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Original article

Fluorinated dual antithrombotic compounds based on 1,4-benzoxazine scaffold

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ABSTRACT

Fluorinated 3,4-dihydro-2*H*-1,4-benzoxazine derivatives possessing both thrombin inhibitory and glycoprotein IIb/IIIa receptor antagonistic activities were prepared as potential dual antithrombotic compounds. Fluorine scan (3-fluorobenzyl, 4-fluorobenzyl, 3,4-difluorobenzyl and 3,5-difluorobenzyl substituted compounds) was performed in order to obtain 6-(carboxymethyl)(3,4-difluorobenzyl) amino compound (**9i**) as the most potent compound with balanced dual activity ($K_{i(Thr)} = 0.33 \pm 0.07$ μM, $IC_{50(GP\ IIb/IIIa)} = 1.1 \pm 0.6$ μM).

1. Introduction

Cardiovascular diseases, with an estimated 17 million deaths yearly which is 29% of all deaths, are the main cause of death and morbidity globally [1]. There is a persistent need to discover novel antithrombotic agents as alternatives to existing treatment strategies [2].

While haemostasis is a physiological process by which the body stops blood loss whenever a blood vessel is severed or ruptured, thrombosis is a pathological process in which haemostatic mechanisms, i.e., blood coagulation and platelet aggregation, are activated in the absence of bleeding. Therefore, inhibiting coagulation, in which thrombin is one of the most important therapeutic targets, and preventing platelet aggregation at different stages are essential components of most antithrombotic therapeutic strategies. Efficient combination of anticoagulant and antiplatelet activity in the same molecule would produce a novel class of antithrombotic drugs featuring substantial advantages over possible combinations of anticoagulant and antiplatelet agents, including more predictable and less complex pharmacokinetics, lower incidence of side effects, less demanding clinical studies, and more straight forward registration procedure [3].

In recent publications we introduced a new type of low molecular weight peptidomimetic antithrombotic compound possessing both thrombin inhibitory and GP IIb/IIIa receptor antagonistic activities in the same molecule (Fig. 1) [4,5]. Such dual acting potential antithrombotic compounds incorporate *a*) an arginine mimetic which interacts with Asp189 in the thrombin S1 pocket and acts as a cationic centre for binding to the GP IIb/IIIa receptor, *b*) a carboxylate group providing ionic or dipolar interaction with the GP IIb/IIIa receptor, *c*) a central scaffold that interacts with the thrombin YPPW loop and also provides a spacer between the two charged groups required for binding to the GP IIb/IIIa receptor and *d*) an aromatic ring in the proximity of the carboxylate group, required for interaction with the thrombin S3 binding pocket and to provide a hydrophobic interaction with the nonpolar binding site of the GP IIb/IIIa receptor [4,6].

Several heterocyles were used in preparation of new antithrombotic compounds [7] and based on preliminary docking results and possibilities for further functionalization we have chosen a 1,4-benzoxazine scaffold for design of our dual antithrombotic compounds. There are several literature reports on the positive effects of introducing fluorine atoms to the P3 aromatic ring of thrombin inhibitors [8,9] while the influence of fluorination on the activity of GP IIb/IIIa receptor antagonists has not been described. The introduction of fluorine atoms into bioactive compounds should also have favourable effects on physiochemical properties and improved pharmacokinetics [10]. To study the effect of fluorine substituent(s) on anticoagulant and GP IIb/IIIa antagonistic activity, we decided to perform a fluorine scan on our recently described dual antithrombotic compounds based on 1,4-benzoxazine scaffold (Fig. 1) [4] by preparing their 3-

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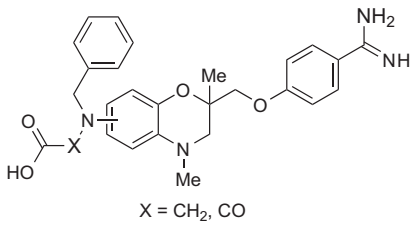


Fig. 1. Recently described compounds possessing both thrombin inhibitory and GP IIb/IIIa receptor antagonistic activities [4].

fluorobenzyl, 4-fluorobenzyl, 3,4-difluorobenzyl and 3,5-difluorobenzyl analogues, bearing a substituent on the position 6 or 7 of the 1,4-benzoxazine scaffold, and possessing either a more flexible acetyl moiety or a more rigid oxalyl moiety, giving altogether 32 compounds.

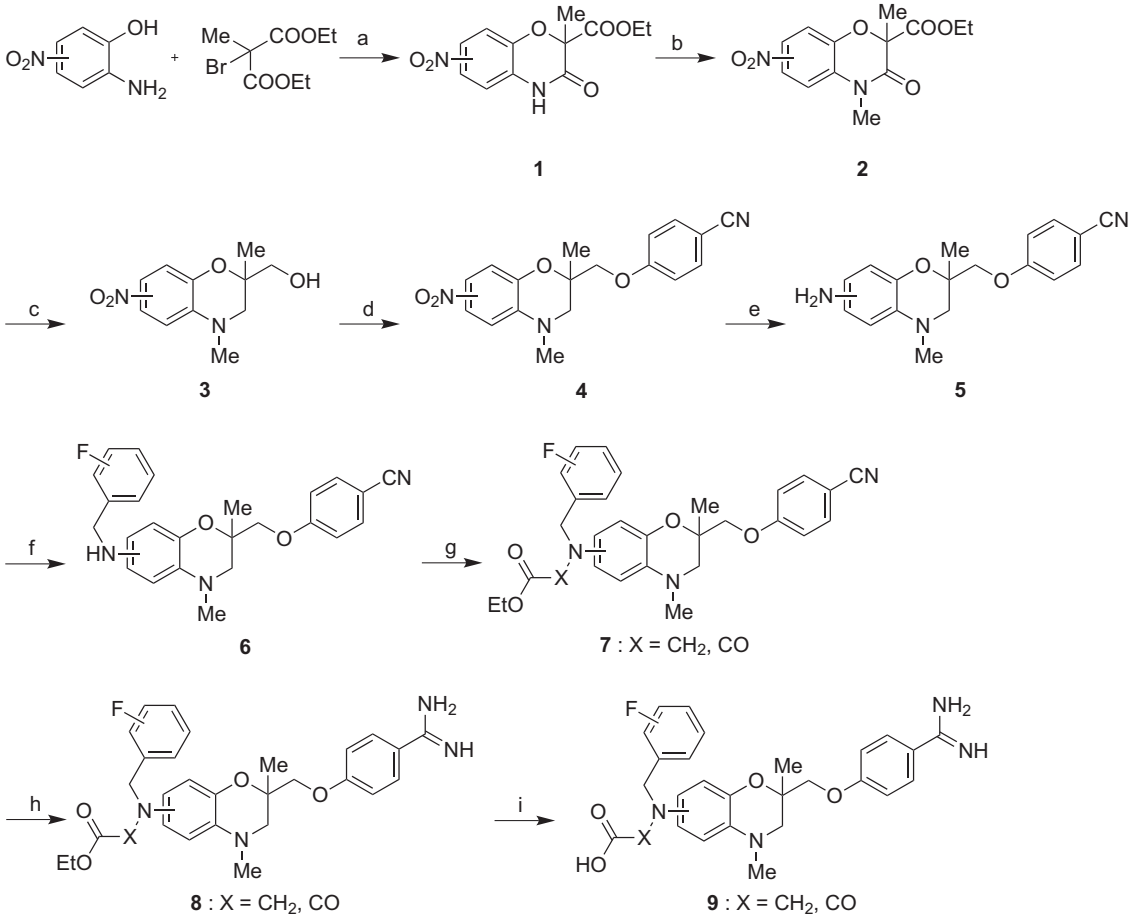
2. Chemistry

The synthesis of target fluorinated dual compounds **8** and **9** is depicted in Scheme 1. The key intermediate 4-((2,4-dimethyl-6/7-nitro-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)methoxy)benzonitrile **4** was prepared in four steps as described from 2-amino-4/5-nitrophenol by cyclization, *N*-methylation and subsequent borane reduction, followed by Mitsunobu reaction with 4-cyanophenol [6]. Nitro group was reduced using catalytic hydrogenation giving amines **5**, which were benzylated at the aromatic amino group with 3-fluorobenzaldehyde, 4-fluorobenzaldehyde, 3,4-difluorobenzaldehyde and 3,5-difluorobenzaldehyde using sodium borohydride as a reducing agent to give amines **6**, which were alkylated with ethyl bromoacetate to afford tertiary amines **7** (X = CH_2) or acylated with ethyloxalyl chloride to give *N*-ethyloxalyl derivatives **7** (X = CO). Target compounds **8** and **9** were prepared from nitriles **7** using the Pinner reaction, followed by alkaline hydrolysis.

3. Results and discussion

3.1. Biological activity

In our previous report [4] we demonstrated basic structure–activity relationships and balancing GP IIb/IIIa receptor antagonists and thrombin inhibitory activities in potential dual antithrombotic compounds based on the central 2*H*-1,4-benzoxazine core. We found out that a methylenoxy spacer provides optimal flexibility between benzamidine moiety and central 1,4-benzoxazine scaffold to ensure desired activity on both targets. To improve balanced dual activity on thrombin and platelet GP IIb/IIIa receptor, a compromise concerning flexibility and bulkiness in the P3 part containing aromatic and carboxylic



Scheme 1. Reagents and conditions: (a) KF, DMF, 60 °C, overnight; (b) CH_3I, KF, DMF, rt, overnight; (c) $Me_2S \times BH_3$, THF, reflux, 4 h; (d) 4-cyanophenol, PPh_3, DIAD, THF, reflux, 48 h; (e) H_2, 10% Pd/C, THF, rt, 2 h; (f) 3-fluorobenzaldehyde or 4-fluorobenzaldehyde or 3,4-difluorobenzaldehyde or 3,5-difluorobenzaldehyde, $NaBH_4$, 1,2-dichloroethane, rt, 6 h; (g) ethyl 2-bromoacetate, BTEAC, K_2CO_3, DMF, 100 °C, 24 h or ethyloxalyl chloride, Et_3N, CH_2Cl_2, rt, 1 h; (h) $HCl_{(g)}$, EtOH, 0 °C, 30 min, then NH_4OAc, EtOH, rt, 24 h; (i) 1.5 M NaOH, H_2O, EtOH, rt, 6 h.

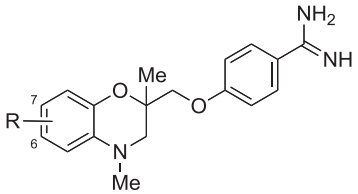
acid moieties was sought. Compounds having a benzyl(carboxymethyl)amino moiety on position 6 of 1,4-benzoxazine scaffold displayed more pronounced GP IIb/IIIa receptor antagonistic activity while compounds possessing the benzyl(carboxymethyl) amino moiety on position 7 showed more pronounced thrombin inhibitory activity (Table 1).

Compound **10d** with a 7-(*N*-ethyloxalyl) substituent had the best thrombin inhibition activity ($K_{i(Thr)} = 42 \pm 4$ nM) among all compounds, which were used as templates for envisaged fluorinated compounds. The comparison of thrombin inhibitory activities of esters **10d** (Table 1) and **8d**, **8h**, **8l** and **8p** (Table 2) showed that in 7-*N*-ethyloxalyl compounds the introduction of fluorine to position 3 of a P3 benzyl group (**8h**) improved activity 2.2-fold ($K_{i\ (Thr)} = 19 \pm 1$ nM), the introduction of fluorine to position 4 of the benzyl group (**8d**) improved activity 1.8-fold ($K_{i\ (Thr)} = 23 \pm 1$ nM), the introduction of two fluorines to positions 3 and 4 of benzyl group (**8l**) improved activity 1.5-fold ($K_{i\ (Thr)} = 28 \pm 3$ nM), while the introduction of two fluorines to positions 3 and 5 of the benzyl group (**8p**) improved the activity 2.4-fold ($K_{i\ (Thr)} = 18 \pm 1$ nM). In 7-*N*-oxalyl compounds the effect of introducing fluorine was less beneficial – in comparison with **10f** the introduction of fluorine to position 3 (**9h**), position 4 (**9d**) or positions 3 and 4 (**9l**) led to slightly reduced activity, while the introduction of two fluorines to positions 3 and 5 (**9p**) improved activity 1.3-fold ($K_{i\ (Thr)} = 150 \pm 20$ nM). In case of 6-substituted compounds, introduction of fluorine to benzyl group resulted in slightly decreased thrombin inhibitory activity. Similar observations can be found concerning the inhibitory activity of compounds **8** and **9** on trypsin and factor Xa. The introduction of fluorine also increased the FXa and trypsin inhibition activity. However, fluorinated compounds are selective towards FXa, while trypsin selectivity is only moderate. The most selective are 7-(*N*-acyl) compounds with good selectivity towards FXa (43- to 123-fold) and good selectivity towards trypsin (22- to 37-fold). 6-Alkylated fluorinated compounds **8** and **9** have even slightly better inhibitory activity on trypsin than thrombin, resulting in no selectivity towards trypsin.

Antagonistic activity on GP IIb/IIIa receptor was also improved with introducing fluorine to the P3 benzyl group. Esters (prodrugs) were not active ($IC_{50} > 100$ μM), while fluorinated acids **9** were slightly more active than their non-fluorinated analogues. A trend of higher potency of 6-substituted compounds as compared to 7-substituted non-fluorinated compounds was also observed in case of fluorinated compounds **9**. When evaluating the effect of fluorine introduction to benzyl group it is noticeable that there is only a moderate increase (0.8- to 1.6-fold) in potency. In the case of 6-(*N*-acyl) acid (**10e**), the most potent non-fluorinated GP IIb/IIIa receptor antagonist ($IC_{50\ (GP\ IIb/IIIa)} = 2.8 \pm 1.5$ μM), introduction of fluorine to position 3 of the P3 benzyl group (**9g**) improved activity 1.3-fold ($IC_{50\ (GP\ IIb/IIIa)} = 1.7 \pm 0.6$ μM), introduction of fluorine to position 4 of the benzyl group (**9c**) improved activity 1.4-fold ($IC_{50\ (GP\ IIb/IIIa)} = 2.0 \pm 0.6$ μM), the introduction of two fluorines to positions 3 and 4 of the benzyl group (**9k**) improved activity 1.25-fold ($IC_{50\ (GP\ IIb/IIIa)} = 2.5 \pm 1.1$ μM), while the introduction of two fluorines to positions 3 and 5 of the benzyl group (**9o**) decreased activity 0.8-fold ($IC_{50\ (GP\ IIb/IIIa)} = 3.4 \pm 1.7$ μM). In the series of acids **9**, 6-(*N*-alkyl) compounds showed better activity than 6-(*N*-acyl) analogues with 3,4-difluorinated compound (**9i**) being the most active ($IC_{50\ (GP\ IIb/IIIa)} = 1.1 \pm 0.6$ μM), followed by 3-fluorinated compound (**9e**) ($IC_{50\ (GP\ IIb/IIIa)} = 1.5 \pm 0.9$ μM), 4-fluorinated compound (**9c**) ($IC_{50\ (GP\ IIb/IIIa)} = 1.8 \pm 0.9$ μM) and 3,5-difluorinated compound (**9m**) being the least potent in the series ($IC_{50\ (GP\ IIb/IIIa)} = 2.5 \pm 1.1$ μM). Completely different situation was found in the case of 7-(*N*-alkyl) acids where 3,5-difluorinated compound (**9n**) was the most active ($IC_{50\ (GP\ IIb/IIIa)} = 45 \pm 28$ μM) and 3,4-difluorinated compound (**9j**) was the least active ($IC_{50\ (GP\ IIb/IIIa)} = 80 \pm 23$ μM) in the series. Compounds **8** and **9** did not show any activity towards $\alpha_V\beta_3$ receptor, thus showing good selectivity towards the structurally most similar glycoprotein receptor.

Balancing activities on both targets is the most demanding goal in a multiple ligands design, which is also reflected in our work. 6-(*N*-alkyl) acids (**9a**, **9e**, **9i**, **9m**) and 6-(*N*-acyl) acids (**9c**, **9g**, **9k**, **9o**) are the most balanced compounds (Fig. 2). 6-(*N*-acyl) acids are more balanced 7-(*N*-acyl) compounds in this series. 3-Fluoro (**9c**) and 4-fluoro (**9g**) compounds have very similar and balanced activity, while 3,4-difluoro compound (**9k**) has weaker activity and 3,5-difluoro compound (**9o**) is the weakest within the series. 6-(*N*-

Table 1
Biological activity of reference 1,4-benzoxazine derivatives: inhibition of serine proteases thrombin, factor Xa, trypsin and inhibition of fibrinogen binding to $\alpha_{IIb}\beta_3$ (GP IIb/IIIa) and $\alpha_v\beta_3$ receptors [4].



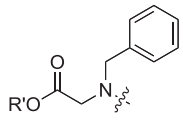
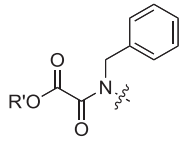
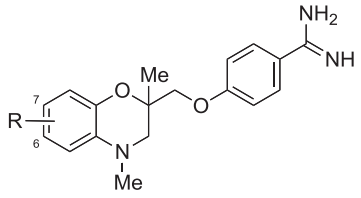
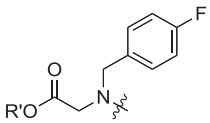
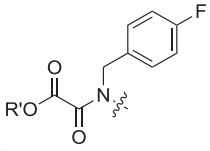
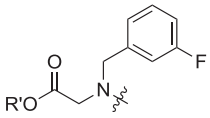
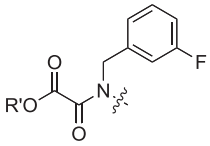
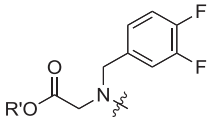
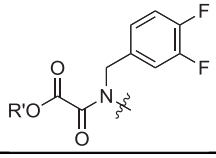
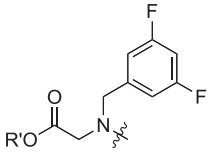
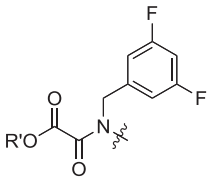
Comp.	R	Substit.	R′	K_i (μM)			IC_{50} (μM)	
				Thrombin	Trypsin	FXa	$\alpha_{IIb}\beta_3$	$\alpha_v\beta_3$
10a		6	Et	0.74 ± 0.11	2.4 ± 0.5	12 ± 3	54 ± 26	>100
10b		7	Et	0.32 ± 0.03	1.7 ± 0.5	2.7 ± 0.8	28 ± 13	>100
10c		6	Et	0.22 ± 0.04	0.59 ± 0.05	16. ± 3	49 ± 23	>100
10d		7	Et	0.042 ± 0.004	0.95 ± 0.08	112 ± 37	52 ± 25	>100
10e		6	H	0.62 ± 0.10	1.2 ± 0.1	32 ± 5	2.8 ± 1.5	>100
10f		7	H	0.19 ± 0.02	1.3 ± 0.3	17 ± 3	39 ± 18	>100

Table 2
Biological activity of fluorinated 1,4-benzoxazine derivatives **8** and **9**: the inhibition of serine proteases thrombin, factor Xa, and trypsin, and the inhibition of fibrinogen binding to $\alpha_{IIb}\beta_3$ (GP IIb/IIIa) and $\alpha_v\beta_3$ receptors.



Comp.	R	Substit.	R′	K_i (μM)			IC_{50} (μM)	
				Thrombin	Trypsin	FXa	$\alpha_{IIb}\beta_3$	$\alpha_v\beta_3$
8a		6	Et	**0.72 ± 0.16**	0.65 ± 0.17	6.66 ± 1.73	>**100**	>100
9a		6	H	**0.75 ± 0.08**	0.52 ± 0.10	7.7 ± 2.0	**2.0 ± 1.1**	>100
8b		7	Et	**0.36 ± 0.09**	0.33 ± 0.09	1.6 ± 0.3	**>100**	>100
9b		7	H	**0.24 ± 0.03**	1.3 ± 0.2	5.6 ± 1.0	**49 ± 24**	>100
8c		6	Et	**0.33 ± 0.08**	0.58 ± 0.11	5.49 ± 0.44	**>10**	>100
9c		6	H	**2.0 ± 0.6**	1.07 ± 0.21	16.3 ± 2.6	**1.8 ± 0.9**	>100
8d		7	Et	**0.023 ± 0.001**	0.52 ± 0.04	2.83 ± 0.33	**>100**	>100
9d		7	H	**0.26 ± 0.04**	1.1 ± 0.2	7.7 ± 0.6	**34 ± 15**	>100
8e		6	Et	**0.80 ± 0.19**	0.65 ± 0.14	6.8 ± 2.1	**>100**	>100
9e		6	H	**0.67 ± 0.07**	0.46 ± 0.10	7.6 ± 2.3	**1.5 ± 0.91**	>100
8f		7	Et	**0.35 ± 0.08**	0.46 ± 0.11	1.2 ± 0.1	**>100**	>100
9f		7	H	**0.32 ± 0.05**	1.6 ± 0.10	6.6 ± 1.0	**60 ± 30**	>100
8g		6	Et	**0.30 ± 0.11**	0.30 ± 0.05	4.9 ± 0.5	**>100**	>100
9g		6	H	**1.7 ± 0.6**	0.80 ± 0.23	18 ± 3	**2.2 ± 0.9**	>100
8h		7	Et	**0.019 ± 0.001**	0.42 ± 0.05	1.6 ± 0.2	**>100**	>100
9h		7	H	**0.24 ± 0.04**	0.78 ± 0.10	4.1 ± 0.2	**33 ± 14**	>100
8i		6	Et	**0.83 ± 0.23**	0.65 ± 0.15	8.0 ± 1.8	**>100**	>100
9i		6	H	**0.33 ± 0.07**	0.56 ± 0.04	6.5 ± 2.0	**1.1 ± 0.6**	>100
8j		7	Et	**0.43 ± 0.09**	0.73 ± 0.25	1.5 ± 0.3	**>100**	>100
9j		7	H	**0.81 ± 0.21**	1.3 ± 0.1	4.4 ± 0.6	**80 ± 37**	>100
8k		6	Et	**0.37 ± 0.07**	0.64 ± 0.16	4.7 ± 0.5	**>100**	>100
9k		6	H	**3.0 ± 0.6**	5.3 ± 0.7	14 ± 3	**2.5 ± 1.1**	>100
8l		7	Et	**0.028 ± 0.003**	0.65 ± 0.04	1.6 ± 0.4	**>100**	>100
9l		7	H	**0.29 ± 0.09**	1.3 ± 0.1	3.6 ± 0.7	**31 ± 13**	>100
8m		6	Et	**0.95 ± 0.20**	0.72 ± 0.13	9.8 ± 3.8	**>100**	>100
9m		6	H	**0.76 ± 0.10**	0.72 ± 0.03	9.8 ± 3.8	**2.5 ± 1.1**	>100
8n		7	Et	**0.48 ± 0.08**	0.67 ± 0.12	15 ± 3	**>100**	>100
9n		7	H	**0.20 ± 0.06**	1.3 ± 0.3	4.0 ± 0.8	**45 ± 28**	>100
8o		6	Et	**0.29 ± 0.08**	0.79 ± 0.29	6.0 ± 1.2	**>100**	>100
9o		6	H	**5.0 ± 1.3**	5.3 ± 0.9	14 ± 3	**3.4 ± 1.7**	>100
8p		7	Et	**0.018 ± 0.001**	0.52 ± 0.04	0.79 ± 0.11	**>100**	>100
9p		7	H	**0.15 ± 0.02**	0.59 ± 0.11	1.6 ± 0.3	**31 ± 15**	>100

Bold numbers annotate that thrombin was desired target and trypsin and FXa activities are there to demonstrate selectivity, as well that $\alpha_{IIb}\beta_3$ receptor was desired target and that $\alpha_v\beta_3$ activities demonstrate selectivity.

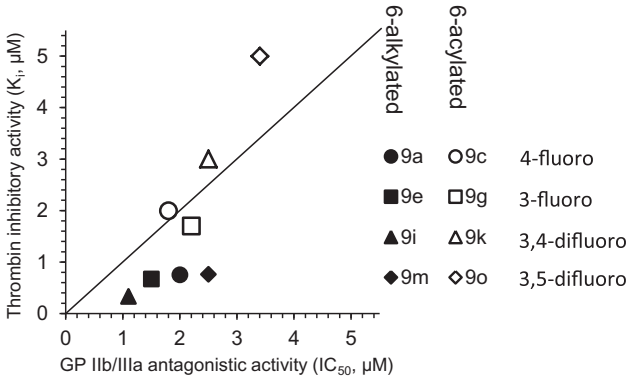


Fig. 2. Balance between thrombin inhibitory activity and GP IIb/IIIa antagonistic activity. Well-balanced compounds are closer to the diagonal line.

alkyl) acids constitute the most potent series with 3,4-difluoro compound (**9i**) being the most potent ($K_{i\ (Thr)} = 0.33 \pm 0.07$ μM, $IC_{50\ (GP\ IIb/IIIa)} = 1.1 \pm 0.6$ μM), while 3,5-difluoro compound (**9m**) has a slightly reduced thrombin inhibitory activity ($K_{i\ (Thr)} = 0.76 \pm 0.10$ μM) and 2.3-fold reduced GP IIb/IIIa antagonistic activity ($IC_{50\ (GP\ IIb/IIIa)} = 2.5 \pm 1.1$ μM) compared to **9i**. Compound **9i** is thus the most potent and balanced designed multiple ligand in this work, exerting moderate selectivity towards factor Xa (20-fold) and trypsin (1.7-fold) and a good selectivity towards $\alpha_V\beta_3$ receptor.

3.2. Molecular modelling

The biological results are explained by the docking both enantiomers of compound **9i** to the thrombin active site [11] and to the GP IIb/IIIa receptor-binding site [12] using CDOCKER (a CHARMM-based docking program) systematically posing the flexible ligands within a static site and conducting low-level energy calculations for each pose. Only binding of the best-posed enantiomer to each target is shown in Fig. 3. (*R*)-**9i** binds to the thrombin active site in a similar manner to dabigatran. The distance between the C-atoms of the Asp189 carboxylate and the amidine group in (*R*)-**9i** was 4.0 Å, and two hydrogen bonds linked the amidine hydrogens and carboxylate oxygen atoms. An additional hydrogen bond was formed between the benzamidine and the Gly219 backbone. The 3,4-dihydro-2*H*-1,4-benzoxazine scaffold was located in the S2 binding pocket. Due to substitution on the position 6, 1,4-benzoxazine scaffold is not optimally placed compared to 7-substituted compound, this is also the reason for 6-substituted compound being less active on thrombin than the 7-substituted compounds. The oxygen of the 1,4-oxazine ring forms a hydrogen bond with the Gly216 backbone, which is often regarded as a key interaction in the thrombin active site [13]. However, the distance of oxygen to Gly216 in the case of docking *R*-isomer of **9i** is not optimal. When docking *S* isomer of **9i** 3,4-difluorobenzyl is placed in the lipophilic S3 binding pocket, with the P3 carboxylate stretching outward from the thrombin surface. The fluorination of aromatic rings changes the quadrupole moment, which profoundly affects aromatic–aromatic interactions [14]. In the case of **9i** the key aromatic–aromatic interaction of the P3 benzyl group is with Trp215 and it is the reason why the different fluorine substitution pattern results in different activity against thrombin.

The analysis of the binding mode of (*S*)-**9i** in the binding site of GP IIb/IIIa receptor shows that it takes an extended conformation. The benzamidine moiety forms hydrogen bonds with Asp224 and Phe160 from the αIIb subunit of the GP IIb/IIIa receptor and makes hydrophobic interactions with Tyr190, Phe231, and Phe191 of the αIIb subunit. The oxygen atom of the 1,4-oxazine ring forms a hydrogen bond with Ala218. The carboxylic acid moiety makes electrostatic interactions with the Mg^{2+} atom and hydrogen bonds to the amide proton of Asn215 of the β3 subunit of the GP IIb/IIIa receptor. The carboxymethyl moiety in *N*-alkyl derivatives is in proximity of Ser213 of the β3 subunit and placed more optimally than carboxycarbonyl moiety in *N*-acyl derivatives (not shown) which seems to be too sterically hindered, resulting in some cases in a reduced activity. 3,4-Diluorobenzyl moiety of (*S*)-**9i** makes aromatic interactions with the Tyr122 of the β3 and Phe160 of the αIIb subunit.

4. Conclusion

In conclusion, we have described the design, synthesis, and the dual activity of several novel fluorinated 3,4-dihydro-2*H*-1,4-benzoxazine compounds capable of acting both as thrombin inhibitors and GP IIb/IIIa receptor antagonists and analyzed the structure–activity relationship of fluorine substitution on combined anticoagulant and fibrinogen receptor antagonistic activity. Influence of introduction of fluorine was not unequivocal for improving activity on both targets. In case of 6-substitued compounds, introduction of fluorine to benzyl group resulted in slightly decreased thrombin inhibitory activity, while it leaded in most cases to improved GP IIb/IIIa antagonistic activity. We found optimal fluorine substitution pattern (3,4-difluoro derivative) to improve balanced dual activity on thrombin and platelet GP IIb/IIIa receptor. Compound **9i**, which has the most potent and well balanced dual antithrombotic activity, close to the nanomolar range, can serve as a lead for the next generation of dual antithrombotic agents.

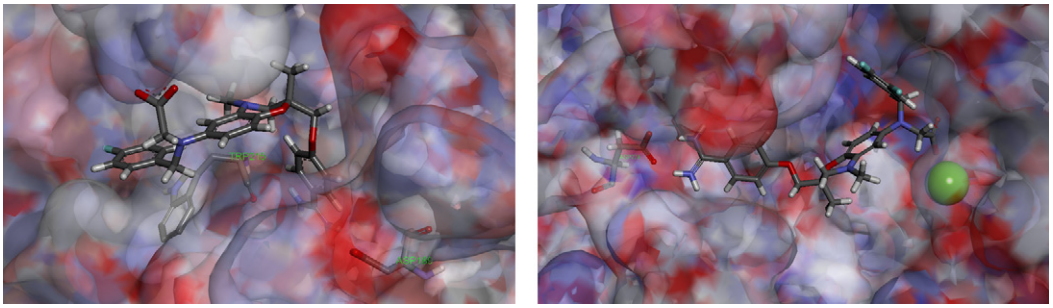


Fig. 3. Compounds **9i** (*R* isomer) docked in the thrombin active site (left) and **9i** (*S* isomer) docked in the GP IIb/IIIa receptor-binding site (right).

5. Experimental

5.1. Chemistry

General: Chemicals were obtained from Acros, Aldrich Chemical Co. and Fluka and used without further purification. THF was distilled immediately prior to use. Analytical TLC was performed on silica gel Merck 60 F_{254} plates (0.25 mm), using visualization with ultraviolet light. Column chromatography was carried out on silica gel 60 (particle size 240–400 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE DPX_{300} spectrometer (^{1}H NMR at 300.132 MHz and ^{13}C NMR at 75.475 MHz) and on a Bruker AVANCE III (^{1}H NMR at 400.130 MHz and ^{13}C NMR at 100.613 MHz) spectrometer respectively in $CDCl_3$ or DMSO-d_6 solution with TMS as the internal standard. All chemical shift values are reported in parts per million (ppm), the coupling constants (J) are given in hertz, and the splitting patterns are appointed as: s (singlet), d (doublet), dd (double doublet), t (triplet) and m (multiplet). For each class of compounds ^{13}C NMR spectra are included (**6a**, **7p**, **8i**, **9d**), which were assigned using gradient COSY, HSQC and HMBC experiments. IR spectra were recorded on a Perkin–Elmer 1600 FT-IR spectrometer. Microanalyses were performed on a Perkin–Elmer C, H, N Analyzer 240 C. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. In some cases solvatomorphs are included in CHN analyses, which were also observed (but not listed for clarity) in NMR spectra. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. HPLC Analyses were performed on an Agilent Technologies HP 1100 instrument with G1365B UV–VIS detector (254 nm), using an Eclips Plus C18 column (4.6 × 150 mm) at flow rate 1 mL/min. The eluant was a mixture of 0.1% TFA in water (A) and methanol (B). Gradient was 40% B to 80% B in 15 min. Purifications of final compounds by reverse phase column chromatography were performed using a Flash Purification System ISOLERATM. The eluant was a mixture of 0.1% TFA in water (A) and methanol (B). Gradient was 40% B to 80% B in 30 column volumes.

Synthesis of compounds **2**, **3**, **4** and **5** were performed using already known synthetic pathways [4,5].

5.1.1. A typical procedure for reductive amination (aldimine formation/reduction in methanol) – *6a, 6b, 6e, 6f, 6i, 6j, 6m, 6n*

4-Fluorobenzaldehyde (1.33 g, 10.7 mmol) and amine **5b** (3 g, 9.7 mmol) were mixed in absolute methanol (50 ml) at room temperature (rt) under argon atmosphere and molecular sieves. The mixture was stirred at rt for 5 h, until the aldimine formation was completed. The resulting aldimine in methanol was carefully treated with solid $NaBH_4$ (587 mg, 15.5 mmol). The reaction mixture was stirred for additional 2 h. The solvent was filtered, evaporated in vacuo, crude residue was dissolved in dichloromethane (50 ml) and washed successively with saturated solution of $NaHCO_3$ (3 × 50 ml) and brine (1 × 50 ml). The organic solution was dried over Na_2SO_4 and the solvent evaporated under reduced pressure. The oily product was purified by column chromatography using dichloromethane/methanol (20:1) as eluant to obtain 2.2 g (54%) of **6b** as brown oil.

5.1.2. A typical procedure for preparation of N-alkylated compounds – *7a, 7b, 7e, 7f, 7i, 7j, 7m, 7n*

Ethyl 2-bromoacetate (1.36 g, 8.2 mmol) was added to a stirred suspension of **6b** (2 g, 4.8 mmol) and potassium carbonate (2 g, 14.4 mmol) in dimethylformamide (50 mL) and the reaction mixture heated for 24 h at 100 °C. The suspension was filtered and the filtrate evaporated under reduced pressure. The oily residue was dissolved in ethyl acetate (50 mL) and washed successively with 10% citric acid (3 × 50 mL), saturated solution of $NaHCO_3$ (2 × 50 mL) and brine (1 × 50 mL). The organic solution was dried over Na_2SO_4 and the solvent evaporated under reduced pressure. The oily product was purified by column chromatography using petroleum ether/ethyl acetate (2.5:1) as eluant to obtain 1.52 g (63%) of **7b** as a yellow oil.

5.1.3. A typical procedure for preparation of N-acylated compounds – *7c, 7d, 7g, 7h, 7k, 7l, 7o, 7p*

Ethyl oxalyl chloride (786 mg, 5.76 mmol) was added to a solution of **6b** (2 g, 4.8 mmol) and triethylamine (583 mg, 5.76 mmol) in dichloromethane (50 ml) and the mixture stirred for 2 h. The solvent was removed under reduced pressure, the residue dissolved in ethyl acetate (50 ml) and washed successively with a 10% citric acid solution (3 × 50 ml), saturated $NaHCO_3$ solution (3 × 50 ml) and brine (1 × 50 ml). The organic phase was dried over Na_2SO_4 and the solvent evaporated under reduced pressure. The oily product was purified by column chromatography using petroleum ether/ethyl acetate (1.5:1) as eluant to obtain 1.7 g (66%) of **7d** as yellow oil.

5.1.4. A typical procedure for preparation of amidines from nitriles (Pinner reaction) – *8a–p*

Gaseous HCl was slowly introduced over 30 min into a solution of the nitrile **7d** (872 mg, 1.68 mmol) in anhydrous ethanol (30 mL). The reaction mixture was closed tightly and stirred for 24 h at room temperature. The solvent was evaporated in vacuo and the residue washed 3 times with anhydrous diethyl ether. The iminoether obtained was dissolved in anhydrous EtOH (30 mL), ammonium acetate (390 mg, 5.05 mmol) was added and the reaction mixture stirred for 24 h at room temperature. The solvent was evaporated and crude product was purified by reverse phase column chromatography with gradient using methanol/trifluoroacetic acid (40–80% in 30 min.) as eluant. After evaporation of methanol white crystals were precipitated from trifluoroacetic acid, filtered off and dried to yield 386 mg (43%) of **8d** as a white solid.

5.1.5. A typical procedure for alkaline hydrolysis of alkyl esters – *9a–p*

To a solution of **8d** (326 mg, 0.61 mmol) in tetrahydrofuran (3 mL) and methanol (1 ml), 1 M LiOH (3.66 ml, 3.66 mmol) was added and the reaction mixture stirred at room temperature for 2 h. Solvent was evaporated under vacuum and the resulting aqueous solution neutralized with 0.1% trifluoroacetic acid (TFA) until the product started to precipitate (pH = 2). The product was purified, if necessary, by reverse phase column chromatography using methanol/0.1% TFA as eluant to obtain 124 mg (40%) of **9d** as a white solid.

5.1.6. 4-((6-((4-Fluorobenzyl)amino)-2,4-dimethyl-3,4-dihydro-2H-1,4-benzoxazin-2-yl)methoxy)benzonitrile (*6a*)

Synthesized according to the *A typical procedure for reductive amination* (5.1.1). The oily product was purified by column chromatography using dichloromethane/methanol (20:1) as eluant to give brown oil, yield 2.2 g (53%); ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.32 (s, 3H, 2-CH_3), 2.75 (s, 3H, N–CH_3), 2.96 (d, J = 11.5 Hz, 1H, 3-H), 3.19 (d, J = 11.5 Hz, 1H, 3-H), 4.01 (d, J = 9.1 Hz, 1H, CH_2O), 4.07 (d, J = 9.1 Hz, 1H, CH_2O), 4.16 (d, J = 6.1 Hz, 2H, Ph–CH_2), 5.66 (t, J = 6.0 Hz, 1H, NH), 5.86 (dd, J = 8.7 Hz, J = 2.4 Hz, 1H, Ar–H^7), 6.05 (d, J = 2.3 Hz, 1H, Ar–H^5), 6.43 (d, J = 8.4 Hz, 1H, Ar–H^8), 7.11–7.15 (m, 4H, Ar–$H^{2'}$, Ar–$H^{6'}$, Ph–$H^{3''}$, Ph–$H^{5''}$), 7.39 (d, J = 8.4 Hz, 2H, Ph–$H^{2''}$, Ph–$H^{6''}$), 7.73 (d, J = 8.9 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm 161.93 (C-1′), 160.97 (d, $^1J_{C-F}$ = 242 Hz, C-1″), 143.24 (C-6), 136.96 (dd, $^4J_{C-F}$ = 2.9 Hz, C-1″), 135.30 (C-4a),

134.13 (C-3′, C-5′), 133.88 (C-8a), 129.04 (d, $^3J_{C-F}$ = 8.0 Hz, C-2″, C-6″), 119.05 (CN), 115.81 (C-8), 115.71 (C-2′, C-6′), 114.82 (d, $^2J_{C-F}$ = 21.0 Hz, C-2″, C-6″), 103.07 (C-4′), 102.19 (C-7), 102.19 (C-7), 97.73 (C-5), 70.93 (CH_2O), 54.16 (C-3), 46.56 (Ph–$\underline{CH_2}$), 38.17 (*N*–CH_3), 20.97 (2-CH_3); IR (KBr, ν, cm^{-1}): 3423, 2221, 1604, 1508, 1219, 1036; HPLC: 95.4%, t_r 13.5 min; HRMS (ESI) *m/z* calcd for $C_{25}H_{25}FN_3O_2$ $[M+H]^+$ 418.1931, found 418.1942. Anal. Calcd. for $C_{25}H_{24}FN_3O_2$: C, H, N.

5.1.7. Ethyl 2-((2-((4-cyanophenoxy)methyl)-2,4-dimethyl-3,4-dihydro-2H-1,4-benzoxazin-6-yl)(4-fluorobenzyl)amino)acetate (**7a**)

Synthesized according to the *A typical procedure for preparation of N-alkylated compounds* (5.1.2). The brown oil was purified by column chromatography using petroleum ether/ethyl acetate (2.5:1) as eluant to give yellow oil, yield 1.45 g (60%); ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 1.28 (t, J = 7.1 Hz, 3H, CH_2–$\underline{CH_3}$), 1.46 (s, 3H, 2-CH_3), 2.78 (s, 3H, *N*–CH_3), 3.02 (d, J = 11.5 Hz, 1H, 3-H), 3.25 (d, J = 11.5 Hz, 1H, 3-H), 3.90 (d, J = 9.0 Hz, 1H, $\underline{CH_2}$–CH_3), 4.01 (s, 2H, *N*–CH_2–CO), 4.10 (d, J = 9.0 Hz, 1H, $\underline{CH_2}$–CH_3), 4.20 (d, J = 7.1 Hz, 1H, CH_2O), 4.24 (d, J = 7.1 Hz, 1H, CH_2O), 4.56 (s, 2H, Ph–CH_2), 6.00–6.15 (m, 2H, Ar–H^8, Ar–H^7), 6.67 (d, J = 9.1 Hz, 1H, Ar–H^5), 6.96 (d, J = 8.9 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 6.99–7.07 (m, 2H, Ph), 7.24–7.36 (m, 2H, Ph), 7.58 (d, J = 8.9 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); IR (KBr, ν, cm^{-1}): 3421, 2220, 1654, 1507, 1253, 1019; HPLC: 100.0%, t_r 22.4 min; HRMS (ESI) *m/z* calcd for $C_{29}H_{31}FN_3O_4$ $[M+H]^+$ 504.2299, found 504.2292. Anal. Calcd. for $C_{29}H_{30}FN_3O_4$: C, H, N.

5.1.8. Ethyl 2-((2-((4-cyanophenoxy)methyl)-2,4-dimethyl-3,4-dihydro-2H-1,4-benzoxazin-7-yl)(3,5-difluorobenzyl)amino)-2-oxoacetate (**7p**)

Synthesized according to the *A typical procedure for preparation of N-acylated compounds* (5.1.3). The brown oil was purified by column chromatography using petroleum ether/ethyl acetate (1.5:1) as eluant to give yellow oil, yield 1.41 g (55%) of a yellow oil; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 0.85 (t, J = 7.1 Hz, 3H, CH_2–$\underline{CH_3}$), 1.36 (s, 3H, 2-CH_3), 2.85 (s, 3H, *N*–CH_3), 3.08 (d, J = 11.1 Hz, 1H, 3-H), 3.29 (d, J = 11.0 Hz, 1H, 3-H), 3.96 (q, J = 7.1 Hz, 2H, $\underline{CH_2}$–CH_3), 4.10 (d, J = 7.1 Hz, 1H, CH_2O), 4.14 (d, J = 7.0 Hz, 1H, CH_2O), 4.88 (s, 2H, Ph–CH_2), 6.57–6.62 (m, 2H, Ar–H^6, Ar–H^8), 6.68 (d, J = 9.2 Hz, 1H, Ar–H^5), 6.90 (d, J = 6.2 Hz, 2H, Ph–$H^{2''}$, Ph–$H^{6''}$), 7.12 (d, J = 8.9 Hz, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.16 (tt, J = 9.4 Hz, J = 2.4 Hz, 1H, Ph–$H^{4''}$), 7.76 (d, J = 8.8 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm 162.40, 161.86, 161.75 ($\underline{CO}$–COO, CO–$\underline{COO}$, C-1′), 162.30 (dd, $^1J_{C-F}$ = 247 Hz, $^3J_{C-F}$ = 13.2 Hz, C-3″, C-5″), 142.12 (C-8a), 141.01 (t, $^3J_{C-F}$ = 9.0 Hz, C-1″), 135.41 (C-4a), 134.16 (C-3′, C-5′), 128.70 (C-7), 120.27 (C-6), 119.01 (CN), 115.65 (C-2′, C-6′), 114.48 (C-8), 111.85 (C-5), 110.89 (dd, $^2J_{C-F}$ = 18.3 Hz, $^4J_{C-F}$ = 6.6 Hz, C-2″, C-6″), 103.21 (C-4′), 103.04 (t, $^2J_{C-F}$ = 25.7 Hz, C-4″), 74.56 (C-2), 70.89 (CH_2O), 61.17 ($\underline{CH_2}$–CH_3), 53.27 (C-3), 50.19 (Ph–$\underline{CH_2}$), 38.06 (*N*–CH_3), 20.59 (2-CH_3), 13.33 (CH_2–$\underline{CH_3}$); IR (KBr, ν, cm^{-1}): 3504, 2225, 1741, 1666, 1509, 1204, 1033; HPLC: 100.0%, t_r 19.08 min; HRMS (ESI) *m/z* calcd for $C_{29}H_{28}F_2N_3O_5$ $[M+H]^+$ 536.1997, found 536.2006. Anal. Calcd. for $C_{29}H_{27}F_2N_3O_5$: C, H, N.

5.1.9. Ethyl 2-((2-((4-carbamimidoylphenoxy)methyl)-2,4-dimethyl-3,4-dihydro-2H-1,4-benzoxazin-6-yl)(3,4-difluorobenzyl)amino)acetate (**8i**)

Synthesized according to the *A typical procedure for preparation of amidines from nitriles* (5.1.4). The brown crude product was purified by reverse phase column chromatography using gradient of methanol/0.1% TFA (40–80%) as eluant to give white solid, yield 353 mg (39%); mp 159–162 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.20 (t, J = 7.1 Hz, 3H, CH_2–$\underline{CH_3}$), 1.34 (s, 3H, 2-CH_3), 2.72 (s, 3H, *N*–-CH_3), 3.00 (d, J = 11.6 Hz, 1H, 3-H), 3.23 (d, J = 11.5 Hz, 1H, 3-H), 4.04 (d, J = 9.9 Hz, 1H, CH_2O), 4.12 (q, J = 7.2 Hz, 2H, $\underline{CH_2}$–CH_3,), 4.13 (d, J = 9.9 Hz, 1H, CH_2O), 4.21 (s, 2H, *N*–CH_2–CO), 4.52 (s, 2H, Ph–CH_2), 5.84 (dd, J = 8.6, 2.8 Hz, 1H, Ar–H^7), 5.95 (d, J = 2.7 Hz, 1H, Ar–H^5), 6.50 (d, J = 8.6 Hz, 1H, Ar–H^8), 7.18 (d, J = 8.9 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.20 (overlapped, 1H, Ph–$H^{6''}$), 7.34–7.44 (m, 2H, Ph–$H^{2''}$, Ph–$H^{5''}$), 7.80 (d, J = 8.8 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$), 6.97 (br s, 2H, amidino-H), 9.15 (br s, 2H, amidino-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm: 171.25 (COO), 164.57 (C(=NH)NH_2), 162.81 (C-1′), 149.41 (dd, $^1J_{C-F}$ = 244.7 Hz, $^2J_{C-F}$ = 12.3 Hz, C-3″), 148.12 (dd, $^1J_{C-F}$ = 243.9 Hz, $^2J_{C-F}$ = 12.4 Hz, C-4″), 142.54 (C-8a), 137.97 (pseudo t, $^3J_{C-F}$ = 4.0 Hz, C-1″), 135.25 (C-6), 134.61 (C-4a), 130.13 (C-3′, C-5′), 123.28 (dd, $^3J_{C-F}$ = 6.2 Hz, $^4J_{C-F}$ = 3.1 Hz, C-6″), 123.29 (dd, $^3J_{C-F}$ = 6.2 Hz, $^4J_{C-F}$ = 3.1 Hz, C-6″), 119.85 (C-4′), 117.26 (d, $^2J_{C-F}$ = 16.9 Hz, C-5″), 115.84 (C-8), 115.62 (d, $^2J_{C-F}$ = 17.6 Hz, C-2″), 114.97 (C-2′, C-6′), 102.56 (C-7), 97.72 (C-5), 73.55 (C-2), 71.01 (CH_2O), 60.26 ($\underline{CH_2}$–CH_3), 54.91 (Ph–$\underline{CH_2}$), 54.03(C-3), 53.65 (N–$\underline{CH_2}$–CO), 38.05 (*N*–CH_3), 20.96 (2-CH_3), 14.14 (CH_2–$\underline{CH_3}$); IR (KBr, ν, cm^{-1}): 3417, 1672, 1515, 1266, 1186, 1140, 1039; HPLC: 100.0%, t_r 17.8 min; HRMS (ESI) *m/z* calcd for $C_{29}H_{33}F_2N_4O_4$ $[M+H]^+$ 539.2470, found 539.2481. Anal. Calcd. for $C_{29}H_{32}F_2N_4O_4 \times 1/4CF_3COOH \times 5/4H_2O$: C, H, N.

5.1.10. 2-((2-((4-Carbamimidoylphenoxy)methyl)-2,4-dimethyl-3,4-dihydro-2H-1,4-benzoxazin-7-yl)(4-fluorobenzyl)amino)-2-oxoacetic acid (**9d**)

Synthesized according to the *A typical procedure for alkaline hydrolysis of alkyl esters* (5.1.5). White solid was obtained, yield 124 mg (40%); mp 174–177 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.29 (s, 3H, 2-CH_3), 2.76 (s, 3H, *N*–CH_3), 2.91 (d, J = 11.8 Hz, 1H, 3-H), 3.09 (d, J = 11.7 Hz, 1H, 3-H), 3.88 (d, J = 10.1 Hz, 1H, CH_2O), 3.92 (d, J = 10.1 Hz, 1H, CH_2O), 4.74 (d, J = 15.3 Hz, 1H, Ph–$\underline{CH_2}$), 4.83 (d, J = 15.3 Hz, 1H, Ph–$\underline{CH_2}$), 6.58 (d, J = 2.1 Hz, 1H, Ar–H^8), 6.61 (d, J = 8.7 Hz, 1H, Ar–H^5), 6.66 (dd, J = 8.6 Hz, J = 2.1 Hz, 1H, Ar–H^6), 7.00 (d, J = 8.8 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.13 (t, J = 8.8 Hz, 2H, Ph), 7.22 (dd, J = 8.5 Hz, J = 7.7 Hz, 2H, Ph), 7.77 (d, J = 8.8 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$), 9.02 (br s, 2H, amidino-H), 9.92 (br s, 2H, amidino-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm: 167.88 ($\underline{CO}$–COOH), 164.33 (CO–$\underline{COOH}$), 162.34, 162.28 (C-1′, C(=NH)$\underline{NH_2}$), 161.14 (d, $^1J_{C-F}$ = 242.4 Hz, C-1″), 140.90 (C-4a, C-8a), 134.07 (d, $^4J_{C-F}$ = 2.2 Hz, C-4″), 133.96 (C-7), 129.74 (C-3′, C-5′), 129.34 (d, $^3J_{C-F}$ = 8.1 Hz, C-2″, C-6″), 120.08 (C-4′), 119.24 (C-6), 115.04 (d, $^2J_{C-F}$ = 21.3 Hz, C-3″, C-5″), 114.88 (C-2′, C-6′), 114.15 (C-8), 112.05 (C-5), 74.90 (C-2), 71.40 (CH_2O), 53.43 (C-3), 49.21 (Ph–$\underline{CH_2}$), 38.14 (*N*–CH_3), 21.16 (2-CH_3); IR (KBr, ν, cm^{-1}): 3366, 1608, 1511, 1489, 1265, 1183, 1041, 843; HPLC: 97.6%, t_r 12.6 min; HRMS (ESI) *m/z* calcd for $C_{27}H_{28}FN_4O_5$ $[M+H]^+$ 507.2044, found 507.2057. Anal. Calcd. for $C_{27}H_{27}FN_4O_5 \times 3/4HCl$: C, H, N.

5.2. Docking studies

The binding mode for the ligands to both targets was studied by CDOCKER, a docking tool based on the CHARMm force field, which is incorporated into Discovery Studio 3.0 (Accelrys Software Inc.). All ligands were docked in all possible stereoisomeric forms and were ionized at pH = 7.5 (protonated benzamidine moiety and anionic carboxylate moiety). The crystal structure of thrombin (PDB entry code: 1KTS) and GP IIb/IIIa (PDB entry code: 2VDM) was extracted from the Brookhaven Protein Database. Ligands were removed from the crystal structure prior to docking studies. Ligands were docked in a sphere with radius 15 Å in the active site. In CDOCKER, random ligand conformations are generated through molecular dynamics, and a variable number of rigid-body rotations/translations are applied to each conformation to generate the initial ligand poses. The conformations are further refined by grid-based simulated annealing in the receptor active site, which makes the

results accurate (2000 heating steps; heating target temperature was 700 K, 5000 cooling steps, cooling target temperature was 300 K). The CDOCKER interaction energy between the ligands and macromolecule was finally computed. The final minimization was performed using full potential.

A total of 100 dockings were performed (*Top Hits*), and ligands with RMSD less than 2 were joined in clusters. The ligand with the lowest docked energy was chosen for interpreting the docking results.

5.3. Biochemical evaluation

5.3.1. Enzyme assay for inhibition of serine proteases

The enzyme amidolytic method for determining inhibition was based on the spectrophotometric determination of absorbance of the product formed after amide bond cleavage of a chromogenic substrate in the presence of the enzyme. K_i, which is a quantitative measure of inhibitor potency, was determined from the kinetics of substrate hydrolysis with and without the addition of the inhibitor [15]. Measurements (spectrophotometer, BioTek Synergy H4) were performed in 96-well microtiter plates with a final volume of 200 μL. Thrombin was tested at a final concentration of 0.5 NIH E/mL with the substrate S-2238 (Chromogenix) at 20 and 40 μM final concentration, and factor Xa at the final concentration of 1 mBAEE E/mL with the substrate S-2222 (Chromogenix) at 100 and 200 μM final concentrations.

Trypsin was assayed at a final concentration of 0.5 nkat/mL through the use of the substrate S-2222 (Chromogenix) at 50 and 100 μM final concentrations. Inhibitors were dissolved in DMSO (concentration of stock solutions, 10 mmol/L) and diluted with distilled water to concentrations from 0.5 to 100 μM. Reaction rates were measured in the presence and the absence of the inhibitor. Then 50 μM HBSA buffer, the 50 μM solution of each inhibitor concentration (or of HBSA buffer in case of measurement without inhibitor), and 50 μM of enzyme solution were pipetted into the microtiter wells. The plate was incubated for 15 min at 25 °C and 50 μL of chromogenic substrate then added. Absorbance at 405 nm at 25 °C was measured every 10 s. Measurements were carried out in triplicate with three concentrations of the inhibitor and two concentrations of the substrate. For every combination of concentrations, K_i was calculated from the change of absorbance in the initial, linear part of the curve according to the method of Cheng and Prusoff and the final result was given as their average value. *Dabigatran* (thrombin, $K_i = 6.3 \pm 1.1$ nM) was used as control.

5.3.2. Inhibition of in vitro binding of fibrinogen to isolated GP IIb/IIIa and GPV/IIIa receptors

Binding affinities to GP IIb/IIIa and GPV/IIIa receptor were measured by a solid-phase competitive displacement assay [16]. Human fibrinogen (100 mg) was dissolved in aqueous NaCl (0.3 M, 5 mL) at 30 °C and then it was diluted with 0.1 M $NaHCO_3$ in water to a final concentration of 1 mg/mL. Biotin *N*-hydroxysuccinimide ester (2 mg) was dissolved in DMF (2 mL) and added to 6 mL of fibrinogen solution. The reaction mixture was incubated for 90 min at 30 °C and dialyzed for 3 h at room temperature against *buffer 1* (3 L, 20 mM Tris, 150 mM NaCl, pH 7.4). After dialysis, the solution was centrifuged for 5 min at 5400 rpm and Tween-20 (0.005%) added to give the stock solution. Human integrin (10 μL of GP IIb/IIIa receptor solution (Calbiochem), 1 μL of GPV/IIIa receptor solution (Calbiochem)) was diluted in 10.2 mL of *buffer 2* (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4) and adsorbed to 96-well (100 μL/well) high-binding microtiter plates (Greiner, Lumitrac 600) overnight at 4 °C. Nonspecific receptor-binding sites were blocked with 1% BSA in *buffer 2* (200 μL/well). Following incubation for 1 h at room temperature, the plates were washed twice with *buffer 3* (*buffer 2* containing 0.1% Tween-20). The potential antagonists were serially diluted with *buffer 2* containing 0.1% of BSA and test solutions added (50 μL/well) together with biotinylated fibrinogen (50 μL/well, 1:42 dilution of stock solution in *buffer 2* containing 0.1% of BSA) to each well. The plates were incubated for 2 h at room temperature, and then washed twice with *buffer 3*. Peroxidase-conjugated antibiotin goat antibody (100 μL/well of a 1:1000 dilution of purchased solution (Calbiochem) in *buffer 2* containing 0.1% of BSA) was added to each well and incubated for another hour. The microtiter plates were washed three times with *buffer 3*. Finally, chemiluminescent substrate (POD, Roche Diagnostics, Boehringer Mannheim) (100 μL/well) was added and the luminescence measured with a BioTek Synergy H4 multimode research reader three times over 10 min. Positive controls received no inhibitors, while negative controls received no receptor. *Tirofiban* ($IC_{50\ (GP\ IIb/IIIa)} = 0.37 \pm 0.11$ nM) was used as the internal standard. Assays were performed in duplicate and repeated at least three times on various days. The mean experimental data were fitted to the sigmoid model and IC_{50} values calculated from the dose–response curve (OriginPro, OriginLab, version 7.5).

Compounds **10**, which were already published, were tested again in the same test system on all targets as newly synthesised compounds in order to be able to fully compare determined biological activity.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2012.01.059. These data include MOL files and InChiKeys of the most important compounds described in this article.

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