and selective FXa inhibitor and warrants further evaluation as a potential candidate for the prevention and treatment of thromboembolic diseases. Additionally, myocardial ischemia (MI) is known to induce the formation of thrombosis, and thrombus is also a cause of MI[18-20]. Thus, compounds possessing both anticoagulant and cardioprotective effects in MI are desirable. The most excellent compound **16g** was selected to investigate its effect on hypoxia–reoxygenation -induced H9C2 cell viability. MTT results showed that **16g** could inhibite hypoxia– reoxygenation-induced H9C2 cell viability loss.

2. Drug design

Over the past decades, the development of synthetic, direct fXa inhibitors has undergone four phases [21]. Although these fXa inhibitors possess various scaffolds, most of them bind to

the active site in a characteristic L-shaped conformation[21]. In other word, they have a three-component system including a central scaffold and two hydrophobic fragments (P1 and P4) which provides a similar non-linear geometry considered to play a crucial role in fXa recognition. Many novel fXa inhibitors such as apixaban and rivaroxaban adopt very similar conformations including the length and orientation of P1 and P4. As shown in **Fig.2**A, apixaban (pink, PDB code 2P16) and rivaroxaban (green, PDB code 2W26) bind with fXa in a quite similar conformational space. Although their scaffolds could not be overlaid and formed significantly different interactions with fXa, their aromatic rings in P4 fragments superimposed very well, and length and orientation of P1 and P4 are also very similar. In addition, the binding conformation of betrixaban with fXa was predicted by glide in Schrodinger 2009 which has been demonstrated to successfully predict the binding modes of many reported inhibitors to fXa. It was also found that the P1 and P4 fragments of betrixaban and rivaroxaban, especially P1 group, adopted very similar conformations (**Fig. 2**B).

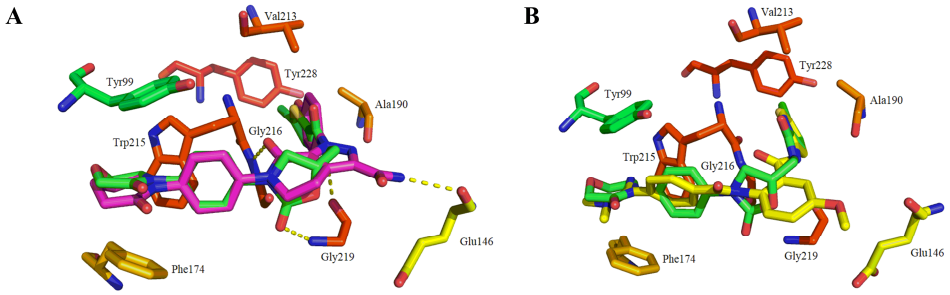


Fig.2. The binding mode of apixaban, rivaroxaban and betrixaban with the active sites of fXa. (A): Superimposition of structures of rivaroxaban (green, PDB code 2W26) and apixaban (pink, PDB code 2P16); (B). Overlay of the binding conformations of rivaroxaban and betrixaban. PDB entry 2W26 was used for molecular docking simulation of betrixaban. Hydrogen atoms have been hidden for clarity. The figures were prepared using PyMol (www.pymol.org).

Based on these modeling and findings, it was postulated that the highly basic amidine group of betrixaban can be replaced by less basic P4 moieties of rivaroxaban, apixaban and edoxaban. Meanwhile, the P1 fragment of betrixaban also can be changed to other substituted aromatic or heteroaromatic rings. As a result, a novel series of anthranilamide derivatives were designed (**Fig. 3**), synthesized and evaluated for their fXa inhibitory activity.

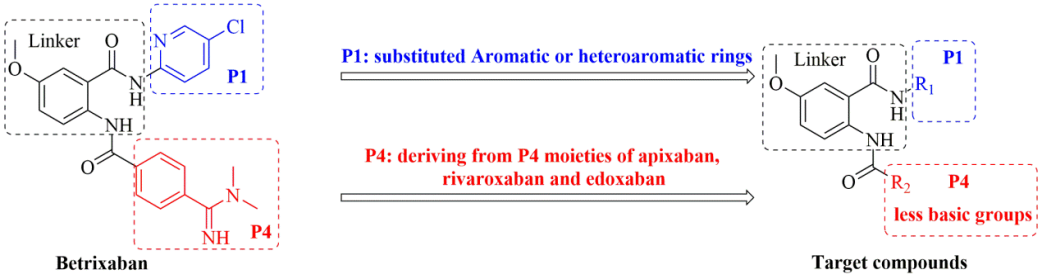
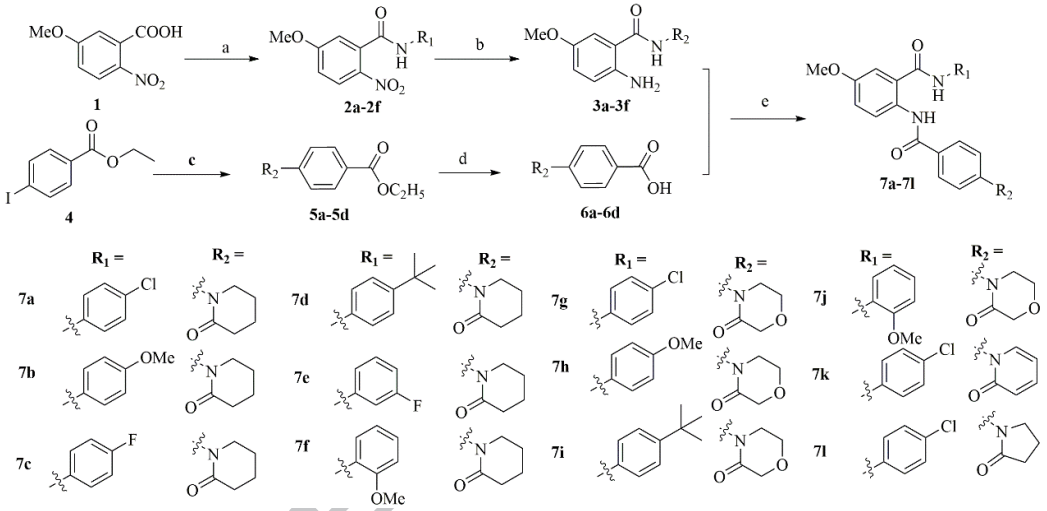


Fig. 3. Design of the target compounds

3. Results and discussion

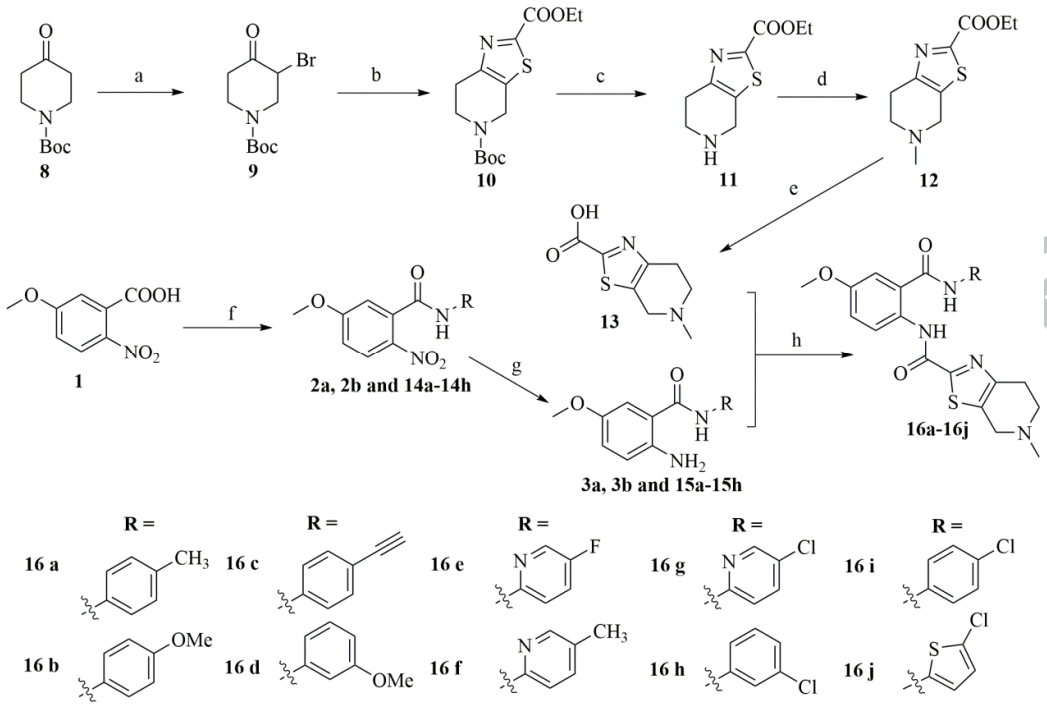
3.1 Chemistry

The synthetic procedure for **7a-7l** is shown in **Scheme 1**. To synthesize intermediates **3a-3f**, commercial available carboxylic acid **1** was used as the starting material. This material was treated with aromatic amines and EDCI in the presence of DIPEA to form intermediates **2a-2f**, which were reduced by using hydrogen in the presence of Pd/C catalyst (10%) in anhydrous EtOH to be converted to **3a-3f**. The intermediates **5a-5d** was formed by the copper-catalysed coupling reaction of the corresponding β-Lactam with commercially available **4** in the presence of *N, N'*-dimethyl ethylenediamine as a ligand[22]. **5a-5d** were hydrolyzed in aqueous sodium hydroxide to yield **6a-6d**, respectively, which were treated with **3a-3f** and EDCI in the presence of DIPEA to obtain products **7a-7l**.



Scheme 1. Synthesis routes and structures of intermediates and target compounds **7a-7l**. (a) Arylamine, EDCI, DIPEA, CH_2Cl_2, 0-r.t., 12 h; (b) H_2, 10% Pd/C, ethonal, r.t., 15 h; (c) Morpholin-3-one or Piperidin-2-one, N,N'-dimethyl ethylenediamine, CuI, K_3PO_4, N_2, PhMe, reflux, 16 h; (d) NaOH, $MeOH/H_2O$, 0 °C, 1 h; (e) EDCI, DIPEA, DMF, 0-r.t., 24 h.

The synthetic procedure for **16a-16j** is presented in **Scheme 2**. In this route, intermediates **15a-15h** were synthesized in the same method as that described for **3a-3f**. The synthesis of carboxylic acid **13** started with compound **8**, which was transformed to intermediate **9** by bromination[23]. Treatment of **9** with ethyl thioxamate and then deprotection with trifluoroacetic acid afforded amine **11**. Subsequent reaction with methyl iodide afforded intermediate 1**2**. After hydrolysis of **12** with aqueous sodium hydroxide, the key intermediate **13** was obtained in good yield. Then, compounds **16a-16j** were synthesized from **13** and **3a**, **3b** and **15a-15h** following the approach used for compounds **7a-7l**.



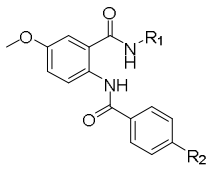
Scheme 2. Synthesis routes and structures of intermediates and target compounds **16a-16j**. (a) Bromine, CH_2Cl_2, 0-r.t., 0.5 h; (b) Ethyl thiooxamate, $NaHCO_3$, IPA, reflux, 12 h; (c) F_3CCO_2H, CH_2Cl_2, 0 ºC-r.t., 3 h; (d) iodomethane, CH_2Cl_2, 30 ºC, 18 h; (e) 1 M NaOH, MeOH, r.t., 1.5 h; (f) Arylamine, EDCI, DIPEA, CH_2Cl_2, 0-r.t., 12 h; (g) H_2, 10% Pd/C, ethonal, r.t., 15 h; (h) EDCI, DIPEA, DMF, 0-r.t., 48 h.

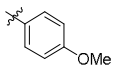
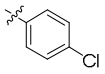
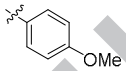
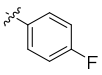
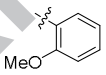
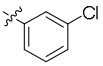
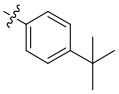
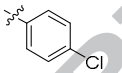
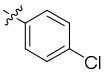
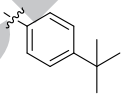
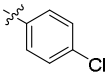
3.2 Biological activities and discussion

3.2.1 In vitro fXa inhibition activities studies

All the designed compounds were evaluated for their human fXa enzyme inhibitory activity. Shown in **Table1** are IC_{50} values of the targeted compounds **7a-7j**. The assay data displayed compounds with morpholin-3-one as P4 fragment showed slightly increase of activity compared with molecules possessing piperidin-2-one, pyridin-2(1H)-one or pyrrolidin-2-one as P4 surrogate (**7a-7f** vs **7g-7j**, **7g vs 7e**, **7k**, **7l**). In general, compounds (**7e** and **7g**) with 4-chlorophenyl as the P1 surrogate showed strong inhibitory activity. Derivative **7d** with 3-chlorophenyl as the P1 fragment showed a sharply decreased fXa inhibitory activity compared with that of **7e** and **7g**. Additionally, compared with 4-chlorophenyl derivatives, a loss of potency to varying extents was observed for the methoxy, fluoro and tert-butyl derivatives. The results were consistent with our precious study of SAR which the Cl–π interaction formed by the chlorine atom and the phenyl ring of Tyr228 plays a crucial role in potency[16].

Table 1. fXa inhibitory effect of compounds **7a-7l**.



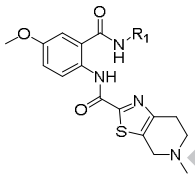
Compd.	R_1	R_2	IC_{50} (nM)[a]	Compd.	R_1	R_2	IC_{50} (nM)[a]
7a			246 ± 47	**7g**			18 ± 5
7b			513 ± 113	**7h**			77 ± 15
7c			1079 ± 208	**7i**			2087 ± 676
7d			272 ± 65	**7j**			1543 ± 592
7e			45 ± 11	**7k**			179 ± 42
7f			3189 ± 790	**7l**			653 ± 136
Betrixaban	**-**	-	4.5 ± 1.8				

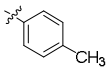
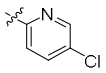
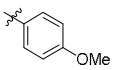
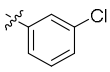
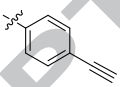
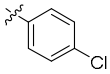
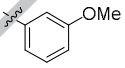
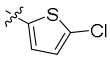
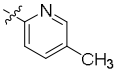
[a] The data represent the mean of at least three independent determinations.

Further modifications were made to P1 surrogates with 5-methyl-4,5,6,7-tetrahydrothiazolo [5,4-c]pyridin as P4 substituent. Overall, these compounds had a moderate

to high potency against fXa. Among them, the derivatives (**16e-16g**) with substituted pyridine as P1 fragment were more potent than their corresponding phenyl and chlorothiophene derivatives. Compound **16g** with 5-chloropyridin as the P1 surrogate displayed the strongest potency against fXa with the IC_{50} value of 3.5 nM. Moreover, 5-fluoropyridin (**16e**) and 5-methylpyridin (**16f**) derivatives also exhibited excellent potency. When the substituted phenyls were used as P1 fragment, 4-chlorophenyl (**16i**) was found to be the best surrogate. Compounds with 4- methyl phenyl (**16a**) or 4-ethynylphenyl (**16c**) as the P1 substituent also showed a potent fXa inhibitory activity. However, changing 4-chlorophenyl to 3-chlorophenyl (**16h**) resulted in a 66-fold loss of potency, whereas 4-methoxy and 3-methoxy derivatives (**16c** and **16d**) lost potency by about 49 and 200-fold, respectively. Additionally, Compound **16j** with 5-chlorothiophen as the P1 surrogate displayed low potency.

Table 2. fXa inhibitory effect of compounds **16a-16j**.



Compd.	R_1	IC_{50} (nM)[a]	Compd.	R_1	IC_{50} (nM)[a]
16a		30 ± 7.0	**16g**		3.5 ± 1.0
16b		234 ± 63	**16h**		315 ± 63
16c		20 ± 5.1	**16i**		4.8 ± 1.4
16d		981 ± 185	**16j**		138 7± 362
16e		24 ± 6.3	**Betrixaban**		4.5 ± 1.8
16f		10 ± 3.2			

[a] The data represent the mean of at least three independent determinations.

3.2.2 Selectivity and in vitro clotting activity assay

Based on the results of fXa inhibition activities, compounds **16f**, **16g** and **16i** were selected to evaluate their selectivity against related serine proteases. All tested compounds were found to be more than 1,000 fold selective versus fIIa, fVIIa, fIXa, and trypsin (**Table 3**). In addition, the *in vitro* clotting activities of these compounds were measured by the prothrombin time (PT) assay and activated partial thromboplastin time (aPTT). Compounds **16f** displayed moderate clotting activity judged by 2 × PT value of 5.6 μM and 2 × aPTT value of 2.2 μM. For its counterpart, **16g** and **16i** demonstrated potent activity in both assays. Especially, compound **16g,** which exhibited the best clotting activity with 2 × PT value of 0.8 μM and 2 × aPTT value of 0.5 μM, was better than betrixaban.

Table 3 Selectivity and anticoagulant activity of compounds selected.

Compd.	Thrombin IC_{50}(μM)[a]	fVIIa IC_{50}(μM)[a]	fIXa IC_{50}(μM)[a]	trypsin IC_{50}(μM)[a]	2 × PT (μM)[b]	2 × aPTT (μM)[c]
16g	4.2	12	36	9.1	0.8	0.5
16i	7.1	4.7	26	18	1.3	0.8
16f	32	11	42	54	5.6	2.2
Betrixaban	18				2.4	1.1

[a]The data represent the mean of at least three independent determinations.

[b]PT values are defined as the inhibitor concentration required to double fibrin formation the time and using rabbit plasma.

[c]aPPT values are defined as the inhibitor concentration required to double activated partial thromboplastin time using rabbit plasma.

*3.2.3 In vivo antithrombotic activity in rats and bleeding risk of **16g** in rats.*

As a result of the excellent *in vitro* potency and selectivity, compound **16g** was selected to further evaluate its *in vivo* antithrombotic effect in $FeCl_3$-induced venous thrombosis (VT), arteriovenous shunt (AV-SHUNT) thrombosis model in rats and bleeding risk in a rat tail-bleeding time study. As shown in **Fig. 4**, **16g** displayed significant *in vivo* antithrombotic activity in both thrombosis models in a dose-dependent manner. In VT model (**Fig. 4**A), **16g** reduced thrombus weight by 23%, 36% and 54% at 1 mg/kg, 5 mg/kg and 10 mg/kg compared with 27%, 38% and 59% for betrixaban, respectively. In AV-SHUNT thrombosis model (**Fig. 4**B), compound **16g** was found to be more potent than betrixaban. Reduction in thrombus weight was 30%, 50% and 67% at 1 mg/kg, 5 mg/kg and 10 mg/kg respectively. Betrixaban reduced thrombus weight by 24%, 43% and 61% at 1 mg/kg, 5 mg/kg and 10

mg/kg, respectively. Additionally, a rat tail-bleeding time assay was implemented to evaluate the bleeding risk of **16g**. The test results appearing in **Fig. 5** showed that **16g** and betrixaban prolonged bleeding time in a dose-dependent manner, and **16g** had safer profile than that of betrixaban at the doses of 1 mg/kg and 5 mg/kg. When administrated with 10 mg/kg, **16g** and betrixaban prolonged the bleeding time to the same extent.

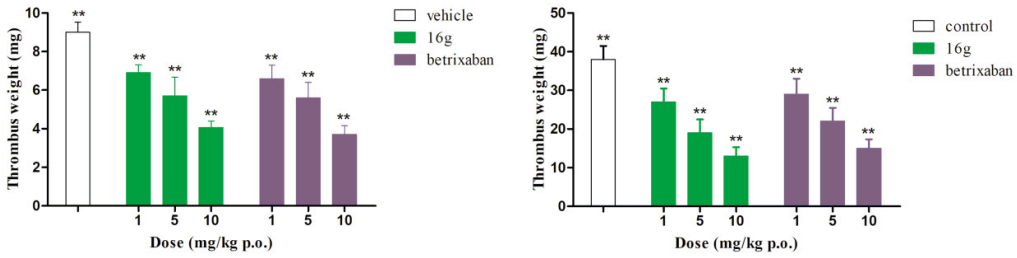


Fig. 4. *In vivo* antithrombotic activity of **16g** and betrixaban in rats (A): Antithrombotic profile of **16g** and betrixaban in $FeCl_3$-induced venous thrombosis model; (B): Antithrombotic profile of **16g** and betrixaban in the rat arteriovenous shunt model. Values are mean ± SEM (n = 8). *P < 0.05, **P < 0.01, and ***P < 0.001 compared to vehicle group by Student's t-test.

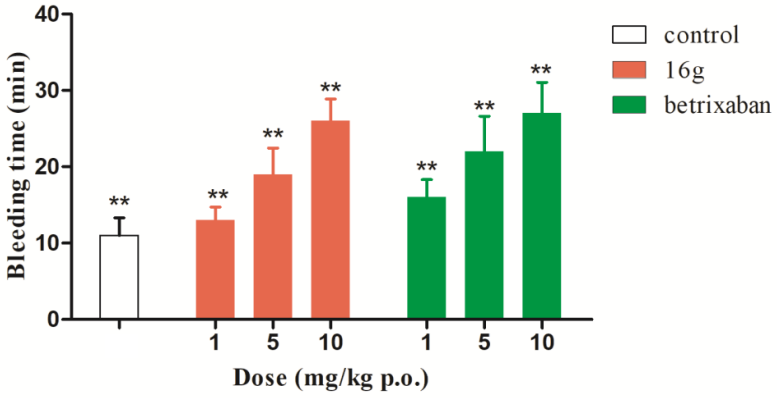


Fig. 5. Effect of **16g** and betrixaban on rat tail-bleeding time. Values are mean ± SEM (n = 8). *P < 0.05, **P < 0.01, and ***P < 0.001 compared to vehicle group by Student's t-test.

*3.2.4 PK profiles of compound **16g**.*

Given the excellent *in vitro* potency, the PK profiles of compound **16g** were studied in rats with the data for betrixaban included for comparison. As summarized in **Table 4**, compound **16g** displayed favorable PK profiles, with high plasma exposure ($AUC_{0-\infty}$ = 1160 ng·h/mL), high maximal plasma concentration (C_{max} = 221 ng/mL), lower clearance (CL_p = 0.92 L/h/kg) and good oral bioavailability (F = 51.3%) after oral administration. These values were comparable or better than that of betrixaban.

Table 4. PK profiles of **16g** and betrixaban in male rats[a].

Compd.	Route	Dose (mg/kg)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{0-\infty}$ (ng·h/mL)	CLp (L/h/kg)	Vss (L/kg)	F (%)
16g	p.o.	3	221	3.2	9.6	1160			51.3
	i.v.	1			8.8	2258	0.92	0.201	
Betrixaban	p.o.	3	146	2.6	8.6	571			30.2
	i.v.	1			7.8	1832	1.36	0.191	

[a]Abbreviations: p.o., per oral; i.v., intravenous**;** C_{max}, peak plasma concentration of the compound after administration; T_{max}, time to reach Cmax; $T_{1/2}$, elimination half-life; AUC, area under the concentration-time curve; CL_p, plasma clearance; Vss, volume of distribution at steady state; **F**, bioavailability.

3.2.5 MTT assay

Compound **16g** was selected to investigate its effect on hypoxia–reoxygenation-induced H9C2 cell viability. MTT results showed that hypoxia–reoxygenation significantly inhibited H9C2 cell viability, which was remarkably alleviated by **16g**. By contrast, the assay demonstrated H9C2 cell viability could not be improved by betrixaban.
or better than rivaroxaban

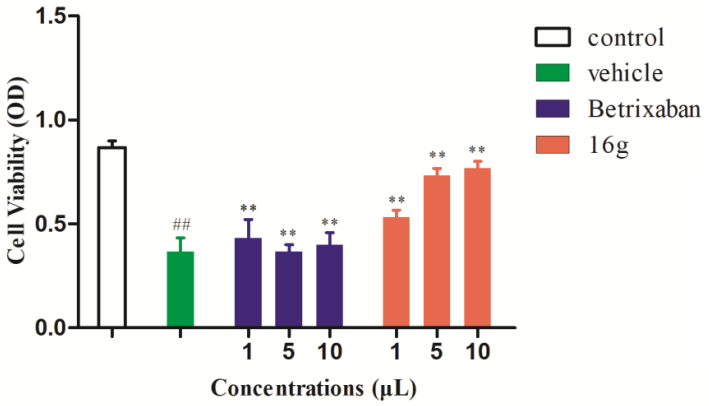


Fig. 6. MTT results. All values given are the mean ± SDs. ##P < 0.01 vs. control group. **P < 0.01 vs. hypoxia–reoxygenation vehicle group.

*3.3 Docking study for compound **16g***

To elucidate the binding mode compound **16g** to fXa, molecular docking was conducted using glide in Schrodinger 2009 with the crystal structure of fXa (PDB code: 2w26). As shown in **Fig.7**A, The 5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine as P4 fragment occupy S4 pocket which is formed by Tyr99, Trp215, and Phe174 and did not interact with any residues

in the S4 pocket. For the central scaffold, two hydrogen bonds are formed with the residues in this region of the enzyme. The carbonyl oxygen forms a hydrogen bond with Gly216. The NH of the scaffold carboxamide forms a hydrogen bond with the carbonyl oxygen of Gly219. The 5-chloropyridin is orientated to the S1 pocket and interacts with the aromatic ring of Tyr228 located at the bottom of the S1 pocket to form the key Cl-π interaction, which is considered to be important for the potent fXa inhibitory activity[24]. In addition, when the binding conformations of **16g** and rivaroxaban were overlaid within the same coordinate system (**Fig.7B**), it can be found that they adopted very similar conformations. The two P4 fragments have similar length and orientation and their P1groups superimposed very well. All the results above confirmed the reliability of our drug design strategy.

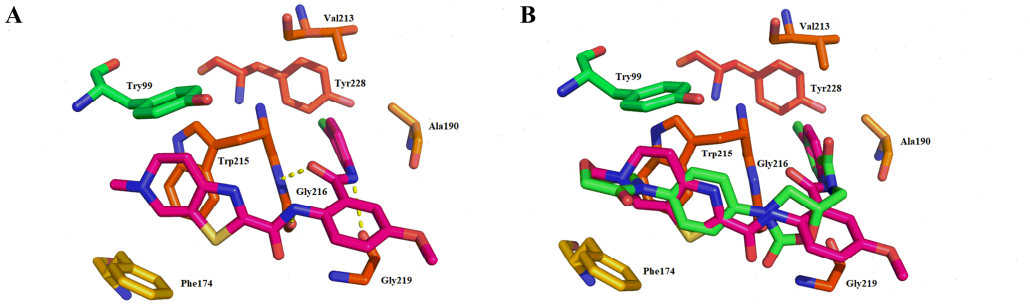


Fig.7. The docking mode of **16g** with the active sites of fXa (PDB code 2w26). (A) 3D interactions of **16g** with the active sites of fXa; (B) Superimposition of the binding conformations of **16g** and rivaroxaban.

4. Conclusion

In summary, a series of novel anthranilamide derivatives were designed and synthesized as potent and selective fXa inhibitors by utilizing structure-based design strategies. Most of the target compounds displayed some degree of fXa inhibitory activity. Of these molecules, the compound **16g** was found to possess the best fXa inhibition with an IC_{50} value of 3.5 nM, significant selectivity against other serine protease, and pronounced *in vitro* and *in vivo* anticoagulant efficacy. The bleeding risk evaluation exhibited that **16g** had a safer profile than that of betrixaban at 1 mg/kg and 5 mg/kg dose. The pharmacokinetic profiles of **16g** were studied and presented favorable results. In addition, in MTT assay, **16g** displayed significant ability to improve hypoxia–reoxygenation-induced H9C2 cell viability. The effects of **16g** on myocardial injury are being evaluated by our lab and will be reported in due course.

5. Experimental section

5.1 Docking study

Ligand molecules for docking studies were drawn using ChemBioDraw Ultra 16.0 software. The compounds were energy minimized and refined with CHARMm force field using Prepare Ligands tool of DS3.0. Docking simulation was performed with Glide in Schrodinger 2009[25]. The crystal complex was downloaded from the protein data bank (PDB code: 2w26) and processed with water removed and hydrogen added at pH=7.0. Then a 10-Å

box was defined by the geometrical center of the ligand binding site. Protein structure was prepared using the "protein preparation wizard" to generate the grid file in the binding site. Other parameters were in default.

5.2 Chemistry

5.2.1 General chemistry

Solvents, reagents and starting materials were obtained from commercial sources in the appropriate grade and used without further purification. NMR spectra were recorded on Bruker AVANCE AV-300 spectrometer (300 MHz for ^{1}H, 75 MHz for ^{13}C) using DMSO-d_6 or $CDCl_3$. Chemical shifts were reported in δ (ppm). Multiplicity was indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad). Coupling constants were reported in Hertz (Hz). Mass spectrometry (MS): HewlettePackard 1100 LC/MSD spectrometer, in m/z; Elemental analyses were carried out on CHN-O-Rapid instrument. For thin layer chromatography (TLC), Yantai pre-coated TLC plates (HSGF 254) were used, and compounds were visualized with a UV light at 254 nm. Column chromatography separations were performed on silica gel (300–400 mesh) eluting with n-hexane/ethyl acetate (8/1 to 1/1) or dichloromethane/methanol (100/1, 60/1 or 30/1).

*5.2.2 N-(4-chlorophenyl)-5-methoxy-2-nitrobenzamide(**2a**)*

To a mixture of compound **1** (2 g, 10.1 mmol) and EDCI (2.3 mg, 12.2 mmol) in CH_2Cl_2 (25 mL) cooled to 0 °C was added dropwise a solution of 4-chloroaniline (1.3 g, 10.1 mmol) and DIPEA (2.5 mL) in CH_2Cl_2 (10 mL). The reaction mixture was stirred at 0 °C for 15 min and then kept at room temperature for 12 h, and quenched by the addition of water (20 mL). The organic layer was washed with 15 mL brine, dried over anhydrous Na_2SO_4 and filtered, and then the filtrate was concentrated in vacuo. The resulted residue was chromatographed on silica gel eluting with n-hexane/ethyl acetate (5/1-2/1) to give the desired compound **2a** as faint yellow solid (2.3 g, 74 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.73 (s, 1H), 8.20 (d, J = 8.9 Hz, 1H), 7.70 (d, J = 8.9 Hz, 2H), 7.42 (d, J = 8.8 Hz, 2H), 7.29 – 7.20 (m, 2H), 3.94 (s, 3H).

*5.2.3 5-methoxy-N-(4-methoxyphenyl)-2-nitrobenzamide (**2b**)*

Compound **2b** was synthesized from **1** (1.7 g, 8.6 mmol) and 4-methoxyaniline (1.1 g, 8.6 mmol) by the similar manner to that described for **2a** as yellow solid (1.5 g, 75 %).^{1}H NMR (300 MHz, DMSO-d_6): δ 9.83 (s, 1H), 8.16 (d, J = 9.1 Hz, 1H), 8.01 (d, J = 7.7 Hz, 1H), 7.26 – 7.12 (m, 3H), 7.07 (d, J = 7.5 Hz, 1H), 6.98 (t, J = 7.5 Hz, 1H), 3.93 (s, 3H), 3.80 (s, 3H).

*5.2.4 N-(4-fluorophenyl)-5-methoxy-2-nitrobenzamide (**2c**)*

Compound **2c** was synthesized from **1** (1.8 g, 9.1 mmol) and 4-fluoroaniline (1.0 g, 9.1 mmol) by the similar manner to that described for **2a** as yellow solid (2.0 g, 75 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.68 (s, 1H), 8.25 (d, J = 8.9 Hz, 1H), 7.73 (d, J = 8.9 Hz, 2H), 7.48 (d, J = 8.8 Hz, 2H), 7.15 (m, 2H), 3.93 (s, 3H).

*5.2.5 N-(4-(tert-butyl)phenyl)-5-methoxy-2-nitrobenzamide (**2d**)*

Compound **2d** was synthesized from **1** (1.8 g, 9.1 mmol) and 4-(tert-butyl)aniline (1.4 g, 9.1 mmol) by the similar manner to that described for **2a** as yellow solid (2.7 g, 90 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 8.32 (d, J = 9.0 Hz, 1H), 7.71 (d, J = 8.6 Hz, 2H), 7.52-7.47 (m, 3H), 7.22 (dd, J = 9.1, 2.8 Hz, 1H), 3.87 (s, 3H), 1.31 (s, 9H).

*5.2.6 N-(3-fluorophenyl)-5-methoxy-2-nitrobenzamide (**2e**)*

Compound **2e** was synthesized from **1** (2.0 g, 10.1 mmol) and 3-fluoroaniline (1.1 g, 10.1 mmol) by the similar manner to that described for **2a** as yellow solid (1.6 g, 65 %).^{1}H NMR (300 MHz, DMSO-d_6): δ 10.72 (s, 1H), 8.11 (d, J = 9.0 Hz, 1H), 7.78 (s, 1H), 7.54 (d, J = 8.2 Hz, 1H), 7.48– 7.7.35 (m, 4H), 3.94 (s, 3H).

*5.2.7 5-methoxy-N-(2-methoxyphenyl)-2-nitrobenzamide (**2f**)*

Compound **2f** was synthesized from **1** (2.0 g, 10.1 mmol) and 2-methoxyaniline (1.3 g, 10.1 mmol) by the similar manner to that described for **2a** as yellow solid (2.7 g, 86 %).^{1}H NMR (300 MHz, DMSO-d_6): δ 10.58 (s, 1H), 8.32 (d, J = 9.6 Hz, 1H), 7.51-7.46 (m, 4H), 7.17 (d, J = 8.2 Hz, 1H), 6.91(s 1H), 3.91(s, 3H), 3.76 (s, 3H).

*5.2.8 2-amino-N-(4-chlorophenyl)-5-methoxybenzamide (**3a**)*

A mixture of compound **2a** (1.0 g, 3.3 mmol) and Pd/C (10%, 0.1 g) in ethanol (15 mL) was stirred under 1 atm of H_2 at room temperature for 15 h. The reaction was filtered through a celite pad, and the filtrate was concentrated to give crude product, which was chromatographed on silica gel eluting with CH_2Cl_2/MeOH (100/1-60/1) to give the desired compound **3a** as white solid (0.7 g, 80 %). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.11 (s, 1H), 7.74 (d, J = 8.6 Hz, 2H), 7.39 (d, J = 8.6 Hz, 2H), 7.17 (s, 1H), 6.94 (s, 1H), 6.74 (s, 1H), 5.93 (s, 2H), 3.73 (s, 3H).

*5.2.9 2-amino-5-methoxy-N-(4-methoxyphenyl)benzamide (**3b**)*

Compound **3b** was prepared from **2b** (1.1 g, 3.6 mmol) by the similar method to that described for **3a** as white solid (0.8 g, 81 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.87 (s, 1H), 7.58 (d, J = 8.9 Hz, 2H), 7.16 (d, J = 2.7 Hz, 1H), 6.96 – 6.86 (m, 3H), 6.71 (d, J = 8.9 Hz, 1H), 5.84 (s, 2H), 3.74 (s, 3H), 3.72 (s, 3H).

*5.2.10 2-amino-N-(4-fluorophenyl)-5-methoxybenzamide (**3c**)*

Compound **3c** was prepared from **2c** (1.0 g, 3.5 mmol) by the similar method to that described for **3a** as white solid (0.6 g, 67 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.64 (s, 1H), 7.67 (d, J = 9.0 Hz, 1H), 7.59 – 7.35 (m, 3H), 7.22 (d, J = 9.0, 2H), 6.96 (s, 1H), 3.86 (s, 3H).

*5.2.11 2-amino-N-(4-(tert-butyl)phenyl)-5-methoxybenzamide (**3d**)*

Compound **3d** was prepared from **2d** (0.7 g, 2.1 mmol) by the similar method to that described for **3a** as white solid (0.4 g, 63%). ^{1}H NMR (300 MHz, DMSO-d6): δ 10.46 (s, 1H), 7.93 (d, J = 8.7 Hz, 2H), 7.63 (d, J = 8.6 Hz, 2H), 7.45 (d, J = 2.8 Hz, 1H), 7.38 (d, J = 8.7 Hz, 1H), 7.22 (s, 1H), 6.01 (s, 2H), 3.87 (s, 3H).

*5.2.12 2-amino-N-(3-fluorophenyl)-5-methoxybenzamide (**3e**)*

Compound **3e** was prepared from **2e** (0.8 g, 2.8 mmol) by the similar method to that described for **3a** as white solid (0.5 g, 70%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.62 (s, 1H), 7.88 (d, J = 9.0 Hz, 2H), 7.66 (m, 1H), 7.51 – 7.38 (m, 5H), 6.96(m, 1H), 5.94(s, 2H), 3.87 (s, 3H).

*5.2.13 2-amino-5-methoxy-N-(2-methoxyphenyl)benzamide (**3f**)*

Compound **3f** was prepared from **2f** (1.0 g, 3.3 mmol) by the similar method to that described for **3a** as white solid (0.4 g, 44%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.75 (s, 1H), 7.73 (m, 1H), 7.28-7.16 (m, 3H), 7.07 (s, 1H), 7.00 (s, 1H), 6.87 (s, 1H), 3.84 (s, 3H), 3.76 (s, 3H).

*5.2.14 ethyl 4-(2-oxopiperidin-1-yl)benzoate (**5a**)*

Compound **4** (5.0 g, 18 mmol) was dissolved in toluene (100 mL), followed by the addition of piperidin-2-one (3.6 g, 36 mmol), anhydrous potassium phosphate (7.8 g, 36 mmol), CuI (0.34 g, 1.8 mmol) and N,N'-dimethylethanediamine (0.39 mL, 3.6 mmol). The mixture was degassed and refilled with N_2 three times and was heated to 115 °C under N_2 for 16 h, cooled, and quenched with water (100 mL). The organics were extracted with ethyl acetate (2 × 75 mL) and dried with Na_2SO4. Purification by silica gel column chromatography (n-hexane/ethyl acetate, 6/1 to 2/1, as eluent) afforded compound **5a** as off-white solid (3.5 g, 65 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07-8.02 (m, 2H), 7.38-7.33 (m, 2H), 4.36 (q, J = 7.1 Hz, 2H), 3.70-3.65 (m, 2H), 2.60-2.55 (m, 2H), 2.03-1.90 (m, 4H), 1.39 (t, J = 7.1 Hz, 3H).

*5.2.15 ethyl 4-(3-oxomorpholino)benzoate (**5b**)*

Compound **5b** was prepared by the similar method to that described for **5a** as faint yellow solid (4.0 g, 69%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.11(d, J = 9.0 Hz, 2H), 7.48(d, J = 9.0 Hz, 2H), 4.43-4.36 (m, 4H), 4.07(t, J = 4.7 Hz, 2H), 3.83 (t, J = 5.2 Hz, 2H), 1.41(t, J = 7.1 Hz, 3H).

*5.2.16 ethyl 4-(2-oxopyridin-1(2H)-yl)benzoate(**5c**)*

Compound **5c** was prepared by the similar method to that described for **5a** as yellow solid (2.1 g, 52%).^{1}H NMR (300 MHz, $CDCl_3$): δ 8.38 (m, 1H), 8.13 (m, 1H), 7.95 – 7.77 (m, 2H), 7.44 (m, 1H), 7.39 – 7.11 (m, 2H), 6.06 (m, 1H), 4.31 (q, J = 7.1 Hz, 2H), 1.41 (t, J = 7.2 Hz, 3H).

*5.2.17 ethyl 4-(2-oxopyrrolidin-1-yl)benzoate(**5d**)*

Compound **5d** was prepared by the similar method to that described for **5a** as white solid (3.6 g, 71%).^{1}H NMR (300 MHz, $CDCl_3$): δ 8.90 – 8.69 (m, 2H), 8.04 – 7.68 (m, 2H), 4.30 (q, J = 7.2Hz, 2H), 3.79 (m, 2H), 2.45 (m, 2H), 2.18 – 1.87 (m, 2H), 1.30 (t, J = 7.2 Hz, 3H).

*5.2.18 4-(2-oxopiperidin-1-yl)benzoic acid (**6a**)*

Compound **5a** (3.0 g, 12.1 mmol) was dissolved in methanol (36 mL). To the solution was added 1 M NaOH (5 mL), and the reaction mixture was stirred at room temperature for 1 h. The

mixture was adjusted to pH 5 with 1 M hydrochloric acid. The resulting precipitate was filtered, washed with H_2O/EtOH (6 mL, v/v, 2:1), and dried to afford the desired product which was pure enough for the next step (2.4 g, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07-8.02 (m, 2H), 7.38-7.33 (m, 2H), 4.36 (q, J = 7.1 Hz, 2H), 3.70-3.65 (m, 2H), 2.60-2.55 (m, 2H), 2.03-1.90 (m, 4H), 1.39 (t, J = 7.1 Hz, 3H).

*5.2.19 4-(3-oxomorpholino)benzoic acid (**6b**)*

Compound **6b** was prepared by the similar method to that described for **6a** as colorless solid (2.1 g, 81%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.96 (s, 1H), 7.95 (d, J = 9.0 Hz, 2H), 7.57 (d, J = 9.0 Hz, 2H), 4.23 (s, 2H), 3.98 (t, J = 4.6 Hz, 2H), 3.80 (t, J = 5.2 Hz, 2H).

*5.2.20 N-(4-chlorophenyl)-5-methoxy-2-(4-(2-oxopiperidin-1-yl)benzamido)benzamide (**7a**)*

To a solution of **6a** (95 mg, 0.43 mmol) and EDCI (91 mg, 0.48 mmol) in DMF (2 mL) at 0 °C was added **3a** (120 mg, 0.43 mmol) and DIPEA (91 μL, 0.52 mmol) in DMF (1 mL). The mixture was stirred for 24 h at room temperature. The reaction was quenched by addition of cold water (3 mL). The resulting precipitate was filtered, washed with water (5 mL), and dried to afford the crude product which was purified by chromatography on silica gel with CH2Cl2/MeOH (30:1) afforded **7a** as colorless solid (170 mg, 82%). ^{1}H NMR (300MHz, DMSO-d_6): δ 11.23 (s, 1H), 10.89 (s, 1H), 8.17 (d, J = 9.0 Hz, 1H), 7.90 (d, J = 8.5 Hz, 2H), 7.81 (d, J = 8.8 Hz, 2H), 7.43 (dd, J = 16.5, 8.8 Hz, 5H), 7.18 (dd, J = 9.0, 2.8 Hz, 1H), 3.87 (s, 3H), 3.66 (t, J = 5.2 Hz, 2H), 2.42 (t, J = 6.0 Hz, 2H), 1.97 – 1.75 (m, 4H); ^{13}C NMR (75 MHz, DMSO -d_6): δ 169.03, 166.80, 164.04, 155.03, 146.30, 137.72, 131.81, 131.21, 128.41, 127.56, 127.46, 125.81, 123.98, 122.52, 117.53, 115.63, 113.92, 55.60, 50.31, 32.66, 22.87, 20.75; MS (ESI) *m/z*: 500.2 $[M+H]^+$; Anal. calcd. for $C_{26}H_{24}ClN_3O_4$: C, 65.34; H, 5.06; N, 8.79. Found: C, 65.31; H, 5.08; N, 8.76.

*5.2.21 5-methoxy-N-(4-methoxyphenyl)-2-(4-(2-oxopiperidin-1-yl)benzamido)benzamide (**7b**)*

Compound **7b** was prepared by the similar method to that described for **7a** as colorless solid (112 mg, 90%). mp: 236-239 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.47 (s, 1H), 10.40 (s, 1H), 8.35 (d, J = 9.1 Hz, 1H), 7.88 (d, J = 8.5 Hz, 2H), 7.61 (d, J = 9.0 Hz, 2H), 7.46 (dd, J = 8.1, 5.7 Hz, 3H), 7.21 (dd, J = 9.1, 2.8 Hz, 1H), 6.95 (d, J = 9.0 Hz, 2H), 3.86 (s, 3H), 3.75 (s, 3H), 3.66 (t, J = 5.3 Hz, 2H), 2.43 (t, J = 6.1 Hz, 2H), 1.94 – 1.76 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.02, 166.57, 163.77, 156.02, 154.80, 146.52, 131.74, 131.66, 131.30, 127.31, 125.89, 124.46, 123.11, 122.83, 117.36, 113.77, 55.55, 55.17, 50.28, 32.66, 22.87, 20.76; MS (ESI) *m/z*: 496.2 $[M+Na]^+$; Anal. calcd. for $C_{27}H_{27}N_3O_5$: C, 68.49; H, 5.75; N, 8.87. Found: C, 68.41; H, 5.78; N, 8.82.

*5.2.22 N-(4-fluorophenyl)-5-methoxy-2-(4-(2-oxopiperidin-1-yl)benzamido)benzamide (**7c**)*

Compound **7c** was prepared by the similar method to that described for **7a** as colorless solid (114 mg, 83%). mp: 212-215 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.00 (s, 1H), 10.64 (s, 1H), 8.15 (d, J = 9.0 Hz, 1H), 7.89 (d, J = 8.6 Hz, 2H), 7.67 (dd, J = 11.6 Hz, 2.1 Hz, 1H), 7.57 – 7.32 (m, 5H), 7.22 (dd, J = 9.0, 2.9 Hz, 1H), 6.96 (td, J = 8.4, 2.2 Hz, 1H), 3.86 (s, 3H), 3.66 (t, J = 5.3 Hz, 2H), 2.43 (t, J = 6.1 Hz, 2H), 1.97 – 1.78 (m, 4H); ^{13}C NMR (75

MHz, DMSO-d_6): δ 169.03, 166.74, 163.92, 154.94, 146.50, 134.84, 131.74, 131.39, 127.38, 125.86, 125.05, 123.55, 122.98, 122.87, 117.35, 115.36, 115.07, 113.93, 55.56, 50.29, 32.66, 22.87, 20.76; MS (ESI) *m/z*: 484.2 $[M+Na]^+$; Anal. calcd. for $C_{26}H_{24}FN_3O_4$: C, 67.67; H, 5.24; N, 9.11. Found: C, 67.63; H, 5.28; N, 9.14.

*5.2.23 N-(4-(tert-butyl)phenyl)-5-methoxy-2-(4-(2-oxopiperidin-1-yl)benzamido)benzamide (**7d**)*

Compound **7d** was prepared by the similar method to that described for **7a** as colorless solid (88 mg, 90%). mp: 238-242 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.39 (s, 1H), 10.46 (s, 1H), 8.32 (d, J = 9.0 Hz, 1H), 7.89 (d, J = 8.5 Hz, 2H), 7.62 (d, J = 8.6 Hz, 2H), 7.46 (dd, J = 11.7 Hz, 5.6 Hz, 3H), 7.38 (d, J = 8.6 Hz, 2H), 7.22 (dd, J = 9.1, 2.8 Hz, 1H), 3.86 (s, 3H), 3.66 (s, 2H), 2.42 (t, J = 6.0 Hz, 2H), 1.86 (d, J = 2.6 Hz, 4H), 1.28 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.00, 166.77, 163.80, 154.82, 146.65, 146.52, 135.79, 131.70, 131.54, 127.32, 125.88, 125.23, 124.74, 123.23, 120.90, 117.30, 113.96, 55.55, 50.27, 33.96 32.66, 31.13, 22.87, 20.77; MS (ESI) *m/z*: 522.3 $[M+Na]^+$; Anal. calcd. for $C_{30}H_{33}N_3O_4$: C, 72.12; H, 6.66; N, 8.41. Found: C, 72.17; H, 6.58; N, 8.46.

*5.2.24 N-(3-fluorophenyl)-5-methoxy-2-(4-(2-oxopiperidin-1-yl)benzamido)benzamide (**7e**)*

Compound **7e** was prepared by the similar method to that described for **7a** as colorless solid (91 mg, 69%). mp: 202-205 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.98 (s, 1H), 10.62 (s, 1H), 8.15 (d, J = 9.0 Hz, 1H), 7.88 (d, J = 9.0 Hz, 2H), 7.66 (d, J = 9.0 Hz, 2.1 Hz, 1H), 7.52 – 7.37 (m, 5H), 7.20 (m, 1H), 6.94 (m, 1H), 3.86 (s, 3H), 3.65 (t, J = 5.3 Hz, 2H), 2.42 (t, J = 6.1 Hz, 2H), 1.85 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.04, 166.87, 164.05, 155.22, 146.41, 140.29, 131.60, 130.84, 130.20, 127.47, 125.84, 124.10, 117.15, 116.26, 113.99, 110.66, 107.08, 55.57, 50.30, 32.66, 22.87, 20.77; MS (ESI) *m/z*: 484.2 $[M+H]^+$; Anal. calcd. for $C_{26}H_{24}FN_3O_4$: C, 67.67; H, 5.24; N, 9.11. Found: C, 67.72; H, 5.28; N, 9.13.

*5.2.25 5-methoxy-N-(2-methoxyphenyl)-2-(4-(2-oxopiperidin-1-yl)benzamido)benzamide (**7f**)*

Compound **7f** was prepared by the similar method to that described for **7a** as colorless solid (96 mg, 79%). mp: 221-224 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.49 (s, 1H), 9.77 (s, 1H), 8.26 (d, J = 9.0 Hz, 1H), 7.87 (d, J = 9.0 Hz, 2H), 7.69 (d, J = 9.0 Hz, 1H), 7.47 (dd, J = 8.1, 5.7 Hz, 3H), 7.21 (m, 2H), 6.95 (m, 2H), 3.85 (s, 3H), 3.70 (s, 3H), 3.64 (s, 2H), 2.42 (s, 2H), 1.86 (s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.03, 166.78, 163,45, 156.13, 150.80, 146.89, 134.71, 132.56, 128.31, 127.67, 125.93, 125.87, 125.45, 123.83, 123.45, 121.78, 120.22, 115.36, 112.87, 55.55, 55.27, 50.29, 32.66, 22.85, 21.76; MS (ESI) *m/z*: 496.2 $[M+Na]^+$; Anal. calcd. for $C_{27}H_{27}N_3O_5$: C, 68.49; H, 5.75; N, 8.87. Found: C, 68.43; H, 5.72; N, 8.84.

*5.2.26 N-(4-chlorophenyl)-5-methoxy-2-(4-(3-oxomorpholino)benzamido)benzamide (**7g**)*

Compound **7g** was prepared by the similar method to that described for **7a** as colorless solid (54 mg, 50%). mp: 202- 204 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.28 (s, 1H), 10.50 (s, 1H), 8.23 (dd, J = 22.8, 9.0 Hz, 1H), 8.01 – 7.86 (m, 2H), 7.74 (dd, J = 11.9, 8.4 Hz, 2H), 7.62 (dd, J = 8.6, 1.9 Hz, 2H), 7.41 (dd, J = 6.8, 4.9 Hz, 2H), 7.27 – 7.07 (m, 2H), 4.25 (s, 2H), 4.08 – 3.94 (m, 2H), 3.86 (d, J = 1.4 Hz, 3H), 3.83 – 3.71 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.81, 166.12, 163.74, 154.97, 144.43, 138.48, 132.04, 131.29, 128.61, 128.52, 127.60, 127.55, 124.91, 122.37, 121.02, 117.32, 113.97, 67.73, 63.38, 55.56, 48.44; MS (ESI)

m/z: 478.1 $[M-H]^+$; Anal. calcd. for $C_{25}H_{22}ClN_3O_5$: C, 62.57; H, 4.62; N, 8.76. Found: C, 62.51; H, 4.68; N, 8.81.

*5.2.27 5-methoxy-N-(4-methoxyphenyl)-2-(4-(3-oxomorpholino)benzamido)benzamide (**7h**)*

Compound **7h** was prepared by the similar method to that described for **7a** as colorless solid (89 mg, 42%). mp: 191- 193 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.54 (s, 1H), 10.53 (s, 1H), 8.33 (d, J = 9.0 Hz, 1H), 7.93 (d, J = 8.7 Hz, 2H), 7.63 (dd, J = 8.6, 6.3 Hz, 4H), 7.49 (d, J = 2.6 Hz, 1H), 7.20 (dd, J = 9.1, 2.8 Hz, 1H), 6.94 (d, J = 9.0 Hz, 2H), 4.24 (s, 2H), 3.99 (s, 2H), 3.87 (s, 3H), 3.84 – 3.77 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.56, 166.13, 163.67, 155.98, 154.87, 144.43, 132.08, 131.59, 131.37, 127.50, 124.91, 124.63, 123.23, 122.85, 117.44, 113.80, 113.73, 67.73, 63.38, 55.57, 55.17, 48.43; MS (ESI) *m/z*: 498.2 $[M+Na]^+$; Anal. calcd. for $C_{26}H_{25}N_3O_6$: C, 65.68; H, 5.30; N, 8.84. Found: C, 65.65; H, 5.81; N, 8.87.

*5.2.28 N-(4-(tert-butyl)phenyl)-5-methoxy-2-(4-(3-oxomorpholino)benzamido)benzamide (**7i**)*

Compound **7i** was prepared by the similar method to that described for **7a** as colorless solid (87 mg, 63%). mp: 208-211 °C; ^{1}H NMR (300MHz, DMSO-d_6): δ 11.42 (s, 1H), 10.46 (s, 1H), 8.33 (d, J = 9.0 Hz, 1H), 7.93 (d, J = 8.7 Hz, 2H), 7.63 (d, J = 8.6 Hz, 4H), 7.45 (d, J = 2.8 Hz, 1H), 7.38 (d, J = 8.7 Hz, 2H), 7.22 (dd, J = 9.1, 2.9 Hz, 1H), 4.25 (s, 2H), 3.99 (dd, J = 5.9, 4.0 Hz, 2H), 3.87 (s, 3H), 3.83 – 3.76 (m, 2H), 1.28 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.77, 166.11, 163.68, 154.86, 146.66, 144.44, 135.79, 132.02, 131.50, 127.49, 125.23, 124.90, 124.79, 123.26, 120.90, 117.28, 113.98, 67.73, 63.37, 55.54, 48.42, 34.06, 31.13; MS (ESI) *m/z*: 524.3 $[M+Na]^+$; Anal. calcd. for $C_{29}H_{31}N_3O_5$: C, 69.44; H, 6.23; N, 8.38. Found: C, 69.49; H, 6.19; N, 8.32.

*5.2.29 5-methoxy-N-(2-methoxyphenyl)-2-(4-(3-oxomorpholino)benzamido)benzamide (**7j**)*

Compound **7j** was prepared by the similar method to that described for **7a** as colorless solid (101 mg, 61%). mp: 203-206 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.57 (s, 1H), 9.81 (s, 1H), 8.28 (d, J = 9.0 Hz, 1H), 7.93 (d, J = 8.6 Hz, 2H), 7.72 (d, J = 6.8 Hz, 1H), 7.61 (d, J = 8.6 Hz, 2H), 7.54 (d, J = 2.4 Hz, 1H), 7.31 – 7.16 (m, 2H), 7.07 (d, J = 7.5 Hz, 1H), 6.99 (t, J = 7.6 Hz, 1H), 4.24 (s, 2H), 4.06 – 3.93 (m, 2H), 3.86 (s, 3H), 3.83 – 3.75 (m, 2H), 3.71 (s, 3H); ^{13}C NMR (75 MHz, DMSO-*d*) δ 166.51, 166.11, 163.97, 155.24, 151.84, 144.49, 131.83, 131.32, 127.56, 126.38, 126.04, 124.97, 124.89, 123.93, 120.22, 117.89, 113.60, 111.43, 67.73, 63.36, 55.54, 55.50, 48.42; MS (ESI) *m/z*: 498.2 $[M+H]^+$; Anal. calcd. for $C_{26}H_{25}N_3O_6$: C, 65.68; H, 5.30; N, 8.84. Found: C, 65.60; H, 5.35; N, 8.87.

*5.2.30 N-(4-chlorophenyl)-5-methoxy-2-(4-(2-oxopyridin-1(2H)-yl)benzamido)benzamide(**7k**)*

Compound **7k** was prepared by the similar method to that described for **7a** as faint yellow solid (76 mg, 46%). mp: 215-218 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.54 (s, 1H), 10.50 (s, 1H), 8.30 (m, 1H), 8.13 (m, 1H), 7.81 – 7.65 (m, 5H), 7.56 (m 1H), 7.31 (m, 5H), 6.08 (m, 1H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, DMSO-*d*) δ 166.58, 166.70, 165.82, 160.16, 144.92, 139.17, 138.48, 136.72, 136.46, 134.70, 130.37, 128.67, 128.23, 125.66, 123.46, 123.25, 122.88, 121.71, 121.23, 115.24, 110.25, 56.18; MS (ESI) *m/z*: 474.1 $[M+H]^+$; Anal. calcd. for : $C_{26}H_{20}ClN_3O_4$: C, 65.90; H, 4.25; N, 8.87; Found: C, 65.81; H, 4.30; N, 8.81.

*5.2.31 N-(4-chlorophenyl)-5-methoxy-2-(4-(2-oxopyrrolidin-1-yl)benzamido)benzamide(**7l**)*

Compound **7l** was prepared by the similar method to that described for **7a** as white yellow solid (91 mg, 74%). mp: 187-191 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.92 (s, 1H), 10.60 (s, 1H), 8.10 (d, J = 9.0 Hz, 1H), 7.83 (d, J = 9.0 Hz, 2H), 7.61 (d, J = 9.0 Hz, 2.1 Hz, 1H), 7.50 – 7.33(m, 5H), 7.18 (m, 1H), 6.92 (m, 1H), 3.89 – 3.65 (m, 5H), 2.45 (m, 2H), 2.30 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.16, 166.80, 164.82, 155.62, 146.20, 140.20, 131.51, 130.62, 130.41, 127.32, 125.75, 124.32, 117.20, 116.31, 113.63, 110.76, 108.14, 55.68, 45.14, 31.46, 18.87; MS (ESI) *m/z*: 464.1 $[M+H]^+$; Anal. calcd. for : $C_{26}H_{20}ClN_3O_4$: C, 64.73; H, 4.78; N, 9.06; Found: C, 64.79; H, 4.70; N, 8.76.

*5.2.32 tert-butyl 3-bromo-4-oxopiperidine-1-carboxylate (**9**)*

To a solution of *N*-(tert-Butoxycarbonyl)-4-piperidone (8 g, 48.2 mmol) in CH_2Cl_2 (80 mL) was added dropwise a solution of bromine (7.06 g, 44.17 mmol) in CH_2Cl_2 (80 mL) over a period of 1 h. After addition, the reaction mixture was stirred for 0.5 h at room temperature. The reaction mixture was quenched by the addition of 10% aqueous sodium bisulfite. The layers were separated, and the organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and filtered, and then the filtrate was concentrated in vacuo. The residue was recrystallized from ethyl acetate/petroleum (16 mL, v/v, 1:1) to afford **9** as colorless solid (4.5 g, 41%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.35 (br, 1H), 4.03 (br, 2H), 3.50 (br, 2H), 3.03 (br, 1H), 2.43 (m, 1H), 1.52 (s, 9H).

*5.2.33 5-(tert-butyl) 2-ethyl 6,7-dihydrothiazolo[5,4-c]pyridine-2,5(4H)-dicarboxylate (**10**)*

Compound **9** (4.0 g, 14.4 mmol) was dissolved in IPA (25 mL). Ethyl thioxamate (1.60 g, 12.0 mmol) and $NaHCO_3$ (2.01 g, 24.0 mmol) were added. The mixture was refluxed for 12 h, followed by concentration. The residue was taken in water and extracted with dichloromethane. The organic layer was washed with water, brine and dried over anhydrous Na_2SO_4 and concentrated to the desired compound (2.2 g, 60%), which is pure enough for the next step. mp: 138-140 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.71(s, 2H), 4.48(q, J = 12Hz, 2H,), 3.72(m, 2H), 2.96(t, J = 5.4Hz, 2H), 2.41(t, J = 6.3Hz, 2H), 1,51(s, 9H), 1,44(t, J = 1.2Hz, 3H,); MS (ESI) *m/z*: 335.1 $[M+Na]^+$.

*5.2.34 Ethyl 4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine-2-carboxylate (**11**)*

To a solution of **10** (2.23 g, 7.1 mmol) in AcOEt (15 mL) was added trifluoroacetic acid (1.6 mL, 21.3 mmol). The mixture was stirred at room temperature for 3 h. Then, the solution was evaporated, and the residue was triturated with Et_2O, filtrated and dried in vacuo to give compound **11** as a paint yellow solid (1.62 g, 91%), which is pure enough for the next step. mp: 148-151 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.32 (s, 2H), 4.21 (m, 5H), 3.32 (s, 2H), 2.76 (s, 2H), 1.41 (s, 2H), 1.32 (s, 3H); MS (ESI) *m/z*: 213.2 $[M+H]^+$.

*5.2.35 Ethyl 5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine-2-carboxylate (**12**)*

To a solution of **11** (1.6 g, 7.1 mmol) and triethyl amine (1.5 mL, 10.6 mmol eq) in CH_2Cl_2 (15 mL) was added iodomethane (0.48 mL, 7.8 mmol) and the reaction mixture was stirred for l8 h at 30 °C. Reaction mixture was then diluted with CH_2Cl_2 (10 mL) and washed with water. Organic layer was then separated, dried and concentrated under reduced pressure to afford the

desired compound (1.4 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$,): δ 4.91-4.78（m, 2H）, 4.49 (d, J = 7.1 Hz, 2H) 3.99-3.80（m, 2H）, 3.09-3.00（m, 2H）, 2.46（s, 3H）, 1.44（t, J = 7.1 Hz, 3H）.

*5.2.36 5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine-2-carboxylic acid (**13**)*

Compound **12** (1.3 g, 5.8 mmol) was dissolved in methanol (10 mL). To this solution was added 1 M NaOH (5 mL), and the reaction mixture was stirred at 0 °C for 1.5 h. The mixture was adjusted to pH 4 with 0.5 M hydrochloric acid. The methanol was removed in vacuo, and the resulting precipitate was filtered, washed with H_2O/EtOH (9 mL, v/v, 2:1), and dried to afford the desired product. ^{1}H-NMR (DMSO-*d*6): δ2.36 (3H, s), 2.63-2.76 (4H, m), 3.54 (2H, s).

*5.2.37 N-(4-ethynylphenyl)-5-methoxy-2-nitrobenzamide (**14a**)*

Compound **14a** was prepared by the similar method to that described for **2a** as yellow solid (0.56 g, 75%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.75 (s, 1H), 8.20 (d, J = 9.1 Hz, 1H), 7.77 – 7.67 (m, 3H), 7.31 – 7.17 (m, 1H), 7.16 (d, J = 8.1 Hz, 2H), 3.94 (s, 3H), 2.55 (s, 1H).

*5.2.38 5-methoxy-N-(3-methoxyphenyl)-2-nitrobenzamide (**14b**)*

Compound **14b** was prepared by the similar method to that described for **2a** as faint yellow solid (0.51 g, 67%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.54 (s, 1H), 8.19 (d, J = 9.6 Hz, 1H), 7.35 (s, 1H), 7.31 – 7.16 (m, 4H), 6.70 (d, J = 8.2 Hz, 1H), 3.94 (s, 3H), 3.75 (s, 3H).

*5.2.39 5-methoxy-2-nitro-N-(p-tolyl)benzamide (**14c**)*

Compound **14c** was prepared by the similar method to that described for **2a** as faint yellow solid (0.61 g, 83%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.46 (s, 1H), 8.18 (d, J = 10.0 Hz, 1H), 7.54 (d, J = 8.4 Hz, 2H), 7.27 – 7.20 (m, 2H), 7.16 (d, J = 7.8 Hz, 2H), 3.94 (s, 3H), 2.28 (s, 3H).

*5.2.40 N-(5-fluoropyridin-2-yl)-5-methoxy-2-nitrobenzamide (**14d**)*

Compound **14d** was prepared by the similar method to that described for **2a** as tan solid (0.43 g, 32 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.22 (s, 1H), 8.36 (d, J = 2.2 Hz, 1H), 8.28 – 8.14 (m, 2H), 7.82 (td, J = 8.9, 3.1 Hz, 1H), 7.26 – 7.17 (m, 2H), 3.93 (s, 4H).

*5.2.41 5-methoxy-N-(5-methylpyridin-2-yl)-2-nitrobenzamide (**14e**)*

Compound **14e** was prepared by the similar method to that described for **2a** as yellow solid (0.48 g, 38 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.00 (s, 1H), 8.22 – 8.11 (m, 2H), 8.08 (d, J = 8.5 Hz, 1H), 7.72 – 7.56 (m, 1H), 7.29 – 7.13 (m, 2H), 3.93 (s, 3H), 2.28 (s, 3H).

*5.2.42 N-(5-chloropyridin-2-yl)-5-methoxy-2-nitrobenzamide (**14f**)*

Compound **14f** was prepared by the similar method to that described for **2a** as yellow solid (0.37 g, 28 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.30 (s, 1H), 8.40 (s, 1H), 8.19 (t, J = 8.2 Hz, 2H), 8.06 – 7.87 (m, 1H), 7.24 (s, 1H), 7.20 (d, J = 2.5 Hz, 1H), 3.93 (s, 3H).

*5.2.43 N-(3-chlorophenyl)-5-methoxy-2-nitrobenzamide (**14g**)*

Compound **14g** was prepared by the similar method to that described for **2a** as faint yellow solid (0.63 g, 81 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.78 (s, 1H), 8.19 (s, 1H), 7.87 (s, 1H),

7.56 – 7.15 (m, 5H), 3.94 (s, 3H).

*5.2.44 N-(5-chlorothiophen-2-yl)-5-methoxy-2-nitrobenzamide (**14h**)*

Compound **14h** was prepared by the similar method to that described for **2a** as tan solid (0.25 g, 52 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.65 (s, 1H), 8.05 (d, J = 9.1 Hz, 1H), 8.01 (s, 1H), 7.64 (s, 1H), 7.14 (dd, J = 9.0, 2.8 Hz, 1H), 7.05 (d, J = 2.5 Hz, 1H), 3.90 (s, 3H).

*5.2.45 2-amino-N-(4-ethynylphenyl)-5-methoxybenzamide (**15a**)*

Compound **15a** was prepared by the similar method to that described for **3a** as faint yellow solid (0.32 g, 65 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.92 (s, 1H), 7.86 – 7.69 (m, 1H), 7.69 – 7.49 (m, 1H), 7.45 (d, J = 7.9 Hz, 1H), 7.16 (s, 2H), 6.90 (d, J = 7.5 Hz, 1H), 6.82 – 6.51 (m, 1H), 5.85 (s, 2H), 3.73 (s, 3H), 2.58 (s, 1H).

*5.2.46 2-amino-5-methoxy-N-(3-methoxyphenyl)benzamide (**15b**)*

Compound **15b** was prepared by the similar method to that described for **3a** as faint yellow solid (0.51 g, 89 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.96 (s, 1H), 7.38 (s, 1H), 7.35 – 7.20 (m, 2H), 7.15 (s, 1H), 6.95 – 6.85 (m, 1H), 6.76 – 6.61 (m, 2H), 5.83 (s, 2H), 3.74 (s, 3H), 3.73 (s, 3H).

*5.2.47 2-amino-5-methoxy-N-(p-tolyl)benzamide (**15c**)*

Compound **15c** was prepared by the similar method to that described for **3a** as faint yellow solid (0.45 g, 85 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.91 (s, 1H), 7.57 (d, J = 8.1 Hz, 2H), 7.24 – 7.04 (m, 3H), 6.90 (dd, J = 8.9, 2.6 Hz, 1H), 6.71 (d, J = 9.2 Hz, 1H), 5.84 (s, 2H), 3.72 (s, 3H), 2.28 (s, 4H).

*5.2.48 2-amino-N-(5-fluoropyridin-2-yl)-5-methoxybenzamide (**15d**)*

Compound **15d** was prepared by the similar method to that described for **3a** as faint yellow solid (0.32 g, 72 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.67 (s, 1H), 8.38 (d, J = 9.0 Hz, 1H), 8.12 (m, 1H), 7.77 (m, 1H), 7.28 (d, J = 8.9 Hz, 1H), 6.95 – 6.84 (m, 1H), 6.73 (d, J = 8.5 Hz, 1H), 6.00 (s, 2H), 3.73 (s, 3H).

*5.2.49 2-amino-5-methoxy-N-(5-methylpyridin-2-yl)benzamide (**15e**)*

Compound **15e** was prepared by the similar method to that described for **3a** as faint yellow solid (0.21g, 76 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.47 (s, 1H), 8.20 (s, 1H), 7.99 (d, J = 8.3 Hz, 1H), 7.63 (dd, J = 8.3, 2.0 Hz, 1H), 7.28 (d, J = 2.7 Hz, 1H), 6.88 (dd, J = 8.8, 2.7 Hz, 1H), 6.72 (d, J = 8.8 Hz, 1H), 5.98 (s, 2H), 3.73 (s, 3H), 2.28 (s, 3H).

*5.2.50 2-amino-N-(5-chloropyridin-2-yl)-5-methoxybenzamide (**15f**)*

Compound **15f** was prepared by the similar method to that described for **3a** as faint yellow solid (0.19 g, 63%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.74 (s, 1H), 8.42 (d, J = 2.5 Hz, 1H), 8.15 (d, J = 9.0 Hz, 1H), 7.93 (dd, J = 9.0, 2.7 Hz, 1H), 7.28 (d, J = 2.8 Hz, 1H), 6.91 (dd, J = 8.9, 2.8 Hz, 1H), 6.78 – 6.70 (m, 1H), 6.03 (s, 2H), 3.74 (s, 3H).

*5.2.51 2-amino-N-(3-chlorophenyl)-5-methoxybenzamide (**15g**)*

Compound **15g** was prepared by the similar method to that described for **3a** as faint yellow

solid (0.32 g, 86%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.20 (s, 1H), 7.95 (s, 1H), 7.69 (s, 1H), 7.42 (s, 1H), 7.32 – 7.11 (m, 2H), 7.00 (s, 1H), 6.79 (s, 1H), 5.93 (s, 2H), 3.78 (s, 3H).

*5.2.52 2-amino-N-(5-chlorothiophen-2-yl)-5-methoxybenzamide (**15h**)*

Compound **15h** was prepared by the similar method to that described for **3a** as faint yellow solid (0.15 g, 61%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.13 (s, 1H), 7.83 (d, J = 8.8 Hz, 1H), 7.62 (s, 1H), 7.33 (s, 1H), 7.02 (m, 1H), 6.89 (d, J = 2.5 Hz, 1H), 5.91 (s, 2H), 3.75 (s, 3H).

*5.2.53 N-(4-methoxy-2-(p-tolylcarbamoyl)phenyl)-5-methyl-4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine-2-carboxamide (**16a**)*

To a solution of **15c** (100 mg, 0.39 mmol) and EDCI (82 mg, 0.43 mmol) in DMF (2 mL) at 0 °C was added **13** (77 mg, 0.39 mmol) and DIPEA (82 μL, 0.47 mmol) in DMF (1 mL). The mixture was stirred for 48 h at room temperature. The reaction was quenched by addition of cold water (3 mL). The resulting precipitate was filtered, washed with water (5 mL), and dried to afford the crude product which was purified by chromatography on silica gel with CH_2Cl_2/MeOH (60/1-30/1) afforded **16a** as colorless solid (91 mg, 53%). mp: 183-186 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.53 (s, 1H), 10.52 (s, 1H), 8.37 (d, J = 9.1 Hz, 1H), 7.52 – 7.07 (m, 5H), 6.73 (s, 1H), 3.85 (s, 3H), 3.76 (s, 3H), 3.68 (s, 2H), 2.94 – 2.82 (m, 2H), 2.74 (t, J = 5.5 Hz, 2H), 2.38 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.28, 160.38, 157.19, 154.94, 150.20, 135.78, 133.56, 133.37, 130.34, 129.03, 124.61, 122.74, 121.17, 117.31, 114.04, 55.54, 51.87, 51.43, 44.45, 26.45, 20.49; MS (ESI) *m/z*: 459.2 $[M + Na]^+$; Anal. calcd. for $C_{23}H_{24}N_4O_3S$: C, 63.28; H, 5.54; N, 12.83. Found: C, 63.32; H, 5.57; N, 12.88.

*5.2.54 N-(4-methoxy-2-((4-methoxyphenyl)carbamoyl)phenyl)-5-methyl-4,5,6,7-tetrahydroth iazolo[5,4-c]pyridine-2-carboxamide (**16b**)*

Compound **16b** was prepared by the similar method to that described for **16a** as faint yellow solid (105 mg, 60%). mp: 206-210 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.63 (s, 1H), 10.48 (s, 1H), 8.39 (d, J = 8.9 Hz, 1H), 7.56 (d, J = 8.3 Hz, 2H), 7.41 (s, 1H), 7.19 (d, J = 7.5 Hz, 3H), 3.85 (s, 3H), 3.67 (s, 2H), 2.85 (s, 2H), 2.73 (s, 2H), 2.37 (s, 3H), 2.29 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.09, 160.39, 157.18, 156.03, 154.92, 150.20, 133.55, 131.25, 130.39, 124.45, 122.81, 122.65, 117.29, 113.96, 113.79, 55.55, 55.18, 51.88, 51.44, 44.46, 26.45; MS (ESI) *m/z*: 475.1 $[M+Na]^+$; Anal. calcd. for $C_{23}H_{24}N_4O_4S$: C, 61.05; H, 5.35; N, 12.38. Found: C, 61.12; H, 5.41; N, 12.32.

*5.2.55 N-(2-((4-ethynylphenyl)carbamoyl)-4-methoxyphenyl)-5-methyl-4,5,6,7-tetrahydrothi azolo[5,4-c]pyridine-2-carboxamide (**16c**)*

Compound **16c** was prepared by the similar method to that described for **16a** as faint yellow solid (65 mg, 70%). mp: 221-226 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.64 (s, 1H), 10.49 (s, 1H), 8.39 (d, J = 8.9 Hz, 1H), 7.72 (s, 1H), 7.58 (d, J = 7.6 Hz, 1H), 7.51 (s, 1H), 7.41 (s, 1H), 7.21 (d, J = 7.7 Hz, 2H), 3.85 (s, 3H), 3.67 (s, 2H), 2.85 (s, 2H), 2.73 (s, 2H), 2.58 (d, J = 7.2 Hz, 1H), 2.37 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ166.28, 158.75, 157.19, 154.93, 150.20, 133.55, 132.24, 130.34, 127.85, 124.51, 122.72, 121.23, 120.70, 117.30, 114.09, 81.24, 75.44, 55.55, 51.87, 51.44, 44.45, 26.44; MS (ESI) *m/z*: 445.1 $[M-H]^+$; Anal. calcd. for $C_{24}H_{22}N_4O_3S$: C, 64.56; H, 4.97; N, 12.55. Found: C, 64.58; H, 4.92; N, 12.51.

*5.2.56 N-(4-methoxy-2-((3-methoxyphenyl)carbamoyl)phenyl)-5-methyl-4,5,6,7-tetrahydroth iazolo[5,4-c]pyridine-2-carboxamide (**16d**)*

Compound **16d** was prepared by the similar method to that described for **16a** as faint yellow solid (89 mg, 74%). mp: 194-198 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.56 (s, 1H), 10.49 (s, 1H), 8.37 (d, J = 7.7 Hz, 1H), 7.32 (dd, J = 34.7, 17.4 Hz, 5H), 6.74 (s, 1H), 4.81 (d, J = 13.8 Hz, 2H), 3.81 (m, 8H), 2.90 (m, 2H), 2.11 (s 3H); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 166.25, 160.15, 157.08, 155.03, 151.99, 150.23, 139.59, 139.24, 133.63, 130.12, 129.44, 124.75, 122.88, 117.16, 113.11, 109.74, 106.84, 55.57, 55.04, 51.88, 51.46, 44.42, 26.18; MS (ESI) *m/z*: 475.1 [M+Na]$^+$; Anal. calcd. for $C_{23}H_{24}N_4O_4S$: C, 61.05; H, 5.35; N, 12.38. Found: C, 61.12; H, 5.37; N, 12.35.

*5.2.57 N-(2-((5-fluoropyridin-2-yl)carbamoyl)-4-methoxyphenyl)-5-methyl-4,5,6,7-tetrahydr othiazolo[5,4-c]pyridine-2-carboxamide (**16e**)*

Compound **16e** was prepared by the similar method to that described for **16a** as faint yellow solid (49 mg, 41%). mp: 178-182 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.56 (s, 1H), 11.23 (s, 1H), 8.43 (s, 1H), 8.32 (d, J = 8.9 Hz, 1H), 8.12 (d, J = 5.3 Hz, 1H), 7.84 (s, 1H), 7.47 (s, 1H), 7.19 (d, J = 8.4 Hz, 1H), 3.85 (s, 3H), 3.66 (s, 2H), 2.85 (s, 2H), 2.73 (s, 2H), 2.37 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.98, 160.30, 157.29, 154.98, 150.17, 135.62, 135.29, 133.60, 130.42, 125.38, 125.12, 123.98, 123.06, 118.47, 116.80, 113.90, 55.50, 51.87, 51.43, 44.44, 26.44; MS (ESI) *m/z*: 464.1 [M+Na]$^+$; Anal. calcd. for $C_{21}H_{20}FN_5O_3S$: C, 57.13; H, 4.57; N, 15.86. Found: C, 57.11; H, 4.56; N, 15.84.

*5.2.58 N-(4-methoxy-2-((5-methylpyridin-2-yl)carbamoyl)phenyl)-5-methyl-4,5,6,7-tetrahydr othiazolo[5,4-c]pyridine-2-carboxamide (**16f**)*

Compound **16f** was prepared by the similar method to that described for **16a** as faint yellow solid (32 mg, 33%). mp: 186-189 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.63 (s, 1H), 11.03 (s, 1H), 8.33 (d, J = 9.2 Hz, 1H), 8.25 (s, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 7.9 Hz, 1H), 7.47 (s, 1H), 7.18 (d, J = 8.8 Hz, 1H), 3.86 (s, 3H), 3.68 (s, 2H), 2.87 (s, 2H), 2.75 (s, 2H), 2.38 (s, 3H), 2.30 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.98, 160.26, 157.27, 154.95, 150.16, 149.41, 147.74, 138.48, 133.54, 130.46, 129.27, 124.07, 122.93, 118.42, 115.10, 113.76, 55.48, 51.87, 51.43, 44.43, 26.43, 17.31. MS (ESI) *m/z*: 438.2 [M+H]$^+$; Anal. calcd. for $C_{22}H_{23}N_5O_3S$: C, 60.40; H, 5.30; N, 16.01. Found: C, 60.45; H, 5.32; N, 16.07.

*5.2.59 N-(2-((5-chloropyridin-2-yl)carbamoyl)-4-methoxyphenyl)-5-methyl-4,5,6,7-tetrahydr othiazolo[5,4-c]pyridine-2-carboxamide (**16g**)*

Compound **16g** was prepared by the similar method to that described for **16a** as faint yellow solid (51 mg, 37%). mp: 178-182 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.50 (s, 1H), 11.29 (s, 1H), 8.47 (s, 1H), 8.29 (d, J = 9.2 Hz, 1H), 8.13 (d, J = 8.6 Hz, 1H), 8.00 (d, J = 8.8 Hz, 1H), 7.46 (s, 1H), 7.19 (d, J = 8.3 Hz, 1H), 3.85 (s, 3H), 3.67 (s, 2H), 2.86 (s, 2H), 2.74 (s, 2H), 2.38 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 167.17, 160.27, 157.17, 154.99, 150.37, 150.17, 146.38, 137.86, 133.62, 130.36, 126.02, 124.09, 123.09, 118.55, 116.43, 113.94, 55.50, 51.88, 51.43, 44.45, 26.45; MS (ESI) *m/z*: 458.1 [M+H]$^+$; Anal. calcd. for $C_{21}H_{20}ClN_5O_3S$: C, 55.08; H, 4.40; N, 15.29. Found: C, 55.05; H, 4.46; N, 15.34.

*5.2.60 N-(2-((3-chlorophenyl)carbamoyl)-4-methoxyphenyl)-5-methyl-4,5,6,7-tetrahydrothia zolo[5,4-c]pyridine-2-carboxamide (**16h**)*

Compound **16h** was prepared by the similar method to that described for **16a** as faint yellow solid (62 mg, 68%). mp: 201-204 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.42 (s, 1H), 10.67 (s, 1H), 8.32 (s, 1H), 7.84 (s, 1H), 7.64 (s, 1H), 7.40 (s, 2H), 7.22 (s, 2H), 3.85 (s, 3H), 3.67 (s, 2H), 2.86 (s, 2H), 2.74 (s, 2H), 2.37 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 167.17, 160.28, 157.29, 155.08, 150.35, 150.15, 139.98, 137.87, 133.66, 130.35, 126.02, 124.85, 123.17, 120.39, 117.50, 116.43, 114.20, 55.58, 51.86, 51.42, 44.45, 26.43; MS (ESI) *m/z*: 479.1 $[M+Na]^+$; Anal. calcd. for $C_{22}H_{21}ClN_4O_3S$: C, 57.83; H, 4.63; N, 12.26. Found: C, 57.89; H, 4.68; N, 12.21.

*5.2.61 N-(2-((4-chlorophenyl)carbamoyl)-4-methoxyphenyl)-5-methyl-4,5,6,7-tetrahydrothia zolo[5,4-c]pyridine-2-carboxamide (**16i**)*

Compound **16i** was prepared by the similar method to that described for **16a** as colorless solid (87 mg, 74%). mp: 194-197 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.49 (s, 1H), 10.64 (s, 1H), 8.35 (d, J = 9.1 Hz, 1H), 7.73 (d, J = 8.9 Hz, 2H), 7.45 (d, J = 8.8 Hz, 2H), 7.41 (d, J = 2.8 Hz, 1H), 7.22 (dd, J = 9.2, 2.8 Hz, 1H), 3.85 (s, 3H), 3.68 (s, 2H), 2.86 (t, J = 5.1 Hz, 2H), 2.74 (t, J = 5.6 Hz, 2H), 2.38 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.47, 160.31, 157.22, 155.00, 150.18, 137.40, 133.56, 130.18, 128.58, 127.91, 124.71, 123.00, 122.53, 117.44, 114.18, 55.57, 51.86, 51.42, 44.44, 26.42; MS (ESI) *m/z*: 479.1 $[M+H]^+$; Anal. calcd. for $C_{22}H_{21}ClN_4O_3S$: C, 57.83; H, 4.63; N, 12.26. Found: C, 57.81; H, 4.66; N, 12.29.

*5.2.62 N-(2-((5-chlorothiophen-2-yl)carbamoyl)-4-methoxyphenyl)-5-methyl-4,5,6,7-tetrahy drothiazolo[5,4-c]pyridine-2-carboxamide (**16j**)*

Compound **16j** was prepared by the similar method to that described for **16a** as colorless solid (32 mg, 22%). mp: 166-170 °C; ^{1}H NMR (300MHz, DMSO-d_6): δ ^{1}H NMR (300 MHz, DMSO) δ 11.98 (s, 1H), 11.60 (s, 1H), 8.35 (d, J = 8.8 Hz, 1H), 7.44 (s, 1H), 7.23 (s, 1H), 6.96 (s, 1H), 6.75 (s, 1H), 3.85 (s, 3H), 3.72 (s, 2H), 2.91 (s, 2H), 2.80 (s, 2H), 2.41 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 164.27, 161.38, 157.29, 155.01, 151.88, 150.08, 137.25, 133.28, 130.65, 123.52, 123.23, 120.10, 117.80, 113.92, 111.64, 55.68, 51.80, 51.32, 44.29, 40.33, 40.05, 39.77, 39.50, 39.22, 38.94, 26.36; MS (ESI) *m/z*: 461.1 $[M-H]^+$; Anal. calcd. for $C_{20}H_{19}ClN_4O_3S_2$: C, 51.89; H, 4.14; N, 12.10. Found: C, 51.82; H, 4.20; N, 12.17.

5.3 Biology

5.3.1 In vitro enzyme assays.

In vitro enzymatic activity against human fIIa, fVIIa, fIXa, fXa and trypsin was measured using chromogenic or fluorogenic substrates [17]. The final enzyme concentrations were 0.003 U/mL (human fXa from Enzyme Research Laboratories) and 0.125 U/mL (human thrombin fVIIa, fIXa, and trypsin from Sigma Chemical Co.). The reagents containing 25μL of buffer (0.02 M Bis-Tris；0.7M NaCl; pH 6.0),10 μL compound dilutions and enzyme were mixed, centrifuged, and incubated for 30 min at 37 °C in 96-well microtiter plates. The appropriate substrate was added to initiate enzyme reaction. The time course of the reaction was monitored continuously for 20 min at 405 nm in Synergy H1 MultMode Reader (BioTek, USA). The IC_{50} value was determined by nonlinear regression using GraphPad Prism 5.

5.3.2 In Vitro Anticoagulant Activity.

Commercially available kits were used for the measurement of PT and aPTT[26]. All tested Compounds or DMSO (3 μL) were added to citrated rabbit plasma containing 3.8% sodium citrate (100 μL, v/v, 1:9) and incubated for 10 min at 37 °C. Clotting time was recorded using a coagulometer (A52 Semi-auto Coagulometer 2-channel analyzer) according the manufacturer's instructions and each experiment was repeated three times. Anticoagulant activity was defined as the concentration of inhibitor needed to double PT and aPTT.

5.3.3 In vivo venous thrombosis model assay.

The protocol for this assay was approved by the Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China) and similar to the method reported previously with minor modifications[27, 28]. The vena cava was isolated by using a midline abdominal incision, and the surface was cleared by blunt dissection between the renal and iliolumbar veins. Male rats (n = 10 per dose group) were treated orally with **16g** or betrixaban suspended in 0.5% carboxymethylcellulose sodium (CMC-Na) solution at 1, 5 and 10 mg/kg or vehicle w 60 min before thrombus formation. A 1.5×2.5 mm strip of filter paper saturated with 25% $FeCl_3$ in water was kept on the vena cava for 1.5 min. One hour after application of the filter paper, the vena cava was dissected free. The thrombus was excised, cleaned in saline, blotted of excess blood, and weighed.

5.3.4 In vivo AV-shunt model assays

The protocol for this test was approved by the Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China) and similar to the method reported previously with slightly modifications[16, 27, 28]. The right common carotid artery and left jugular vein were cannulated with 80 mm long, saline-filled polyethylene tube. Then the tube was connected with a 50 mm long polyethylene tube containing rough nylon thread (50 × 0.15 mm) which was folded into a double string. Male rats (n = 10 per dose group) were treated orally with **16g** or betrixaban dissolved in 0.5% carboxymethylcellulose sodium (CMC-Na) solution or vehicle was treated orally 90 min before the shunt was opened for 15 min. The nylon thread was withdrawn rapidly, cleaned in saline, dried and weighed.

5.3.5 Tail-Bleeding Model

Betrixaban, **16g** or vehicle was treated orally 90 min before the tails of anesthetized rats according the reported protocols with minor modifications[26]. Compound **16g**, betrixaban or vehicle was given orally 60 min before the tails of anesthetized rats were transected 4 mm from the tip and immersed vertically immediately into saline at 37 °C. The time until continuous blood flow ceased for >30 s was recorded with a maximum observation time of 30 min. The use of rats in this study was approved by the Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China).

5.3.6 Pharmacokinetic Studies

Compounds **16g** (5% DMSO + 5% Tween-80 in 90% saline) and betrixaban (10% DMSO + 10% Tween-80 + 80% PEG400 (40% solution in saline)) were selected to PK studies on male SD rats (200−225 g) with five animals in each group[28]. Test compounds were administered via

the oral route at 3 mg/kg or administered via the intravenous route at 1 mg/kg. Blood samples were collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48 h after the dose via posterior orbital venous plexus and were into microcentrifuge tubes containing heparin sodium. Then blood samples were centrifuged at 3,000 rpm for 10 min to separate plasma. Serial specimens (0.4 mL) were collected via the retrobulbar vein and quantified by liquid chromatography–mass spectrometry (LC-MS). PK parameters were calculated from the mean plasma concentration by noncompartmental analyses of DAS 3.2.8 statistical software. The protocol for this study was reviewed and approved by the Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China).

5.3.7 MTT assay (Cell Viability Assay)

H9C2 cell viability was measured by cell proliferation Kit (Sigma, St. Louis, MO) according the manufacturer's instructions. The MTT cell viability OD value was detected.

Acknowledgment

This research was supported by the Natural Science Foundation of Jiangsu Province (No. BK 20141349) and the China National Key Hi-tech Innovation Project for the R&D of Novel Drugs (No.2013ZX09301303-002).

References

1. Borensztajn, K, CA Spek. *Expert Opin Ther Targets*, 2011; 15: 341-349.

2. Mann, KG, RJ Jenny, S Krishnaswamy. *Annu Rev Biochem*, 1988; 57: 915-956.

3. Riddel, JP, Jr., BE Aouizerat, C Miaskowski, et al. *J Pediatr Oncol Nurs*, 2007; 24: 123-131.

4. Weitz, JI. *J Thromb Haemost*, 2007; 5 Suppl 1: 65-67.

5. Franco Moreno, AI, RM Martin Diaz, MJ Garcia Navarro. *Med Clin (Barc)*, 2017.

6. Burness, CB, CM Perry. *Drugs*, 2014; 74: 243-262.

7. Agnelli, G, HR Buller, A Cohen, et al. *N Engl J Med*, 2013; 369: 799-808.

8. Lip, GY, G Agnelli. *Eur Heart J*, 2014; 35: 1844-1855.

9. Cohen, AT, RA Harrington, SZ Goldhaber, et al. *N Engl J Med*, 2016; 375: 534-544.

10. Rao, PSS, T Burkart. *Blood Rev*, 2017; 31: 205-211.

11. Mekaj, YH, AY Mekaj, SB Duci, et al. *Ther Clin Risk Manag*, 2015; 11: 967-977.

12. Chan, YH, YH Yeh, HT Tu, et al. *Oncotarget*, 2017; 8: 98898-98917.

13. Spinar, J, L Spinarova. *Vnitr Lek*; 63: 424-430.

14. De Gottardi, A, J Trebicka, C Klinger, et al. *Liver Int*, 2017; 37: 694-699.

15. Trikha, R, PR Kowey. *Cardiology*, 2017; 136: 115-124.

16. Xing, J, L Yang, Y Yang, et al. *Eur J Med Chem*, 2017; 125: 411-422.

17. Xing, J, L Yang, H Li, et al. *Eur J Med Chem*, 2015; 95: 388-399.

18. Tang, L, YY Wu, GY Lip, et al. *Lancet Haematol*, 2016; 3: e30-44.

19. Gurbel, PA, US Tantry. *JACC Heart Fail*, 2014; 2: 1-14.

20. Shantsila, E, GY Lip. *Cochrane Database Syst Rev*, 2016; 9: CD003333.

21. Lee, YK, MR Player. *Med Res Rev*, 2011; 31: 202-283.

22. Klapars, A, X Huang, SL Buchwald. *J Am Chem Soc*, 2002; 124: 7421-7428.

23. Provins, L, F Denonne, S Celanire, et al. *ChemMedChem*, 2012; 7: 2087-2092.

24. Roehrig, S, A Straub, J Pohlmann, et al. *J Med Chem*, 2005; 48: 5900-5908.

25. Xing, J, Q Li, S Zhang, et al. *Chem Biol Drug Des*, 2014; 84: 364-377.

26. Perzborn, E, J Strassburger, A Wilmen, et al. *J Thromb Haemost*, 2005; 3: 514-521.

27. Schumacher, WA, JS Bostwick, AB Stewart, et al. *J Cardiovasc Pharmacol*, 2010; 55: 609-616.

28. Xue, T, S Ding, B Guo, et al. *J Med Chem*, 2014; 57: 7770-7791.

Highlights

- A series of anthranilamide derivatives were designed and synthesized.
- Most of the compounds had significant fXa inhibitoy activity.
- **16g** displayed great *in vitro* and *in vivo* antithrombotic activity and low bleeding risk.
- **16g** exhibited satisfactory PK profile.
- **16g** was found to improve hypoxia–reoxygenation-induced H9C2 cell viability.