Supporting Information

Improved Stability of Proline-derived Direct Thrombin Inhibitors through Hydroxyl to Heterocycle Replacement

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Contents of Supporting Information:

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General Information. All reagents were purchased from Aldrich and used without further purification unless otherwise stated. Column chromatography was carried out on flash silica gel (Merck 230-400 mesh). TLC analysis was conducted on ANALTECH silica gel plates. The LC/MS analyses were performed using a MICROMASS ZMD mass spectrometer coupled to an AGILENT 1100 Series HPLC utilizing a YMC ODS-A 4.6 x 50 mm column eluting at 4.5 mL/min with a solvent gradient of 10 to 95% B over 2.5 min, followed by 0.5 min at 95% B: solvent A = 0.06% TFA in water; solvent B = 0.05% TFA in acetonitrile. ^{1}H-NMR spectra were obtained on a 500 MHz VARIAN Spectrometer in $CDCl_3$, CD_3OD, or Acetone-d_6 as indicated and chemical shifts are reported as δ using the solvent peak as reference and coupling constants are reported in hertz (Hz).

All animal studies described herein were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

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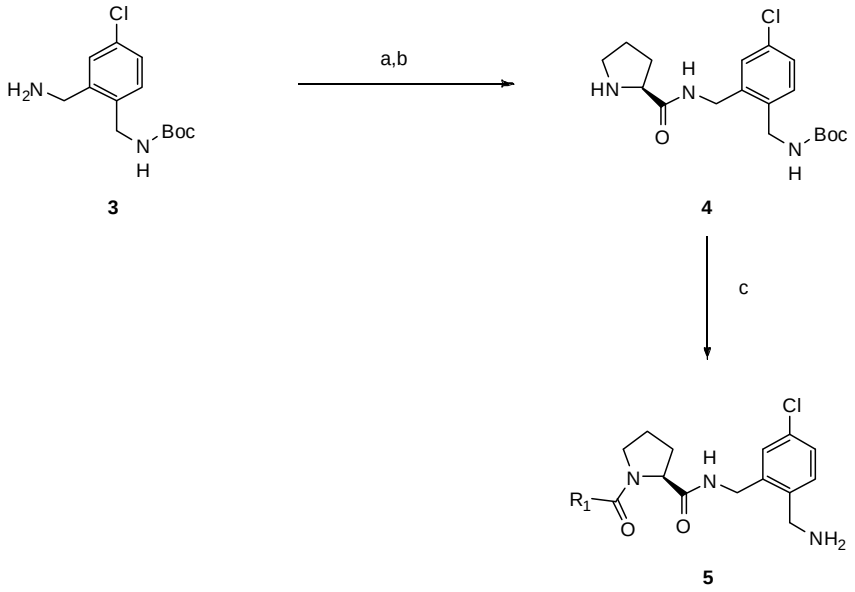
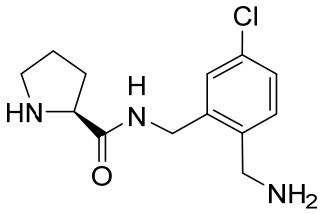
General Scheme:

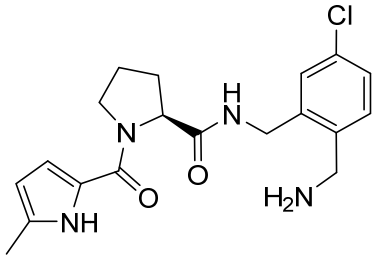


(S)-*N*-(2-(aminomethyl)-5-chlorobenzyl)pyrrolidine-2-carboxamide



For Synthesis of intermediate, see Reference 8.

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(5-methyl-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide (10)



Step 1: 5-Methyl-1H-pyrrole-2-carboxylic acid

A solution of ethyl 5-methyl-1H-pyrrole-2-carboxylate (2.2 g, 14.4 mmol) and 2N NaOH (14.4 mL, 28.7 mmol) in MeOH (46 mL) was heated to 90°C in a sealed tube for 14 hours. The reaction was concentrated and then partitioned between diethyl ether (200 mL) and 0.5M HCl (200 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated to give 1.61 g (90%) of 5-methyl-1H-pyrrole-2-carboxylic acid.

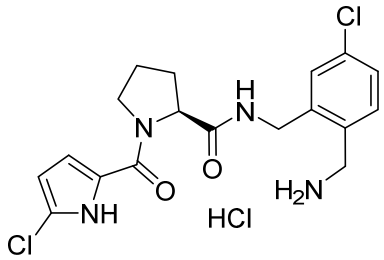
Step 2: (*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(5-methyl-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide hydrochloride

A tube containing 5-methyl-1H-pyrrole-2-carboxylic acid (0.561 g, 4.49 mmol), (*S*)-*tert*-butyl 4-chloro-2-((pyrrolidine-2-carboxamido)methyl)benzylcarbamate (1.5 g, 4.08 mmol),[8] 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (0.860 g, 4.49 mmol) and 1-hydroxybenzotriazole (HOBT) (0.687 g, 4.49 mmol) was treated with a solution of DIEA (1.8 mL, 10.19 mmol) in DCE (27 mL) at rt overnight. The resulting solution was passed through an SPE loaded with 3 mL of water and eluted with DCM. The eluant was concentrated and the residue purified via MPLC (10-65% EAIM/Hex) {EAIM: 80% EtOAc/10% ACN/ 8% IPA/2% MeOH} to 1.79 g of desired product.

Concomitant BOC group removal was accomplished with 25% TFA in DCM (30 mL total volume) overnight at rt. The excess solvent was removed and the residue neutralized by partitioning between DCM and 0.25 N NaOH. The organic layer was dried over $MgSO_4$, filtered and concentrated. The residue was redissolved in diethyl ether and treated with excess 4N HCl in diethyl ether to give the HCl salt after concentration. The salt was redissolved in a minimal amount of DCM with MeOH (<3%) and then hexanes was added to the point of precipitation. The solution was heated to 50°C and then allowed to stand overnight, the crystals were sonicated in the solution and then collected by filtration to give

1.18 g (70%) of **10**. LC/MS: *m/e* 375.18 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 7.45 (m 2H), 7.40 (m, 1H), 6.66 (bs, 1H), 5.95 (bs, 1H), 4.55 (m, 2H), 4.35 (m, 2H), 4.25 (m, 3H), 3.90 (bm, 3H), 2.04 (bm, 4H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(5-chloro-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide hydrochloride (12)



Step 1: 5-Chloro-1H-pyrrole-2-carboxylic acid

A solution of methyl 5-chloro-1H-pyrrole-2-carboxylate (2.1 g, 12.9 mmol), 2N NaOH (12.9 mL, 26 mmol) in MeOH (65 mL) was heated in a sealed tube for 4h to a temperature of 90°C. The reaction was cooled and concentrated. The residue was then partitioned between diethyl ether (500 mL) and 0.5M HCl (200 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated to give 1.88 g (64%) of desired intermediate which was used without further purification.

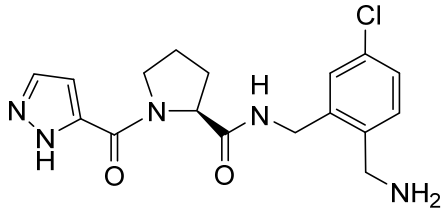
Step 2: (*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(5-chloro-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide hydrochloride

A tube containing 5-chloro-1H-pyrrole-2-carboxylic acid (0.522 g, 3.6 mmol), (*S*)-*tert*-butyl 4-chloro-2-((pyrrolidine-2-carboxamido)methyl)benzylcarbamate (1.2 g, 3.3 mmol), EDC (0.70 g, 3.6 mmol) and HOBT (0.5 g, 3.6 mmol) was treated with a solution of DIEA (1.4 mL, 8.2 mmol) in DCE (22 mL) at rt overnight. The resulting solution was passed through an SPE loaded with 3 mL of water and eluted with DCM. The eluant was concentrated and the residue purified via MPLC (20-65% EAIM/Hex) {EAIM: 80% EtOAc/10% ACN/ 8% IPA/2% MeOH }to give product.

Boc group removal was accomplished with 25% TFA in DCM (20 mL total volume) overnight at room temp. The excess solvent was removed and the residue neutralized by partitioning between DCM and 0.25N NaOH. The organic layer was dried over magnesium sulfate, filtered and concentrated. The

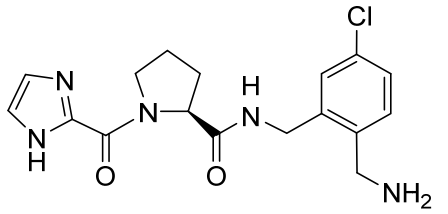
residue was redissolved in diethyl ether and treated with excess HCl in diethyl ether to give 1.41 g (70%) of the HCl salt after concentration. LC/MS: *m/e* 395.05 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 8.80 (bs, 1H), 7.44 (m, 3H), 6.74 (bs, 1H), 6.12 (bs, 1H), 4.58 (m, 1H), 4.55 (m, 1H), 4.31 (m, 1H), 4.25 (m, 2H), 3.89 (m, 2H), 2.26 (bm, 1H), 2.11 (bm, 1H), 2.04 (bm, 1H), 1.95 (bm, 1H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(1H-pyrazole-5-carbonyl)pyrrolidine-2-carboxamide(6)



Prepared in an identical manner to compound **10** starting with 1H-pyrazole-3-carboxylic acid. LC/MS: *m/e* 362.13 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 7.70 (m, 1H), 7.49 (m, 1H), 7.41 (m, 2H), 6.76 (m, 1H), 4.57 (d, J = 14.67 Hz, 1H), 4.53 (m, 1H), 4.35 (m, 1H), 4.26 (mm, 2H), 4.07 (m, 2H), 2.29 (m, 1H), 2.09 (m, 1H), 1.99 (bm, 2H).

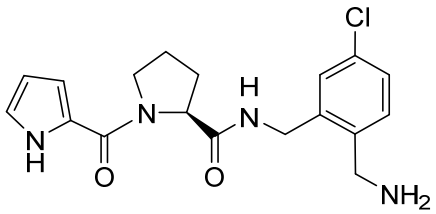
(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(1H-imidazole-2-carbonyl)pyrrolidine-2-carboxamide (7)



Prepared in an identical manner to compound **10** starting with 1H-imidazole-3-carboxylic acid. LC/MS: *m/e* 362.07 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 7.49 (m, 1H), 7.46 (bs, 2H), 7.43 (bm, 1H), 7.39

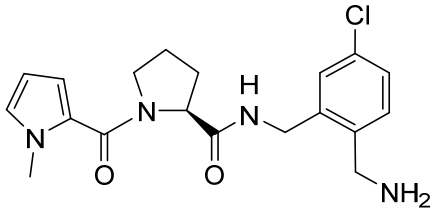
(m, 1H), 7.37 (m, 1H), 7.34 (m, 1H), 7.04 (bs, 1H), 4.57 (m, 2H), 4.36 (m, 1H), 4.26 (m, 2H), 4.08 (m, 2H), 2.34 (m, 1H), 2.12 (m, 1H), 1.99 (bm, 2H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide (8)



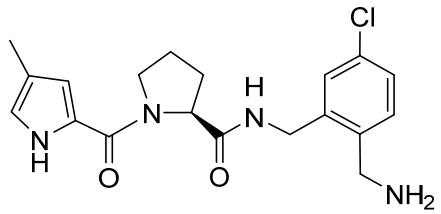
Prepared in an identical manner to compound **10** starting with pyrrole-2-carboxylic acid. LC/MS: *m/e* 361.16 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 10.88 (bs, 1H), 7.44 (m, 4H), 6.98 (bs, 1H), 6.79 (bs, 1H), 6.26 (bs, 1H), 4.53 (bm, 2H), 4.35 (m, 1H), 4.26 (m, 2H), 3.96 (bm, 1H), 3.92 (bm, 1H), 2.27 (bm, 1H), 2.12 (bm, 1H), 2.05 (bm, 1H), 1.95 (bm, 1H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(1-methyl-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide(9)



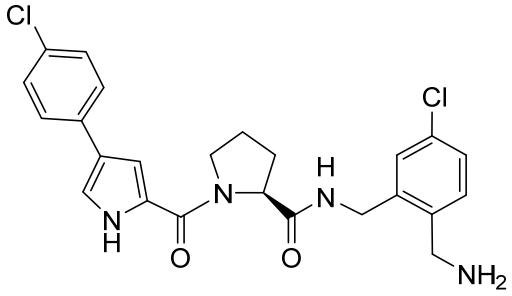
Prepared in an identical manner to compound **10** starting with 1-methyl-1H-pyrrole-2-carboxylic acid. LC/MS: *m/e* 375.14 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 7.52 (bs, 1H), 7.4038-7.43 (m, 2H), 6.82 (bs, 1H), 6.70 (bs, 1H), 6.09 (bs, 1H), 4.47 (m, 3H), 4.25 (ms, 2H), 3.86 (m, 2H), 3.72 (m, 65), 3.76 (bs, 3H), 2.32 (bm, 1H), 2.04 (m, 1H), 1.95 (bm, 2H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(4-methyl-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide (11)



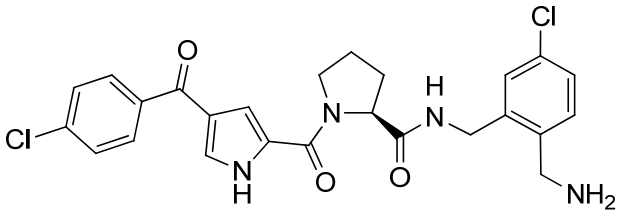
Prepared in an identical manner to compound **10** starting with 1-methyl-1H-pyrrole-2-carboxylic acid. LC/MS: *m/e* 375.10 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 7.47 (m, 1H), 7.40 (m, 2H), 6.75 (bs, 1H), 6.61 (bs, 1H), 4.52 (m, 2H), 4.37 (m, 1H), 4.25 (m, 2H), 3.89 (m, 2H), 2.25 (bm, 1H), 2.11 (bs, 3H), 2.03 (bm, 1H), 1.93 (bm, 2H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(4-(4-chlorophenyl)-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide(13)



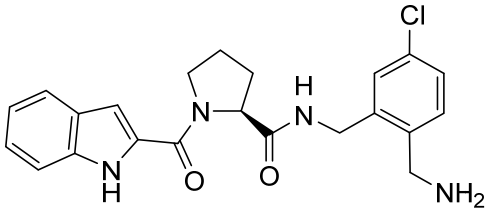
Prepared in an identical manner to compound **10** starting with 4-(4-chlorophenyl)-1H-pyrrole-2-carboxylic acid. LC/MS: *m/e* 471.26 $(M+H)^+$. ^1H NMR (500 MHz; CD_3OD): 7.51 (m, 2H), 7.44 (m, 1H), 7.37 (m, 2H), 7.29 (m, 3H), 7.05 (m, 1H), 4.50 (m, 2H), 4.33 (m, 1H), 4.20 (m, 2H), 3.97 (m, 2H), 2.24 (bm, 1H), 2.07 (bm, 2H), 1.92 (bm, 1H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(4-(4-chlorobenzoyl)-1H-pyrrole-2-carbonyl)pyrrolidine-2-carboxamide (14)



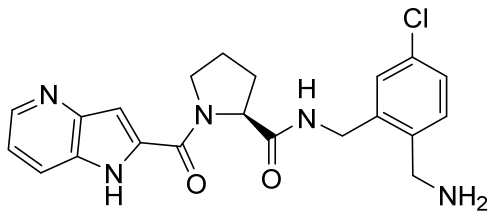
A suspension of 4-(4-chlorobenzoyl)-1H-pyrrole-2-carboxylic acid (30 mg, 0.120 mmol), (*S*)-*tert*-butyl 4-chloro-2-((pyrrolidine-2-carboxamido)methyl)benzylcarbamate (40 mg, 0.100 mmol) and EDC (23 mg, 0.120 mmol) in of pyridine (26 µl, 0.3 mmol)/DIEA (57µl, 0.3 mmol) in DCM (362 µl) at room temp overnight. The reaction was then diluted with DCM, washed water and brine. The organic layer was dried over magnesium sulfate, filtered and concentrated. The crude material was purified via MPLC (10-80% EtOAc/Hex) to obtain product LC/MS: *m/e* 499.25 $(M\text{-}Boc)^+$. The boc protected material was then redissolved in 2 mL of a 2:1 DCM/4N HCl in Dioxane mixture at room temperature for 1 h. Excess solvent was removed on rotovap and the residue was triturated with DCM to provide the HCl salt. LC/MS: *m/e* 499.26 $(M+H)^+$. ^{1}H NMR (500 MHz; CD_3OD): 7.76 (m, 2H), 7.51 (m, 3H), 7.44 (m, 3H), 7.19 (m, 1H), 4.51 (m, 2H), 4.33 (m, 1H), 4.21 (m, 2H), 3.93 (m, 2H), 2.24 (m, 1H), 2.04 (m, 2H), 1.92 (m, 1H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(1H-indole-2-carbonyl)pyrrolidine-2-carboxamide(15)



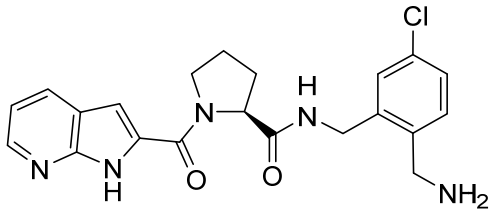
Prepared in an identical manner to compound **10** starting with indole-2-carboxylic acid. LC/MS: *m/e* 411.04 $(M+H)^+$. ^{1}H NMR (500 MHz; DMSO d_6): 11.50 (bs, 1H), 8.72 (m, 1H), 8.36 (bm, 2H), 7.64 (m, 2H), 7.47 (m, 2H), 7.43 (m, 2H), 7.19 (m, 1H), 7.04 (m, 2H), 4.56 (m, 1H), 4.42 (m, 1H), 4.31 (m, 1H), 4.11 (m, 2H), 3.99 (m, 1H), 3.93 (m, 1H), 2.18 (bm, 1H), 2.00 (bm, 2H), 1.83 (bm, 1H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(1H-pyrrolo[3,2-b]pyridine-2-carbonyl)pyrrolidine-2-carboxamide (16)



Prepared in an identical manner to compound **10** starting with 1H-pyrrolo[3,2-b]pyridine-2-carboxylic acid. LC/MS: *m/e* 412.31 $(M+H)^+$. ^{1}H NMR (500 MHz; CD_3OD): 8.70 (d, *J* = 5.38 Hz, 1H), 8.60 (d, *J* = 8.31 Hz, 1H), 7.78 (dd, *J* = 5.87, 8.31 Hz, 1H), 7.53 (m, 1H), 7.45 (m, 1H), 7.40 (m, 1H), 7.36 (s, 1H), 4.67 (m, 1H), 4.59 (m, 1H), 4.39 (m, 1H), 4.28 (m, 2H), 4.07 (m, 2H), 2.38 (bm, 1H), 2.19 (bm, 1H), 2.10 (bm, 1H), 2.03 (bm, 1H).

(*S*)-*N*-(2-(Aminomethyl)-5-chlorobenzyl)-1-(1H-pyrrolo[2,3-b]pyridine-2-carbonyl)pyrrolidine-2-carboxamide (17)



Prepared in an identical manner to compound **10** starting with 1H-pyrrolo[2,3-b]pyridine-2-carboxylic acid. LC/MS: *m/e* 412.09 $(M+H)^+$. ^{1}H NMR (500 MHz; DMSO-d_6): 8.77 (m, 1H), 8.20 (m, 1H), 7.50 (m, 1H), 7.44 (m, 2H), 7.21 (m, 1H), 7.10 (bs, 1H), 4.54 (m, 1H), 4.46 (m, 1H), 4.29 (m, 2H), 4.11 (m, 2H), 3.98 (m, 2H), 2.19 (bm, 1H), 2.01 (bm, 1H), 1.95 (bm, 1H), 1.82 (bm, 1H).

Thrombin assays:

Determination of inhibition constants (K_i). Human α-thrombin (Sekisui) was pre-incubated with inhibitor for 30 minutes at room temperature. The reaction was initiated by the addition of substrate (KPR-AFC, MP Biomedical) and allowed to proceed for 60 minutes at room temperature. Product formation was determined fluorescently (ex 405 nm, em 510 nm). Ki values were calculated using the Cheng-Prusoff equation.

Activated partial thromboplastin time (aPTT), modified pro-thrombin time (PT) measurements

For *in vitro* measurement of aPTT and modified PT, whole blood from healthy human donors was collected into sodium citrate. The blood samples were then centrifuged at 1,500 g for 15 minutes at 4°C to generate plasma. Compounds were spiked into pooled human plasma at various concentrations; vehicle was DMSO at a final concentration of 1%. Activated partial thromboplastin time was determined by standard methods using APTT-XL reagent (Pacific Hemostasis, Fisher Scientific, Pittsburgh, PA, US) and 25mM $CaCl_2$ to trigger clotting on a STAR Evolution Plus coagulation analyzer (Diagnostica Stago, Asnieres sur Seine, France). Prothrombin time was measured in a modified fashion by diluting TriniCLOT PT Excel (Diagnostica Stago) 1:8 with 12.5mM $CaCl_2$ to trigger clotting, samples were run on the same analyzer. Percent increase was determined as compared to vehicle controls.

Rat arterio-venous shunt (AV shunt) thrombosis model

Rat protocols of AV shunt were based on published procedures[1]. Briefly, compounds were administered to male Sprague-Dawley rats (Charles River Laboratories) weighing approximately 275-300 g via a femoral vein (IV) injection at a volume of 3 ml/kg/hour. Compounds were formulated as follows, Dabigatrin and Compound 2 were formulated in 0.9% saline. Compound **10** was formulated in 35% HPßCD in 10mM phosphate buffer. Animals had access to food and water *ad libitum*. AV shunt was performed in thiobutabarbital anesthetized animals. An extracorporeal shunt (Tygon tubing containing a silk thread of known weight) was connected to carotid artery and jugular vein cannulas. After a 5 minute stabilization period, infusion of compound was initiated via the femoral vein. After 15 minutes of drug exposure blood was allowed to flow through the shunt for 15 minutes. The weight of

the thread at the end of the experiment was recorded and thrombus weight calculated via subtracting the weight of the thread prior to the circulation of blood.

[1]Schumacher WA, Bostwick JS, Stewart AB, Steinbacher TE, Xin B, Wong PC. Effect of the Direct Factor Xa Inhibitor Apixaban in Rat Models of Thrombosis and Hemostasis. J Cardiovasc Pharmacol. **2010**, *55*, 609-616.

aPTT, PT measurement and thrombin generation assay

For *ex vivo* measurement of activated partial thromboplastin time (aPTT) and prothrombin time (PT), whole blood was collected into sodium citrate (final concentration, 11 mM). The blood samples were then centrifuged at 2,500 g at 4°C for 15 minutes to generate plasma. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined by standard methods using TriniCLOT aPTT S (Tcoag, Bray, Ireland) and TriniCLOT PT Excel (Tcoag, Bray, Ireland) on a KC4 Delta coagulation analyzer (Tcoag, Bray, Ireland). For aPTT, ellagic acid was used as a trigger. Briefly, 50 µL plasma was incubated with 50 µL trigger for 5 minute at 37^{o}C followed by the addition of 50 µL $CaCl_2$ in order to start the coagulation cascade. For PT measurement, 100 µL TF + $CaCl_2$ mix was directly added to 50 µL plasma in order to start the coagulation cascade. Thrombinoscope (Diagnostica Stago, Parsippany, NJ, USA) TGA was performed using the thrombinoscope software and according to the manufacturer's instructions. In brief, plasma (60 µL) was incubated with 15 µL fXIa (1, 3, 10 pM, Haematologic Technologies Inc., Essex Junction, Vt, USA) or TF (1, 5, 20 pM, Diagnostica Stago, Parsippany, NJ, USA) at 37 °C for 5 minute. This was followed by automatic injection of 15 µL of FluCa buffer to initiate thrombin generation via intrinsic or extrinsic pathways, respectively. Peak thrombin generation was recorded and utilized to measure activities.

Statistical analysis

For mRNA and protein knockdown analysis, efficacy, aPTT, PT, TGA and bleeding studies, Dunnett's multiple comparison t-test was performed using GraphPad Prism Software Version 5.04 (GraphPad Software, Inc, La Jolla, CA, USA).

AV Shunt Data:

	Dabigatran (**1**)		Compound **2**		Compound **10**	
mg/kg/30 min	% inhibition	SEM	% inhibition	SEM	% inhibition	SEM
0.03	32	10	**8**	**13**	**21**	**10**
0.1	80	4	**57**	**11**	**26**	**10**
0.3	96	0.6	**58**	**11**	**34**	**22**
1	NA	NA	**90**	**3**	**63**	**15**
	Dabigatran		Compound **2**		Compound **10**	
mg/kg/30 min	µM level	SD	µM level	SD	µM level	SD
0.03	**.037**	**0.0022**	**0.034**	**0.02**	**0.069**	**0.04**
0.1	**.078**	**0.0094**	**0.126**	**0.09**	**0.147**	**0.07**
0.3	**.188**	**0.0194**	**0.415**	**0.19**	**0.387**	**0.23**
1			**0.738**	**0.47**	**1.044**	**0.36**