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Low molecular weight dual inhibitors of factor Xa and fibrinogen binding to GPIIb/IIIa with highly overlapped pharmacophores

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ABSTRACT

Dual antithrombotic agents acting as anticoagulants and aggregation inhibitors could have substantial advantages over currently prescribed combinations of antithrombotic drugs. Herein, we report compounds with moderate inhibitory activity for factor Xa and fibrinogen GPIIb/IIIa binding (both in the micromolar range). These compounds resulted from our efforts to merge the pharmacophores of selective factor Xa inhibitor rivaroxaban with a mimic of the Arg-Gly-Asp (RGD) sequence of fibrinogen to obtain designed multiple ligands with potential antithrombotic activity. Resulting from this study, a structurally novel class of submicromolar fibrinogen GPIIb/IIIa binding inhibitor bearing 1,2,4-oxadiazol-5(4*H*)-one moiety is also described.

1. Introduction

Thrombotic complications such as myocardial infarction, stroke, unstable angina pectoris, and pulmonary embolism are a major cause of mortality and morbidity worldwide [1]. Due to diverse therapeutic disadvantages of currently prescribed anticoagulant and antiaggregatory drugs, there is a growing need for the development of better antithrombotic agents that would upgrade or replace the existing antithrombotic therapy. Over the past decade, major progress in the prevention of thromboembolic disorders has been made by the introduction of novel inhibitors of thrombin [2,3], factor Xa [4,5] and tissue factor/factor VIIa inhibitors [6,7], and platelet GPIIb/IIIa receptor antagonists [8]. As observed in clinical practice, concomitant use of anticoagulants (e.g., warfarin or heparin) and antiaggregatory drugs (e.g., acetylsalicylic acid or ticlopidine), as well as combinations of thrombin or fXa inhibitors with GPIIb/IIIa antagonists, can display synergistic effects [9,10]. These observations stimulated research toward designed multiple ligands [11] possessing anticoagulant and antiagreggatory activities in a single molecule. Such dual antithrombotic agents could have substantial advantages over combination therapies, including more predictable and less complex pharmacokinetics and pharmacodynamics [11,12], that could render them the antithrombotic drugs of the future.

Predominant strategies for the preparation of designed multiple ligands (DMLs) include the joining of pharmacophores of selective ligands by cleavable or non-cleavable linkers and the overlapping of pharmacophores resulting in fused or merged DMLs [11,12]. The first class of DMLs in the field of antithrombotics were thrombin/fXa and thrombin/factor VIIa inhibitors [13]. This class was followed by dual antiplatelet compounds and finally by anticoagulant/antiplatelet compounds such as thrombin inhibitors/GPIIb/IIIa antagonists, antithrombin III-mediated fXa inhibitors/GPIIb/IIIa antagonists, and thrombin or fXa inhibitors with antiaggregatory activity based on different mechanisms [14]. The main challenge in the development of antithrombotic designed multiple ligands turned out to be the adjustment of desired activity ratios at different targets along with their ability to modulate seemingly unrelated targets and simultaneously possess selectivity over closely related targets [15–18].

In recent publications our group reported on successful preparation of low molecular weight peptidomimetic compounds possessing thrombin inhibitory and GPIIb/IIIa antagonistic activities [15–17] that were designed by overlapping mimetics of the thrombin inhibiting *D*-Phe-Pro-Arg motif and the Arg-Gly-Asp

Abbreviations: CDI, 1,1′-carbonyldiimidazole; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMLs, designed multiple ligands; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; fXa, factor Xa; HOBt, *N*-hydroxybenzotriazole; RGD, Arg-Gly-Asp sequence; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

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(RGD) sequence responsible for binding of fibrinogen to GPIIb/IIIa (Fig. 1).

Successful preparation of compounds possessing inhibitory activity toward thrombin and fibrinogen-GPIIb/IIIa binding [15–18], combined with a hypothesis that targeting fXa could be a more effective strategy than targeting thrombin for anticoagulation [19–22], led us to the idea of combining pharmacophores of fXa inhibitors and GPIIb/IIIa antagonists to yield a low molecular weight, dual acting compound that would inhibit fXa and fibrinogen binding to GPIIb/IIIa. During the course of our work, a report that the antithrombotic activity of EP224283, which is a dual fXa inhibitor/GPIIb/IIIa antagonist prepared by linking fXa inhibitor idraparinux and GPIIb/IIIa antagonist tirofiban with polyethyl eneglycol, exceeded that of the coadministered parent compounds [19], strenghtened our belief that targeting fXa and GPIIb/IIIa with the envisaged dual acting compounds might be a viable concept in antithrombotic therapy.

2. Results and discussion

2.1. Design

To prepare compounds combining in the same molecule the capacities to inhibit both fXa and fibrinogen binding to GPIIb/IIIa, we overlapped the pharmacophores of a direct fXa inhibitor rivaroxaban and RGD sequence of fibrinogen (Fig. 2). The presence of various P_1 arginine-mimicking moieties in direct fXa inhibitors, which contribute significantly to the potency of these compounds via a direct electrostatic interaction with Asp189 in the S_1 pocket of fXa, suggested that the 5-chlorothiophene moiety of rivaroxaban could be replaced by a 2-aminothiazole or benzamidine moiety without significant effect on the inhibitory activity. The morpholinone moiety of rivaroxaban was replaced by piperidine carboxylate moieties to find a substitution pattern that would be simultenously well tolerated by the S_4 pocket of fXa and provide a distance of 1.5–1.8 nm between the introduced acidic and basic centers to enable the binding of these modified rivaroxaban analogs to GPIIb/IIIa. The rivaroxaban *N*-phenyl-(*S*)-oxazolidinone core was retained to allow for an L-shape of the molecules, which is needed for directing substituents into the S_1 and S_4 pockets of fXa, and to exploit two hydrogen bonds to Gly219, which are observed with rivaroxaban bound to fXa active site [23].

A small virtual library of ligands with (*S*) and (*R*) configurations of the central oxazolidinone core, and containing a piperidine carboxylate group in different positions as replacement for the morpholinone moiety, and a 2-aminothiazole replacing the 5-chlorothiophene terminus, was constructed and docked into the binding sites of fXa [23] and GPIIb/IIIa [24]. A comparison of docked structures to those in crystal structures of rivaroxaban/fXa [23], tirofiban/GPIIb/IIIa, L-739758/GPIIb/IIIa and eptifibatide/GPIIb/IIIa complexes [24] indicated that our ligands adopted suitable conformations and showed promising interactions with both targets. In the 4-carboxypiperidine series of compounds, we also explored alternative arginine mimetics to the 2-aminothiazole group of previous series, with the aims of optimally exploiting the interaction with Asp189 in the S_1 pocket of fXa, raising the GPIIb/IIIa antagonistic activity, and balancing the activities against both targets. To this end, the 2-aminothiazole group was replaced with benzamidine, 3-amino-4-chlorophenyl and 3-carbamimidoyl-4-chlorophenyl moieties attached to the rest of the molecule via an amide or methylenamino linkage.

2.2. Chemistry

The synthesis of target dual-activity compounds **7**, **8**, and **11**–**20** is depicted in Scheme 1. Both enantiomers of the key intermediate, ethyl 1-(4-(5-(aminomethyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylate (**5**), were prepared by hydrazinolysis of the phthalimido-protected precursor (**4**), which was synthesized in three steps from ethyl 1-(4-nitrophenyl)piperidine-4-carboxylate (**1**) by catalytic hydrogenation, opening of *N*-(2,3-epoxypropyl) phthalimide by the resulting amine **2**, and subsequent cyclization of **3** to oxazolidin-2-one **4**, as described previously [23]. Intermediate **5** was then coupled with *tert*-butyl (5-(chlorocarbonyl)thiazol-2-yl)carbamate, 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid, 3-amino-4-chlorobenzoic acid, or 4-chloro-3-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid to give compounds **6**, **9**, **17**, and **19**, or subjected to reductive amination using 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzaldehyde to give compound **10**. Compounds **7**, **8**, **11**, **12**, **13**, **14**, **15**, **16**, **18**, and **20** were then prepared by removal of protecting groups employing trifluoroacetic acid-mediated cleavage of *tert*-butyl carbamate, alkaline hydrolysis of ethyl ester, and hydrogenolytic cleavage of 1,2,4-oxadiazol-5(4*H*)-one. Compounds **7**, **8**, and **9**–**18** were prepared in both enantiomeric forms, whereas only the *S*-isomer was obtained for **19** and **20**. The synthesis of enantiomers was performed starting from (*S*)- and (*R*)-*N*-(2,3-epoxypropyl)phthalimide with 99% e.e. using non-racemizing conditions applied in the synthesis of rivaroxaban and standard non-racemizing coupling and deprotection procedures.

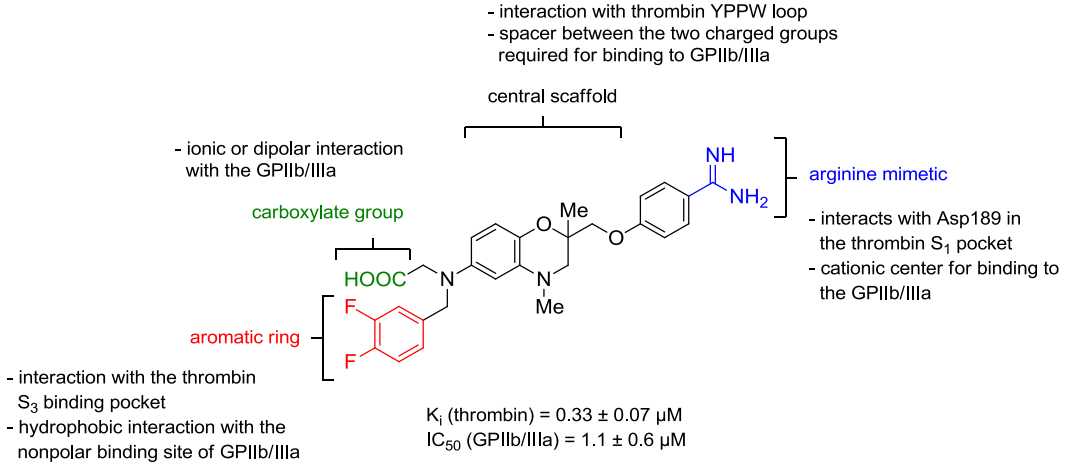


Fig. 1. Dual antithrombotic compound possessing well balanced inhibitory activities toward thrombin and fibrinogen-GPIIb/IIIa binding [17].

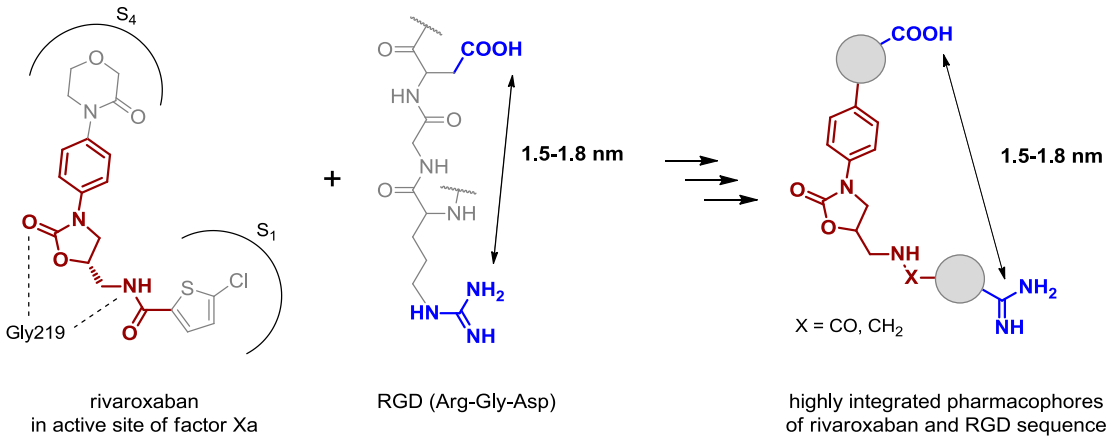
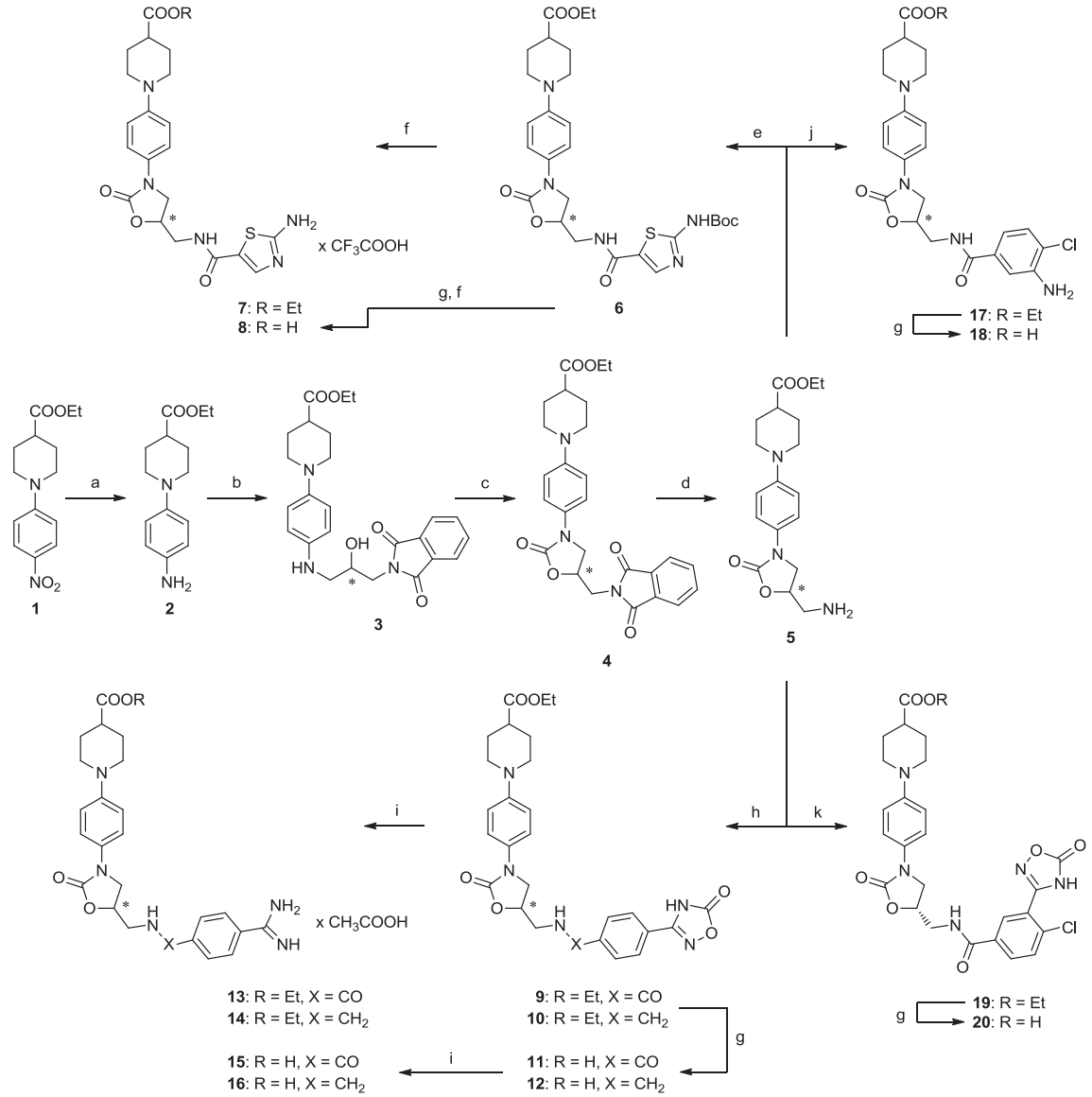


Fig. 2. Combining the pharmacophores of rivaroxaban (left) and the RGD sequence (middle).



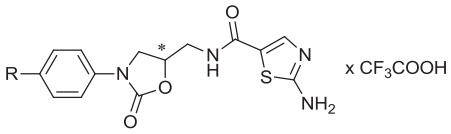
Scheme 1. Reagents and conditions: (a) H_2/Pd–C, EtOH, rt, 10–15 h; (b) (*S*)-*N*-(2,3-epoxypropyl)phthalimide or (*R*)-*N*-(2,3-epoxypropyl)phthalimide, EtOH, reflux, 15 h; (c) CDI, DMAP, THF, 60 °C, 15 h; (d) hydrazine, EtOH, 80 °C, 2 h; (e) *tert*-butyl (5-(chlorocarbonyl)thiazol-2-yl)carbamate, Et_3N, DCM, 0 °C → rt, 15 h; (f) TFA, DCM, 0 °C → rt, 15 h; (g) LiOH, MeOH/H_2O = 1:1, rt, 15 h; (h) 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid, HOBt, EDC, DIEA, DMF, rt, 15 h for **9** or 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzaldehyde, $Na(OAc)_3BH$, DMF, rt, 15 h for **10**; (i) H_2/Pd–C, CH_3COOH, DMF, rt, 15 h; (j) 3-amino-4-chlorobenzoic acid, HOBt, EDC, DIEA, DMF, rt, 15 h; (k) 4-chloro-3-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid, HOBt, EDC, DIEA, DMF, rt, 15 h.

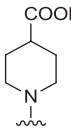
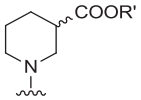
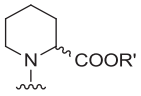
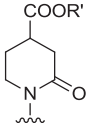
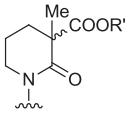
The analogs of compound **7a** in which the 4-ethoxycarbonyl piperidin-1-yl moiety was replaced by 3-ethoxycarbonylpiperidin-1-yl group (i.e., **7b**), 2-ethoxycarbonylpiperidin-1-yl group (**7c**), 4-ethoxycarbonyl-3-oxopiperidin-1-yl group (**7d**), or 3-ethoxcar bonyl-3-methyl-2-oxopiperidin-1-yl group (**7e**) (see Table 1 for formulas) were prepared from (*S*)-*N*-(2,3-epoxypropyl)phthalimide and respective ethyl 1-(4-nitrophenyl)piperidine carboxylates in accordance to synthetic pathway **1** → **8** to give a 1:1 mixture of two diastereomers for each compound (see Supporting Information).

2.3. Biological evaluation

In the first series of compounds (Table 1), the 5-chlorothiophene ring of rivaroxaban was replaced with the weakly basic 2-aminothiazole moiety and P_4 morpholin-3-one with a piperidine ring bearing a carboxylate group at positions 2, 3, or 4. 2-Amino thiazole was expected to be well tolerated in the S_1 subsite of fXa because 2-chlorothiazole and 3-amino-2-chlorothiophene analogs of rivaroxaban have displayed nanomolar potencies [23]. Our intent was to find a P_4 moiety that would provide suitable distance between its acidic group and the amidine motif of the 2-amino thiazole moiety to mimic the RGD pharmacophore and thus enable binding to GPIIb/IIIa. The determined activities of inhibition of fXa, thrombin, and trypsin and of inhibition of fibrinogen binding to GPIIb/IIIa for this series are given in Table 1. A penalty for building GPIIb/IIIa binding activity into rivaroxaban was a substantial reduction in fXa inhibitory potency in the resulting compounds. This effect was mainly due to replacement of the 2-chlorothiophene group with a 2-aminothiazole moiety because 2-chlorothiophene analogs of compounds **7a**–**d** and **8a**–**e** still possessed nanomolar inhibitory constants for fXa [25]. Comparison of fXa inhibitory potencies of ester/acid pairs **7a**/**8a**, **7b**/**8b**, **7f**/**8f** and ester **7c** (the corresponding carboxylic acid could not be obtained in pure form), which differ in the position of substitution on the piperidine ring, showed only slight differences in the potencies of the esters and their corresponding acids. Substitution at the 2-position (**7c**: $K_{i\,(fxa)} = 28.7 \pm 5.6$ μM) proved to be most favorable. In this case, the ethyl carboxylate group of phenylpiperidine-2-carboxylate forces the two rings into a perpendicular arrangement, thus mimicking perfectly the conformation of the (3-oxomorpholine)phenyl moiety in rivaroxaban [23]. Moreover, the same effect of the 2-oxo group can explain why **8e** ($K_{i\,(fxa)} = 11.2 \pm 2.3$ μM) displayed the most potent fXa inhibitory activity in this series of compounds.

Table 1
Biological activity of P_1 2-aminothiazole series of compounds: the inhibition of serine proteases fXa, thrombin and trypsin and the inhibition of fibrinogen binding to GPIIb/IIIa.



Comp.	R	Configuration (*)	R′	K_i (μM)[a]			IC_{50} (μM)[b]
				FXa	Thrombin	Trypsin	GPIIb/IIIa
7a		*S*	Et	54.4 ± 6.5	>100	>100	>300
8a		*S*	H	48.8 ± 4.0	>100	>100	78.4 ± 23.7
7f		*R*	Et	111 ± 19	>100	>100	>300
8f		*R*	H	88.2 ± 16.1	>100	>100	16.8 ± 5.9
7b		*S*	Et	49.3 ± 6.1	>100	>100	>300
8b		*S*	H	47.5 ± 7.5	>100	>100	>300
7c		*S*	Et	28.7 ± 5.6	>100	>100	>300
7d		*S*	Et	38.2 ± 3.3	>100	>100	>300
7e		*S*	Et	55.6 ± 9.1	>100	>100	>300
8e		*S*	H	11.2 ± 2.3	>100	>100	>300
	Rivaroxaban			0.66 ± 0.09 nM	n.d.[c]	n.d.[c]	n.d.[c]
	Tirofiban			n.d.[c]	n.d.[c]	n.d.[c]	0.37 ± 0.11 nM
	Dabigatran			n.d.[c]	6.3 ± 1.1 nM	n.d.[c]	n.d.[c]

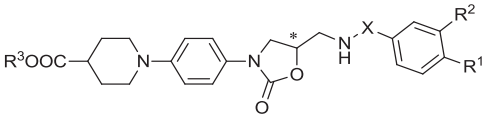
*Denotes the position of a stereogenic center in molecule.
[a] Measurements were carried out in triplicate with three concentrations of the inhibitor and two concentrations of the substrate.
[b] Assays were performed in duplicate and repeated at least three times on various days.
[c] n.d.: not determined.

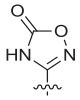
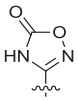
Compounds **7a**–**f** and **8a**–**f** showed no inhibition of the related serine proteases thrombin and trypsin, whereas only the enantiomers **8a** and **8f**, which contain a piperidine-4-carboxylic acid moiety, proved to be active both in the fXa inhibition assay and in the GPIIb/IIIa binding inhibiton assay. The (*R*)-enantiomer **8f** ($IC_{50\ (GPIIb/IIIa)} = 16.8 \pm 5.9$ μM) showed 4.7-fold higher activity than the (*S*)-enantiomer **8a** ($IC_{50\ (GPIIb/IIIa)} = 78.4 \pm 23.7$ μM) for inhibition of fibrinogen binding to GPIIb/IIIa, whereas **8f** was 1.8-fold less potent for inhibition of fXa than the (*S*)-enantiomer **8a** (**8f**: $K_{i\ (fxa)} = 88.2 \pm 16.1$ μM; **8a**: $K_{i\ (fxa)} = 48.8 \pm 4.0$ μM). The better binding observed for the (*R*)-enantiomer **8f** to GPIIb/IIIa was in agreement with our preliminary docking results, which indicated a possible effect of stereochemistry on the binding of this series of compounds to GPIIb/IIIa. The analogs of compounds **8a** and **8f** with a 2-chlorothiophene group replacing the 2-aminothiazole moiety possessed no fibrinogen GPIIb/IIIa binding inhibitory activity ($IC_{50\ (GPIIb/IIIa)} > 300$ μM; results not shown) which demonstrates the importance of the 2-aminothiazole terminus for GPIIb/IIIa binding. In the tested series, the piperidine-4-carboxylic acid moiety endowed **8a** and **8f** with weak dual activity, which is most likely because of an appropriate distance between acidic and basic centers in the molecule, and thus a decision was made to retain the piperidine-4-carboxylate group while exploring 2-aminothiazole surrogates in the next series.

To fully exploit interaction with Asp189 in the S_1 pocket of fXa, increase the binding affinity for GPIIb/IIIa, and balance the activities of compounds on both targets, in the second series of compounds comprising a piperidine-4-carboxylic acid moiety (Table 2), 2-aminothiazole was replaced either with a benzamidine group linked to the rest of the molecule via an amide bond (compounds **13** and **15**) or a methylenamino group (compounds **14** and **16**), or with a 3-amino-4-chlorophenyl moiety (compounds **17** and **18**), while our attempt to prepare a 3-carbamimidoyl-4-chlorophenyl derivative via intermediate **19** failed.

Comparing the activities of the 2-aminothiazole derivative **8a** ($K_{i\ (fxa)} = 48.8 \pm 4.0$ μM; $IC_{50\ (GPIIb/IIIa)} = 78.4 \pm 23.7$ μM) with the corresponding benzamidine compound **15a** ($K_{i\ (fxa)} = 43.0 \pm 5.6$ μM; $IC_{50\ (GPIIb/IIIa)} = 12.0 \pm 1.0$ μM) as well as those of **8f** ($K_{i\ (fxa)} = 88.2 \pm 16.1$ μM; $IC_{50\ (GPIIb/IIIa)} = 16.8 \pm 5.9$ μM) and **15b** ($K_{i\ (fxa)} = 62.9 \pm 9.2$ μM; $IC_{50\ (GPIIb/IIIa)} = 4.7 \pm 0.1$ μM) lead to the conclusion that replacement of the 2-aminothiazole with a benzamidine moiety resulted in 3.6–5.7-fold improvement of GPIIb/IIIa binding, whereas no improvement of fXa inhibitory activity was observed. Moreover, introduction of a benzamidine group rendered compounds **13a**–**16a** nonselective because they gained trypsin inhibitory activity with K_i values in the range 2.2–17.8 μM. Compound **15b** ($K_{i\ (trypsin)} = 3.79 \pm 0.46$ μM; $IC_{50\ (GPIIb/IIIa)} = 4.7 \pm 0.1$ μM) was identified as a well-balanced, low micromolar,

Table 2
Inhibition of serine protease fXa, thrombin, and trypsin, and inhibition of fibrinogen binding to GPIIb/IIIa by benzamidine-, 3-amino-4-chlorophenyl-, and (5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)phenyl compounds **11**–**20**.



Comp.	R^1	R^2	R^3	X	Conf.	K_i (μM)[a]			IC_{50} (μM)[b]
						FXa	Thrombin	Trypsin	GPIIb/IIIa
11a		H	H	CO	*S*	>100	>100	>100	16.0 ± 1.0
11b		H	H	CO	*R*	>100	>100	>100	0.87 ± 0.07
12a		H	H	CH_2	*S*	>100	>100	>100	0.89 ± 0.01
12b		H	H	CH_2	*R*	94.8 ± 11.3	>100	>100	0.26 ± 0.01
13a	 x CH_3COOH	H	Et	CO	*S*	>100	>100	5.65 ± 0.35	14.0 ± 2.0
13b		H	Et	CO	*R*	>100	>100	3.06 ± 0.34	96.0 ± 10.0
14a		H	Et	CH_2	*S*	34.3 ± 7.7	>100	2.17 ± 0.31	55.0 ± 5.0
14b		H	Et	CH_2	*R*	25.6 ± 6.0	>100	6.59 ± 0.55	35.0 ± 8.0
15a	 x CH_3COOH	H	H	CO	*S*	43.0 ± 5.6	>100	8.95 ± 0.77	12.0 ± 1.0
15b		H	H	CO	*R*	62.9 ± 9.2	>100	3.79 ± 0.46	4.7 ± 0.1
16a		H	H	CH_2	*S*	54.1 ± 6.1	53.0 ± 4.6	17.8 ± 1.2	2.2 ± 0.2
17a	Cl	NH_2	Et	CO	*S*	10.8 ± 2.0	>100	>100	>300
17b		NH_2	Et	CO	*R*	82.3 ± 6.4	>100	>100	>300
18a		NH_2	H	CO	*S*	37.0 ± 2.7	>100	>100	25.8 ± 8.1
18b		NH_2	H	CO	*R*	57.7 ± 8.6	>100	>100	12.8 ± 1.0
20			H	CO	*S*	89.7 ± 8.2	>100	>100	15.3 ± 1.1

[a] Measurements were carried out in triplicate with three concentrations of the inhibitor and two concentrations of the substrate.
[b] Assays were performed in duplicate and repeated at least three times on various days.

dual trypsin inhibitor/fibrinogen GPIIb/IIIa binding inhibitor for which no therapeutic relevance has yet been identified. Replacement of the amide bond in **15a** with its reduced methylenamino surrogate (**16a**) [26] resulted in a further 5.5-fold improvement of GPIIb/IIIa binding with a concomitant slight decrease in fXa inhibitory activity (**16a**: $K_{i\ (fxa)} = 54.1 \pm 6.1$ μM; $IC_{50\ (GPIIb/IIIa)} = 2.2 \pm 0.2$ μM). Ester analogs **14a** and **14b** showed a balanced albeit moderate activity on both targets (e.g., **14b**: $K_{i\ (fxa)} = 25.6 \pm 6.0$ μM; $IC_{50\ (GPIIb/IIIa)} = 35.0 \pm 8.0$ μM).

Interestingly, intermediates **11b**–**12b**, which bear a 1,2,4-oxadiazol-5(4*H*)-one moiety as a masked amidino group, showed submicromolar inhibition of fibrinogen binding to GPIIb/IIIa (**11b**: $IC_{50\ (GPIIb/IIIa)} = 0.87 \pm 0.07$ μM; **12a**: $IC_{50\ (GPIIb/IIIa)} = 0.89 \pm 0.01$ μM; **12b**: $IC_{50\ (GPIIb/IIIa)} = 0.26 \pm 0.01$ μM). These compounds present an excellent starting point for optimization toward a structurally novel class of nanomolar GPIIb/IIIa antagonists. Compounds **11b**–**12b** were devoid of fXa inhibitory activity, as expected on the basis of their structure.

Replacement of the 2-aminothiazole group with benzamidine resulted only in improvement of fibrinogen GPIIb/IIIa binding inhibition, whereas fXa inhibitory activity remained almost unchanged. Therefore, we hypothesized that introduction of a chlorine atom, which is a crucial P_1 substituent for the fXa inhibitory activity of rivaroxaban, onto the benzamidine moiety would raise the fXa inhibitory activity of our compounds. Preparation of the synthetically more tractable 3-amidino-4-chlorophenyl analog was attempted but unfortunately failed in the last step, in which the Pd-mediated hydrogenolytic cleavage of 1,2,4-oxadiazol-5(4*H*)-one **20** to an amidino group resulted in partial removal of the chlorine atom, and the low yield of this reaction prevented isolation of a sufficient quantity of the 3-chloro-4-amidino analog. Again, like the *para*-analog **11a**, 1,2,4-oxadiazol-5(4*H*)-one **20** inhibited the binding of fibrinogen to GPIIb/IIIa ($IC_{50\ (GPIIb/IIIa)} = 15.3 \pm 1.1$ μM) and showed no sustantial fXa inhibitory activity. In the second attempt of introducing a chlorine atom, the benzamidine moiety of **15a** and **15b** was replaced with a 3-amino-4-chlorophenyl moiety, which has been identified as a potent benzamidine mimic [27] to obtain the enantiomers **18a** ($K_{i\ (fxa)} = 37.0 \pm 2.7$ μM; $IC_{50\ (GPIIb/IIIa)} = 25.8 \pm 8.1$ μM) and **18b** ($K_{i\ (fxa)} = 57.7 \pm 8.6$ μM; $IC_{50\ (GPIIb/IIIa)} = 12.8 \pm 1.0$ μM).

A comparison of the activities of enantiomeric pairs **8a**/**8f**, **11a**/**11b**, **12a**/**12b**, **14a**/**14b**, **15a**/**15b**, and **18a**/**18b** demonstrates that (*R*)-enantiomers are 2.6-fold–18.4-fold more potent than (*S*)-enantiomers as inhibitors of fibrinogen binding to GPIIb/IIIa. This result is in agreement with our preliminary docking results, whereas stereochemistry does not display a consistent influence on fXa inhibitory activity.

Fig. 3 displays binding configurations of compound **18a** in the active sites of fXa (PDB code: 2W26) and GPIIb/IIIa (PDB code: 2VDM) as predicted by CDOCKER, which is a CHARMM-based docking program [28] that systematically positions the flexible ligands within a static active site and conducts low-level energy calculations for each position. The 3-amino-4-chlorophenyl moiety of compound **18a** occupies the S_1 subsite of fXa in which the position of the chlorine atom matches that of rivaroxaban and the amino group forms a hydrogen bond to Asp189. Due to the bulkiness of the 3-amino-4-chlorophenyl moiety, the central oxazolidinone core of the molecule stretches over that of rivaroxaban. This feature results in one hydrogen bond to Gly219 and influences the improper occupation of the S_4 subsite. The predicted binding conformation of compound **18a** to GPIIb/IIIa is extended and the carboxylate group on the piperidine ring matches that of bound tirofiban. It forms multiple hydrogen bonds to Tyr122, Ser123 and Asn215 in the β3 subunit of the receptor while coordinating to the proximal Mg^{2+}. The central phenyl ring forms a π–π interaction

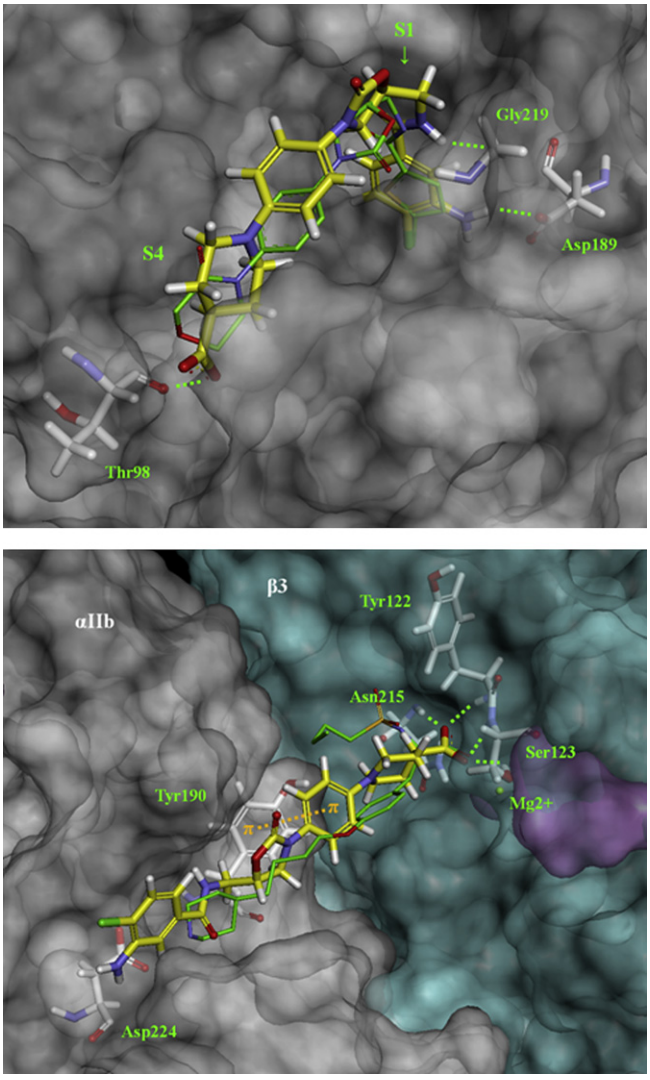


Fig. 3. Compound **18a** docked into the fXa active site (top) and GPIIb/IIIa active site (bottom). The conformations of rivaroxaban in complex with fXa [23] and of tirofiban in complex with GPIIb/IIIa [24] (from the X-ray crystal structures) are indicated in green. The αIIb and β3 subunits of GPIIb/IIIa are shown in gray and blue. The figures were prepared by Discovery Studio 3.1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with Tyr190 of the αIIb subunit, whereas the amino group of 3-amino-4-chlorophenyl moiety fails to establish an expected strong polar interaction with Asp224.

3. Conclusions

We describe the first low molecular weight, dual inhibitors of fXa and fibrinogen binding to platelet fibrinogen receptor (GPIIb/IIIa). These compounds were designed by overlapping the pharmacophores of the selective fXa inhibitor rivaroxaban and the Arg-Gly-Asp (RGD) sequence of fibrinogen. In the first series of compounds, the 5-chlorothiophene ring of rivaroxaban was replaced with a 2-aminothiazole ring, and the piperidine-4-carboxylate moiety was identified as a morpholin-3-one replacement that is well tolerated by the S_4 pocket of fXa while simultaneously enabling weak dual activity. In the next series of compounds, which retain the piperidine-4-carboxylate group, the 2-amino thiazole was replaced with benzamidine and 3-amino-4-chloro phenyl moieties. These modifications led to compound **18a** (K_i

$_{(fxa)}$ = 37.0 μM; $IC_{50\ (GPIIb/IIIa)}$ = 25.8 μM) and **18b** ($K_{i\ (fxa)}$ = 57.7 μM; $IC_{50\ (GPIIb/IIIa)}$ = 12.8 μM), which display balanced, moderate inhibitory activity for both targets and are selective against thrombin and trypsin. As a part of these studies, compounds **11b**, **12a** and **12b** bearing an 1,2,4-oxadiazol-5(4*H*)-one moiety were identified as submicromolar inhibitors of fibrinogen binding to GPIIb/IIIa that deserve further investigation. In conclusion, the present study demonstrates that designing submicromolar dual inhibitors of fXa and fibrinogen binding to GPIIb/IIIa remains a demanding challenge due to constraints imposed by both targets.

4. Experimental

4.1. Chemistry

Chemicals were obtained from Aldrich Chemical Co., Acros, and Alfa Aesar and used without purification. Analytical TLC was performed on silica gel Merck 60 F254 plates (0.25 mm), using visualization with ultraviolet light and ninhydrin. Column chromatography was carried out on flash silica gel (particle size 40–240 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. Optical rotations were measured on a Perkin–Elmer 1241 MC polarimeter. The reported values for specific rotation are average values of 10 successive measurements using an integration time of 5 s. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Bruker AVANCE DPX300 spectrometer (^{1}H NMR at 300.132 MHz and ^{13}C NMR at 75.475 MHz) or Bruker AVANCE III spectrometer (^{1}H NMR at 400.130 MHz and ^{13}C NMR at 100.613 MHz) in $CDCl_3$ or DMSO-d_6 solution. 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 (doublet of doublets), ddd (doublet of doublet of doublets), td (triplet of doublets), t (triplet), dt (doublet of triplets), and m (multiplet). Mass spectra were obtained using a VG Analytical Autospec Q mass spectrometer. IR spectra were obtained on a Perkin–Elmer FTIR System Spectrum BX or Nicolet Nexus 470 FTIR spectrometers. Microanalyses were performed on a Perkin–Elmer C, H, N analyzer 240C. Analyses indicated by the symbols of the elements were within 0.4% of the theoretical values. HPLC analyses were performed on an Agilent Technologies HP 1100 instrument with a G1365B UV–VIS detector (254 nm), a G1316A thermostat, and a G1313A autosampler, using an Agilent Eclipse Plus C18 column (5 μm, 4.6 × 150 mm) at a flow rate of 1 mL/min. The eluant was a mixture of 0.1% trifluoroacetic acid in water (A) and methanol (B). The gradient was 80% A to 20% A over 15 min, then to 10% A over 1 min, then maintaining 10% A for 3 min, then to 80% A over 1 min.

4.1.1. General procedure for the preparation of ethyl 1-(4-nitrophenyl) piperidinecarboxylates *1a*, *1b* and *1c*

To a stirred solution of ethyl isonipecotate (4.62 mL, 30 mmol) or ethyl nipecotate (4.66 mL, 30 mmol) or ethyl pipecolinate (9.38 mL, 60 mmol) and 1-fluoro-4-nitrobenzene (1 eq.) in dimethylsulfoxide (1 M) anhydrous potassium carbonate (1.5 eq.) was added. The resulting mixture was stirred at 55 °C for 15 h and after dilution with cold water extracted with dichloromethane. The organic phase was dried over Na_2SO_3, filtered and concentrated under reduced pressure. Compound **1a** was recrystallized from dichloromethane/hexane (1/19). Compounds **1b** and **1c** were isolated from residue by flash column chromatography using dichloromethane or hexane/EtOAc (5/1) as eluant.

4.1.2. General procedure for catalytic hydrogenation – synthesis of compounds *2a*, *2b*, *2c*, *2d*, and *2e*

Mixture of starting compound (**1a**–**e**) and 10% palladium on activated charcoal in absolute ethanol (0.1 M) was stirred under 1 atm H_2 for 10–15 h at room temperature. The progress of the reaction was monitored by TLC. After completion palladium on activated charcoal was removed by filtration. Filtrate was evaporated under reduced pressure yielding crude amine as red–brown oil. Compounds **2a**–**e** were used without further purification.

4.1.3. General procedure for opening of (S)- or (R)-N-(2,3-epoxypropyl) phthalimides *3a*, *3b*, *3c*, *3d*, *3e*, *3f*

Suspension of starting compound (**2a**–**e**) and (*S*)-*N*-(2,3-epoxypropyl)phthalimide or (*R*)-*N*-(2,3-epoxypropyl)phthalimide (1 eq.) in absolute ethanol (0.2 M) was refluxed for 15 h. The crude product precipitated from clear solution at 0 °C. Compounds **3a**–**f** were purified either by flash column chromatography using EtOAc/hexane as eluant or by recrystallization from absolute ethanol.

4.1.4. General procedure for cyclization to oxazolidin-2-ones *4a*, *4b*, *4c*, *4d*, *4e*, *4f*

N,*N*′-Carbonyldiimidazole (2 eq.) and 4-(dimethylamino)pyridine (catalytic amount) were added to a suspension of starting compound (**3a**–**f**) in tetrahydrofuran (0.1 M). The reaction mixture was stirred for 15 h at 60 °C. The crude product precipitated from clear solution at 0 °C. Compounds **4a**–**4f** were purified either by flash column chromatography using dichloromethane/methanol as eluant or by recrystallization from absolute ethanol.

4.1.5. General procedure for preparation of *5a*, *5b*, *5c*, *5d*, *5e*, *5f* by hydrazinolysis

To a solution of starting compound (**4a**–**f**) in absolute ethanol (0.1 M) hydrazine hydrate (55% solution) (3 eq.) was added. The reaction mixture was stirred at 80 °C for 2 h and afterward at 0 °C for 30 min. Precipitated white crystals of phthalhydrazide were removed by filtration. Filtrate was concentrated under reduced pressure yielding crystals of crude product. Compounds **5a**–**f** were used without further purification.

4.1.6. General procedure for coupling of *5a*–*f* with tert-butyl (5-(chlorocarbonyl)thiazol-2-yl)carbamate – synthesis of compounds *6a*, *6b*, *6c*, *6d*, *6e*, *6f*

Solution of *tert*-butyl (5-(chlorocarbonyl)thiazol-2-yl)carbamate (1.2 eq.) in dichloromethane (0.35 M) was added dropwise to a stirred solution of starting compound (**5a**–**e**) and triethylamine (2 eq.) in dichloromethane (0.075 M) at 0 °C. The reaction mixture was stirred for 15 h at room temperature followed by removal of solvent under reduced pressure to obtain a crude product, which was purified by flash column chromatography using dichloromethane/methanol (19/1) or EtOAc as eluant.

4.1.7. General procedure for trifluoroacetic acid-mediated deprotection of *6a*–*f* – synthesis of compounds *7a*, *7b*, *7c*, *7d*, *7e*, *7f*

To a solution of starting compound (**6a**–**f**) in dichloromethane (0.1 M) at 0 °C trifluoroacetic acid (3 eq.) was added. The reaction mixture was allowed to warm to room temperature and was stirred for 15 h. Solvent and excessive trifluoroacetic acid were removed under reduced pressure to obtain pure products **7a**–**f**.

4.1.8. General procedure for alkaline hydrolysis of ethyl esters and subsequent trifluoroacetic acid-mediated deprotection of *6a*–*f* – synthesis of compounds *8a*, *8b*, *8c*, *8d*, *8e*, *8f*

To a solution of starting compound (**6a**–**f**) in methanol/water (1/1) (0.05 M) lithium hydroxide (1.5 eq.) was added and the reaction mixture was stirred at room temperature. The progress of the reaction was monitored by TLC. After completion methanol was removed under reduced pressure. Water phase was cooled to 0 °C and acidified (pH = 1–2) with addition of conc. hydrochloric acid. Precipitated crystals were separated by filtration, dried in vacuum

and subjected to conditions of General procedure for trifluoroacetic acid-mediated deprotection of **6a–f** (4.1.7.). The crude products were purified by flash column chromatography using dichloromethane/methanol (9/1) as eluant or used without purification.

*4.1.9. Ethyl 1-(4-nitrophenyl)piperidine-4-carboxylate (**1a**)*

Brown crystals of a crude product were recrystallized from dichloromethane/hexane (1/19) to obtain yellow crystals, yield: 7.92 g (95%), mp 90–93 °C. ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 3H, J = 7.2 Hz, CH_2CH_3), 1.75–1.88 (m, 2H, CH_2CHCH_2), 1.99–2.06 (m, 2H, CH_2CHCH_2), 2.53–2.57 (m, 1H, CH_2CHCH_2), 3.06 (dd, 1H, J = 10.7 Hz, 2.7 Hz CH_2NCH_2), 3.10 (dd, 1H, J = 10.7 Hz, 2.7 Hz CH_2NCH_2), 3.85 (t, 1H, J = 3.6 Hz CH_2NCH_2), 3.90 (t, 1H, J = 3.6 Hz CH_2NCH_2), 4.15 (q, 2H, J = 7.1 Hz, CH_2CH_3), 6.81 (d, 2H, J = 9.5 Hz, Ar–H^2,H^6), 8.09 (d, 2H, J = 9.5 Hz, Ar–H^3,H^5). MS (ESI) (%) = 279.1 (M^+, 100). HRMS (ESI) calcd for $C_{14}H_{19}N_2O_4$ 279.1345, found 279.1348. IR (ATR) 2954, 2897, 1723, 1595, 1581, 1513, 1483, 1452, 1404, 1372, 1301, 1250, 1229, 1187, 1165, 1150, 1108, 1038, 1015 cm^{-1}.

*4.1.10. Ethyl 1-(4-aminophenyl)piperidine-4-carboxylate (**2a**)*

Crude product yield: 9.30 g (100%), red–brown oil. ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 3H, J = 7.2 Hz, CH_2CH_3), 1.82–2.04 (m, 4H, CH_2CHCH_2), 2.32–2.42 (m, 1H, CH_2CHCH_2), 2.62–2.70 (m, 2H, CH_2NCH_2), 3.40 (t, 1H, J = 3.7 Hz CH_2NCH_2), 3.44 (t, 1H, J = 3.7 Hz CH_2NCH_2), 4.15 (q, 2H, J = 7.1 Hz, CH_2CH_3), 6.63 (d, 2H, J = 8.8 Hz, Ar–H^2,H^6), 6.81 (d, 2H, J = 8.8 Hz, Ar–H^3,H^5), *NH_2 peak not seen*. MS (ESI) (%) = 249.2 (M^+, 100). HRMS (ESI) calcd for $C_{14}H_{21}N_2O_2$ 249.1603, found 249.1599.

*4.1.11. (S)-Ethyl 1-(4-((3-(1,3-dioxoisoindolin-2-yl)-2-hydroxypropyl) amino)phenyl)piperidine-4-carboxylate (**3a**)*

The crude product was recrystallized from absolute ethanol to give beige crystals, yield: 2.79 g (55%), mp 148–150 °C. $[\alpha]_D^{20}$ +7.3 (c 0.25, DMSO). ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.83–2.05 (m, 4H, CH_2CHCH_2), 2.33–2.43 (m, 1H, CH_2CHCH_2), 2.65 (dd, 1H, J = 10.6 Hz, 2.7 Hz, CH_2NCH_2), 2.69 (dd, 1H, J = 10.8 Hz, 2.7 Hz, CH_2NCH_2), 3.15 (dd, 1H, J = 13.2 Hz, 6.6 Hz, ArNHCH_2), 3.27 (dd, 1H, J = 13.2 Hz, 4.9 Hz, ArNHCH_2), 3.42 (t, 1H, J = 3.6 Hz, CH_2NCH_2), 3.46 (t, 1H, J = 3.6 Hz, CH_2NCH_2), 3.90–3.93 (m, 2H, CON(CO)CH_2CH), 4.23 (q, 3H, J = 7.2 Hz, CH_2CH_3, CHOH), 6.66 (d, 2H, J = 8.8 Hz, Ar–H^2,H^6), 6.86 (d, 2H, J = 8.7 Hz, Ar–H^3,H^5), 7.74–7.79 (m, 2H, Ar–H$^{4'}$,H$^{7'}$), 7.87–7.90 (m, 2H, Ar–H$^{5'}$,H$^{6'}$), *NH and OH peaks not seen*. MS (ESI) (%) = 452.0 (M^+, 70), 406.0 (40), 261.0 (40), 248.0 (100). HRMS (ESI) calcd for $C_{25}H_{30}N_3O_5$ 452.2185, found 452.2177. IR (KBr) 3324, 2944, 1772, 1715, 1516, 1466, 1429, 1395, 1308, 1253, 1172, 1090, 1030 cm^{-1}. Anal. calcd. for $C_{25}H_{29}N_3O_5$: C, 66.50; H, 6.47; N, 9.31; found C, 66.55; H, 6.39; N, 9.55.

*4.1.12. (S)-Ethyl 1-(4-(5-((1,3-dioxoisoindolin-2-yl)methyl)-2-oxooxa zolidin-3-yl)phenyl)piperidine-4-carboxylate (**4a**)*

The crude product was recrystallized from absolute ethanol to give beige crystals, yield: 1.44 g (49%), mp 183–188 °C. $[\alpha]_D^{20}$ −53.4 (c 0.25, DMSO). ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.82–2.07 (m, 4H, CH_2CHCH_2), 2.39–2.49 (m, 1H, CH_2CHCH_2), 2.78 (dt, 2H, J = 11.8 Hz, 3.0 Hz, CH_2NCH_2), 3.58 (t, 1H, J = 3.7 Hz, CH_2NCH_2), 3.62 (t, 1H, J = 3.7 Hz, CH_2NCH_2), 3.88 (dd, 1H, J = 9.1 Hz, 5.9 Hz, ArNCH_2CH), 3.98 (dd, 1H, J = 14.0 Hz, 5.8 Hz, CON(CO) CH_2CH), 4.07–4.21 (m, 4H, CH_2CH_3, ArNCH_2CH, CON(CO)CH_2CH), 4.93–5.02 (m, 1H, ArNCH$_2$CH), 6.94 (d, 2H, J = 9.1 Hz, Ar–H^2,H^6), 7.39 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.76–7.79 (m, 2H, Ar–H$^{4'}$,H$^{7'}$), 7.89–7.91 (m, 2H, Ar–H$^{5'}$,H$^{6'}$). MS (ESI) (%) = 478.0 (M^+, 100). HRMS (ESI) calcd for $C_{26}H_{28}N_3O_6$ 478.1978, found 478.1981. IR (KBr) 3462, 2953, 2811, 1742, 1710, 1516, 1403, 1315, 1223, 1192, 1138, 1088, 1047 cm^{-1}. Anal. calcd. for $C_{26}H_{27}N_3O_6$: C, 65.40; H, 5.70; N, 8.80; found C, 65.17; H, 5.78; N, 8.96.

*4.1.13. (S)-Ethyl 1-(4-(5-(aminomethyl)-2-oxooxazolidin-3-yl) phenyl)piperidine-4-carboxylate (**5a**)*

Crude product yield: 1.05 g (100%), white crystals. ^{1}H NMR (DMSO-d_6) δ 1.27 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.81–1.94 (m, 2H, CH_2CHCH_2), 2.00–2.05 (m, 2H, CH_2CHCH_2), 2.38–2.47 (m, 1H, CH_2CHCH_2), 2.78 (dt, 2H, J = 11.7 Hz, 2.9 Hz, CH_2NCH_2), 2.98 (dd, 1H, J = 13.5 Hz, 5.8 Hz, CHCH_2NH$_2$), 3.08 (dd, 1H, J = 13.6 Hz, 4.2 Hz, CHCH_2NH$_2$), 3.58 (t, 1H, J = 3.7 Hz, CH_2NCH_2), 3.61 (t, 1H, J = 3.7 Hz, CH_2NCH_2), 3.80 (dd, 1H, J = 8.9 Hz, 6.6 Hz, ArNCH_2CH), 4.00 (t, 1H, J = 8.9 Hz, ArNCH_2CH), 4.08 (q, 2H, J = 7.1 Hz, CH_2CH_3), 4.60–4.68 (m, 1H, ArNCH$_2$CH), 6.95 (d, 2H, J = 9.2 Hz, Ar–H^2,H^6), 7.34 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), *NH_2 peak not seen*. MS (ESI) (%) = 348.2 (M^+, 100), 118.1 (60), 77.0 (30). HRMS (ESI) calcd for $C_{18}H_{26}N_3O_4$ 348.1923, found 348.1916.

*4.1.14. (S)-Ethyl 1-(4-(5-((2-((tert-butoxycarbonyl)amino)thiazole-5-carboxamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylate (**6a**)*

The crude product was purified by flash column chromatography using dichloromethane/methanol (19/1) as eluant to give white crystals, yield: 533 mg (48%), mp 269–272 °C. ^{1}H NMR (DMSO-d_6) δ 1.19 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.50 (s, 9H, C(CH_3)$_3$), 1.59–1.72 (m, 2H, CH_2CHCH_2), 1.90 (dd, 2H, J = 13.2 Hz, 3.0 Hz, CH_2CHCH_2), 2.41–2.46 (m, 1H, CH_2CHCH_2), 2.73 (dt, 2H, J = 11.8 Hz, 2.5 Hz, CH_2NCH_2), 3.56–3.60 (m, 4H, CH_2NCH_2, CONHCH_2), 3.78 (dd, 1H, J = 9.0 Hz, 6.1 Hz, ArNCH_2CH), 4.05–4.14 (m, 3H, CH_2CH_3, ArNCH_2CH), 4.74–4.82 (m, 1H, ArNCH$_2$CH), 6.95 (d, 2H, J = 9.1 Hz, Ar–H^2,H^6), 7.35 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 8.00 (s, 1H, Ar–H$^{4'}$), 8.76 (t, 1H, J = 5.7 Hz, CONHCH_2), 11.71 (s, 1H, NHCOO*t*–Bu). MS (ESI) (%) = 574.2 (M^+, 100), 474.2 (30), 259.6 (60). HRMS (ESI) calcd for $C_{27}H_{36}N_5O_7S$ 574.2335, found 574.2324. IR (ATR) 3289, 2932, 1742, 1716, 1623, 1543, 1518, 1433, 1407, 1367, 1307, 1272, 1246, 1216, 1164, 1142, 1095, 1045 cm^{-1}.

*4.1.15. (S)-Ethyl 1-(4-(5-((2-aminothiazole-5-carboxamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylate (**7a**)*

The crude product was purified by flash column chromatography using dichloromethane/methanol (9/1) as eluant to give white crystals, yield: 186 mg (72%), mp 110–113 °C. $[\alpha]_D^{20}$ −29.2 (c 0.21, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.62–1.69 (m, 2H, CH_2CHCH_2), 1.96–2.00 (m, 2H, CH_2CHCH_2), 2.54–2.60 (m, 1H, CH_2CHCH_2), 2.98 (t, 2H, J = 11.2 Hz, CH_2NCH_2), 3.53–3.61 (m, 4H, CH_2NCH_2, CONHCH_2), 3.78 (dd, 1H, J = 9.2 Hz, 6.2 Hz, ArNCH_2CH), 4.06–4.15 (m, 3H, CH_2CH_3, ArNCH_2CH), 4.73–4.82 (m, 1H, ArNCH$_2$CH), 7.16 (d, 2H, J = 8.6 Hz, Ar–H^2,H^6), 7.46 (d, 2H, J = 8.8 Hz, Ar–H^3,H^5), 7.75 (s, 1H, Ar–H$^{4'}$), 8.12 (brs, 2H, NH_2), 8.62 (t, 1H, J = 5.6 Hz, CONHCH_2). ^{13}C NMR (DMSO-d_6) δ 14.05, 26.82, 42.00, 47.53, 50.50, 60.06, 71.36, 114.47, 117.38, 118.33, 119.35, 120.85, 137.03, 154.15, 160.72, 171.15, 173.74, *one alkyl C-atom behind solvent peak*. MS (ESI) (%) = 474.2 (M^+, 100), 237.6 (100), 214.6 (55), 200.6 (32). HRMS (ESI) calcd for $C_{22}H_{28}N_5O_5S$ 474.1811, found 474.1828. IR (KBr) 3326, 3089, 2987, 1735, 1676, 1518, 1430, 1410, 1319, 1202, 1134, 1043 cm^{-1}. HPLC: 96.1%, t_r = 9.0 min.

*4.1.16. (S)-1-(4-(5-((2-Aminothiazole-5-carboxamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylic acid (**8a**)*

The crude product was purified by flash column chromatography using dichloromethane/methanol (9/1) as eluant to give white crystals, yield: 60 mg (39%), mp 275–280 °C. $[\alpha]_D^{20}$ −34.0 (c 0.22, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.63–1.76 (m, 2H, CH_2CHCH_2), 1.91–1.99 (m, 2H, CH_2CHCH_2), 2.39–2.45 (m, 1H, CH_2CHCH_2), 2.84 (t, 2H, J = 10.4 Hz, CH_2NCH_2), 3.51–3.60 (m, 4H, CH_2NCH_2, CONHCH_2), 3.78 (dd, 1H, J = 8.8 Hz, 6.0 Hz, ArNCH_2CH), 4.11 (t, 1H, J = 8.8 Hz, ArNCH_2CH), 4.71–4.80 (m, 1H, ArNCH$_2$CH), 7.06 (d, 2H, J = 8.8 Hz, Ar–H^2,H^6), 7.41 (d, 2H, J = 8.8 Hz, Ar–H^3,H^5), 7.68

(s + brs, 3H, Ar–H$^{4'}$, NH$_2$), 8.49 (t, 1H, J = 5.6 Hz, CONHCH$_2$), 12.27 (brs, 1H, COOH). ^{13}C NMR (DMSO-d_6) δ 27.38, 41.97, 47.67, 49.02, 71.26, 116.80, 119.54, 120.90, 130.87, 141.25, 146.79, 154.23, 161.36, 171.62, 175.81, *one alkyl C-atom behind solvent peak*. MS (ESI) (%) = 446.1 (M$^+$, 15), 85.1 (100). HRMS (ESI) calcd for $C_{20}H_{24}N_5O_5S$ 446.1498, found 446.1482. IR (ATR) 3321, 3134, 1750, 1674, 1621, 1540, 1515, 1486, 1454, 1410, 1388, 1314, 1190, 1134, 1079, 1035 cm^{-1}. HPLC: 99.6%, t_r = 5.9 min.

4.1.17. General procedure for coupling of **5a** and **5f** with 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid (**30**), 3-amino-4-chloroben zoic acid (**31**) or 4-chloro-3-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl) benzoic acid (**34**) – **9a**, **9b**, **17a**, **17b**, **19**

Diisopropylethylamine (2 eq.) and *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (1.3 eq.) were added to a solution of starting compound (**5a** or **5f**), corresponding acid (1 eq.) and 1-hydroxy-1*H*-benzotriazol-hydrate (1.3 eq.) in DMF (0.15 M according to the starting compound). The reaction mixture was stirred for 15 h at room temperature and then concentrated under reduced pressure. The residue was cooled to 0 °C and diluted with methanol. Precipitated crystals were separated by filtration to yield desired products.

4.1.18. (S)-Ethyl 1-(4-(2-oxo-5-((4-(5-oxo-4,5-dihydro-1,2,4-oxadia zol-3-yl)benzamido)methyl)oxazolidin-3-yl)phenyl)piperidine-4-car boxylate (**9a**)

Beige crystals, yield: 1.20 g (53%), mp 273–277 °C. ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, J = 7.1 Hz, CH$_2$CH$_3$), 1.61–1.71 (m, 2H, CH$_2$CHCH$_2$), 1.88–1.92 (m, 2H, CH$_2$CHCH$_2$), 2.45–2.48 (m, 1H, CH$_2$CHCH$_2$), 2.73 (dt, 2H, J = 11.9 Hz, 2.4 Hz, CH$_2$NCH$_2$), 3.59 (td, 2H, J = 12.8 Hz, 3.5 Hz, CH$_2$NCH$_2$), 3.63–3.69 (m, 2H, CONHCH$_2$), 3.83 (dd, 1H, J = 8.9 Hz, 5.8 Hz, ArNCH$_2$CH), 4.06–4.16 (m, 3H, CH$_2$CH$_3$, ArNCH$_2$CH), 4.81–4.87 (m, 1H, ArNCH$_2$CH), 6.96 (d, 2H, J = 9.1 Hz, Ar–H$^{2'}$,H$^{6'}$), 7.36 (d, 2H, J = 9.1 Hz, Ar–H$^{3'}$,H$^{5'}$), 7.91 (d, 2H, J = 8.3 Hz, Ar–H$^{2'}$,H$^{6'}$), 8.02 (d, 2H, J = 8.3 Hz, Ar–H$^{3'}$,H$^{5'}$), 9.03 (t, 1H, J = 5.6 Hz, CONHCH$_2$), 12.93 (brs, 1H, CNHCOO). MS (ESI) (%) = 536.2 (M$^+$, 100). HRMS (ESI) calcd for $C_{27}H_{30}N_5O_7$ 536.2145, found 536.2149. IR (ATR) 3301, 3163, 1822, 1727, 1637, 1546, 1518, 1476, 1435, 1415, 1386, 1309, 1227, 1171, 1140, 1044 cm^{-1}.

4.1.19. (S)-Ethyl 1-(4-(5-((3-amino-4-chlorobenzamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylate (**17a**)

Beige crystals, yield: 0.91 g (73%), mp 146–149 °C. $[\alpha]_D^{20}$ –33.8 (c 0.24, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, J = 7.1 Hz, CH$_2$CH$_3$), 1.66 (ddd, 2H, J = 24.4 Hz, 11.5 Hz, 3.7 Hz, CH$_2$CHCH$_2$), 1.90 (dd, 2H, J = 13.2 Hz, 3.1 Hz, CH$_2$CHCH$_2$), 2.44–2.49 (m, 1H, CH$_2$CHCH$_2$), 2.73 (dt, 2H, J = 11.9 Hz, 2.5 Hz, CH$_2$NCH$_2$), 3.51–3.63 (m, 4H, CH$_2$NCH$_2$, CONHCH$_2$), 3.81 (dd, 1H, J = 9.0 Hz, 5.9 Hz, ArNCH$_2$CH), 4.06–4.13 (m, 3H, CH$_2$CH$_3$, ArNCH$_2$CH), 4.76–4.83 (m, 1H, ArNCH$_2$CH), 5.54 (brs, 2H, NH$_2$), 6.96 (d, 2H, J = 9.2 Hz, Ar–H^2,H^6), 7.00 (dd, 1H, J = 8.3 Hz, 2.1 Hz, Ar–H$^{6'}$), 7.27 (d, 1H, J = 8.4 Hz, Ar–H$^{5'}$), 7.28 (d, 1H, J = 2.1 Hz, Ar–H$^{2'}$), 7.36 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 8.69 (t, 1H, J = 5.8 Hz, CONHCH$_2$). ^{13}C NMR (DMSO-d_6) δ 14.09, 27.44, 42.35, 47.76, 48.33, 59.87, 70.99, 114.52, 115.08, 116.30, 119.56, 119.77, 128.76, 130.10, 133.71, 144.62, 147.58, 154.26, 166.75, 174.22, *one alkyl C-atom behind solvent peak*. MS (ESI) (%) = 501.2 (M$^+$, 100) for ^{35}Cl, 503.2 (M$^+$, 35) for ^{37}Cl. HRMS (ESI) calcd for $C_{25}H_{30}N_4O_5Cl$ 501.1905, found 501.1912. IR (ATR) 3353, 1739, 1649, 1614, 1573, 1538, 1512, 1489, 1429, 1392, 1301, 1258, 1226, 1137, 1087 cm^{-1}. HPLC: 100%, t_r = 19.3 min.

4.1.20. (S)-Ethyl 1-(4-(5-((4-chloro-3-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzamido)methyl)-2-oxooxazolidin-3-yl)phenyl) piperidine-4-carboxylate (**19**)

Crude product yield after purification by flash column chromatography using dichloromethane/methanol (19/1) as eluant: 282 mg (41%). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, J = 7.1 Hz, CH$_2$CH$_3$), 1.66 (ddd, 2H, J = 24.4 Hz, 11.3 Hz, 3.7 Hz, CH$_2$CHCH$_2$), 1.90 (dd, 2H, J = 13.2 Hz, 2.9 Hz, CH$_2$CHCH$_2$), 2.44–2.49 (m, 1H, CH$_2$CHCH$_2$), 2.72 (dt, 2H, J = 11.9 Hz, 2.4 Hz, CH$_2$NCH$_2$), 3.58 (td, 2H, J = 12.6 Hz, 3.3 Hz, CH$_2$NCH$_2$), 3.65 (t, 2H, J = 5.6 Hz, CONHCH$_2$), 3.81 (dd, 1H, J = 9.1 Hz, 6.1 Hz, ArNCH$_2$CH), 4.06 (q, 2H, J = 7.2 Hz, CH$_2$CH$_3$), 4.14 (t, 1H, J = 9.1 Hz, ArNCH$_2$CH), 4.79–4.86 (m, 1H, ArNCH$_2$CH), 6.96 (d, 2H, J = 9.2 Hz, Ar–H^2,H^6), 7.35 (d, 2H, J = 9.2 Hz, Ar–H^3,H^5), 7.84 (d, 1H, J = 8.5 Hz, Ar–H$^{5'}$), 8.09 (dd, 1H, J = 8.5 Hz, 2.2 Hz, Ar–H$^{6'}$), 8.19 (d, 1H, J = 2.2 Hz, Ar–H$^{2'}$), 9.09 (t, 1H, J = 5.7 Hz, CONHCH$_2$), *NHCOON peak not seen*. MS (ESI) (%) = 570.2 (M$^+$, 100) for ^{35}Cl, 572.2 (M$^+$, 35) for ^{37}Cl. HRMS (ESI) calcd for $C_{27}H_{29}N_5O_7Cl$ 570.1756, found 570.1745.

4.1.21. General procedure for reductive amination of **5a** and **5f** – synthesis of **10a**, **10b**

To a solution of starting compound (**5a** or **5f**) and 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzaldehyde (1 eq.) in DMF (0.15 M) Na(OAc)$_3$BH (1.3 eq.) was added and the reaction mixture was stirred for 15 h at room temperature. DMF was almost completely removed under reduced pressure and the residue diluted with cold water. Precipitated crystals were filtered off, washed with ether and recrystallized from absolute ethanol. The product was not completely soluble in hot ethanol.

4.1.22. (S)-Ethyl 1-(4-(2-oxo-5-(((4-(5-oxo-4,5-dihydro-1,2,4-oxadia zol-3-yl)benzyl)amino)methyl)oxazolidin-3-yl)phenyl)piperidine-4-carboxylate (**10a**)

Crude product yield: 212 mg (14%). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, J = 7.1 Hz, CH$_2$CH$_3$), 1.61–1.71 (m, 2H, CH$_2$CHCH$_2$), 1.88–1.92 (m, 2H, CH$_2$CHCH$_2$), 2.45–2.48 (m, 1H, CH$_2$CHCH$_2$), 2.73 (dt, 2H, J = 11.9 Hz, 2.4 Hz, CH$_2$NCH$_2$), 2.88 (d, 2H, J = 5.4 Hz, NHCH$_2$CH), 3.59 (td, 2H, J = 12.8 Hz, 3.5 Hz, CH$_2$NCH$_2$), 3.78 (dd, 1H, J = 8.9 Hz, 6.6 Hz, ArNCH$_2$CH), 3.90 (s, 2H, ArCH$_2$NH), 4.03–4.11 (m, 3H, CH$_2$CH$_3$, ArNCH$_2$CH), 4.71–4.78 (m, 1H, ArNCH$_2$CH), 6.96 (d, 2H, J = 9.2 Hz, Ar–H^2,H^6), 7.38 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.55 (d, 2H, J = 8.3 Hz, Ar–H$^{2'}$,H$^{6'}$), 7.78 (d, 2H, J = 8.3 Hz, Ar–H$^{3'}$,H$^{5'}$), *NH and NHCOON peaks not seen*. MS (ESI) (%) = 522.2 (M$^+$, 100). HRMS (ESI) calcd for $C_{27}H_{32}N_5O_6$ 522.2353, found 522.2365. IR (ATR) 1807, 1723, 1653, 1517, 1479, 1432, 1412, 1388, 1309, 1221, 1174, 1145, 1105, 1042 cm^{-1}.

4.1.23. (S)-1-(4-(2-Oxo-5-((4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzamido)methyl)oxazolidin-3-yl)phenyl)piperidine-4-carboxylic acid (**11a**)

Synthesized according to the alkaline hydrolysis part of the General procedure for alkaline hydrolysis (4.1.8.). The crude product (not completely soluble in hot methanol) was recrystallized from methanol to obtain beige crystals, yield: 556 mg (73%), mp 269–272 °C. $[\alpha]_D^{20}$ –43.9 (c 0.22, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.59–1.69 (m, 2H, CH$_2$CHCH$_2$), 1.88–1.92 (m, 2H, CH$_2$CHCH$_2$), 2.34–2.40 (m, 1H, CH$_2$CHCH$_2$), 2.70 (dt, 2H, J = 11.9 Hz, 2.4 Hz, CH$_2$NCH$_2$), 3.59 (td, 2H, J = 12.8 Hz, 3.5 Hz, CH$_2$NCH$_2$), 3.63–3.69 (m, 2H, CONHCH$_2$), 3.83 (dd, 1H, J = 8.9 Hz, 5.8 Hz, ArNCH$_2$CH), 4.13 (t, 1H, J = 8.9 Hz, ArNCH$_2$CH), 4.81–4.87 (m, 1H, ArNCH$_2$CH), 6.96 (d, 2H, J = 9.1 Hz, Ar–H^2,H^6), 7.36 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.91 (d, 2H, J = 8.3 Hz, Ar–H$^{2'}$,H$^{6'}$), 8.01 (d, 2H, J = 8.3 Hz, Ar–H$^{3'}$,H$^{5'}$), 9.03 (t, 1H, J = 5.5 Hz, CONHCH$_2$), 12.23 (brs, 1H, CNHCOO), *COOH peak not seen*. ^{13}C NMR (DMSO-d_6) δ 27.54, 42.48, 47.82, 48.46, 71.00, 116.27, 119.58, 126.04, 126.39, 128.08, 130.03, 136.87, 147.70, 154.26, 157.60, 160.85, 166.03, 175.92, *one alkyl C-atom behind solvent peak*. MS (ESI) (%) = 508.2 (M$^+$, 100). HRMS (ESI) calcd for $C_{25}H_{26}N_5O_7$ 508.1832, found 508.1829. IR (ATR) 3300, 3149, 2955, 2811, 1822, 1729, 1637, 1597, 1546, 1518, 1476, 1434, 1414, 1387, 1310, 1288, 1211, 1140, 1084, 1037, 1014 cm^{-1}. HPLC: 97.9%, t_r = 9.8 min.

*4.1.24. (S)-1-(4-(2-Oxo-5-(((4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzyl)amino)methyl)oxazolidin-3-yl)phenyl)piperidine-4-carboxylic acid (**12a**)*

Synthesized according to the alkaline hydrolysis part of the General procedure for alkaline hydrolysis (4.1.8.). The crude product (not completely soluble in hot methanol) was recrystallized from methanol to obtain white crystals, yield: 82 mg (25%), mp 177–181 °C. $[\alpha]_D^{20}$ −30.7 (c 0.18, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.59–1.69 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 1.88–1.92 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 2.34–2.42 (m, 1H, $CH_2C\underline{H}CH_2$), 2.71 (dt, 2H, J = 11.9 Hz, 2.3 Hz, $C\underline{H}_2NC\underline{H}_2$), 2.87 (d, 2H, J = 5.4 Hz, $NHC\underline{H}_2CH$), 3.58 (td, 2H, J = 12.5 Hz, 3.5 Hz, $C\underline{H}_2NC\underline{H}_2$), 3.78 (dd, 1H, J = 8.9 Hz, 6.6 Hz, $ArNC\underline{H}_2CH$), 3.89 (s, 2H, $ArC\underline{H}_2NH$), 4.05 (t, 1H, J = 8.9 Hz, $ArNC\underline{H}_2CH$), 4.71–4.78 (m, 1H, $ArNCH_2C\underline{H}$), 6.96 (d, 2H, J = 9.2 Hz, Ar–H^2,H^6), 7.38 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.54 (d, 2H, J = 8.3 Hz, Ar–H$^{2'}$,H$^{6'}$), 7.77 (d, 2H, J = 8.3 Hz, Ar–H$^{3'}$,H$^{5'}$), *COOH, NH and NHCOON peaks not seen*. ^{13}C NMR (DMSO-d_6) δ 27.54, 47.93, 48.48, 50.81, 52.18, 71.70, 116.28, 119.42, 123.48, 125.89, 128.80, 130.15, 143.39, 147.60, 154.32, 159.16, 162.55, 175.96, *one alkyl C-atom behind solvent peak*. MS (ESI) (%) = 494.2 (M$^+$, 100). HRMS (ESI) calcd for $C_{25}H_{28}N_5O_6$ 494.2040, found 494.2047. IR (ATR) 3302, 3075, 1812, 1729, 1637, 1548, 1516, 1478, 1414, 1386, 1308, 1223, 1139, 1043, 1015 cm^{-1}. HPLC: 96.4%, t_r = 6.9 min.

*4.1.25. General procedure for catalytic hydrogenation of 1,2,4-oxadiazol-5(4H)-one – synthesis of compounds **13a**, **13b**, **14a**, **14b***

Mixture of starting compound (**9a**, **9b**, **10a**, **10b**), 10% of palladium on activated charcoal and catalytic amount of DMF in glacial acetic acid (0.05 M) was stirred under 1 atm H_2 for 10–15 h at room temperature. The progress of the reaction was monitored by TLC. After completion palladium on activated charcoal was removed by filtration. Filtrate was evaporated under reduced pressure and product crystallized from ether or ether/methanol.

*4.1.26. (S)-Ethyl 1-(4-(5-((4-carbamimidoylbenzamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylate (**13a**)*

The crude product was recrystallized from ether to obtain white crystals, yield: 130 mg (63%), mp 197–200 °C. $[\alpha]_D^{20}$ −32.7 (c 0.25, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$), 1.61–1.71 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 1.76 (s, 3H, $C\underline{H}_3COOH$), 1.88–1.92 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 2.45–2.48 (m, 1H, $CH_2C\underline{H}CH_2$), 2.73 (dt, 2H, J = 11.9 Hz, 2.4 Hz, $C\underline{H}_2NC\underline{H}_2$), 3.59 (td, 2H, J = 12.8 Hz, 3.5 Hz, $C\underline{H}_2NC\underline{H}_2$), 3.63–3.69 (m, 2H, $CONHC\underline{H}_2$), 3.83 (dd, 1H, J = 8.9 Hz, 5.8 Hz, $ArNC\underline{H}_2CH$), 4.06–4.16 (m, 3H, $C\underline{H}_2CH_3$, $ArNC\underline{H}_2CH$), 4.81–4.87 (m, 1H, $ArNCH_2C\underline{H}$), 6.96 (d, 2H, J = 9.1 Hz, Ar–H^2,H^6), 7.36 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.87 (d, 2H, J = 8.3 Hz, Ar–H$^{2'}$,H$^{6'}$), 8.01 (d, 2H, J = 8.3 Hz, Ar–H$^{3'}$,H$^{5'}$), 9.09 (t, 1H, J = 5.5 Hz, $CONHCH_2$), *CNH(NH$_2$) peaks not seen*. ^{13}C NMR (DMSO-d_6) δ 14.10, 23.96, 27.44, 42.48, 47.77, 48.30, 59.88, 71.02, 116.28, 119.56, 127.61, 127.68, 130.05, 132.24, 137.73, 147.59, 154.26, 165.43, 165.89, 174.22, 175.56, *one alkyl C-atom behind solvent peak*. MS (ESI) (%) = 494.2 (M$^+$, 25), 247.6 (100). HRMS (ESI) calcd for $C_{26}H_{32}N_5O_5$ 494.2403, found 494.2404. IR (ATR) 2953, 1728, 1640, 1553, 1516, 1408, 1308, 1221, 1172, 1142, 1042, 1014 cm^{-1}. HPLC: 100%, t_r = 8.6 min.

*4.1.27. (S)-Ethyl 1-(4-(5-(((4-carbamimidoylbenzyl)amino)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylate (**14a**)*

The crude product was recrystallized from ether/methanol to obtain bright orange crystals, yield: 44 mg (34%), mp 193–196 °C. $[\alpha]_D^{20}$ −23.9 (c 0.17, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.20 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$), 1.61–1.72 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 1.74 (s, 3H, $C\underline{H}_3COOH$), 1.88–1.92 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 2.45–2.48 (m, 1H, $CH_2C\underline{H}CH_2$), 2.73 (dt, 2H, J = 11.9 Hz, 2.4 Hz, $C\underline{H}_2NC\underline{H}_2$), 2.78 (d, 2H, J = 5.2 Hz, $NHC\underline{H}_2CH$), 3.59 (td, 2H, J = 12.8 Hz, 3.5 Hz, $C\underline{H}_2NC\underline{H}_2$), 3.78 (dd, 1H, J = 8.9 Hz, 6.6 Hz, $ArNC\underline{H}_2CH$), 3.85 (s, 2H, $ArC\underline{H}_2NH$), 4.02–4.11 (m, 3H, $C\underline{H}_2CH_3$, $ArNC\underline{H}_2CH$), 4.68–4.74 (m, 1H, $ArNCH_2C\underline{H}$), 6.96 (d, 2H, J = 9.2 Hz, Ar–H^2,H^6), 7.38 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.55 (d, 2H, J = 8.3 Hz, Ar–H$^{2'}$,H$^{6'}$), 7.75 (d, 2H, J = 8.3 Hz, Ar–H$^{3'}$,H$^{5'}$), *CNH(NH$_2$) and NH peaks not seen*. ^{13}C NMR (DMSO-d_6) δ 14.10, 24.52, 27.45, 47.93, 48.35, 51.05, 52.32, 59.88, 72.21, 116.31, 119.37, 127.47, 127.79, 130.27, 146.48, 147.48, 154.43, 165.62, 174.23, 176.22. MS (ESI) (%) = 480.3 (M$^+$, 20), 240.6 (100). HRMS (ESI) calcd for $C_{26}H_{34}N_5O_4$ 480.2611, found 480.2612. IR (ATR) 2952, 1725, 1612, 1563, 1516, 1408, 1308, 1222, 1170, 1141, 1109, 1044, 1015 cm^{-1}. HPLC: 94.8%, t_r = 6.7 min.

*4.1.28. General procedure for alkaline hydrolysis of ethyl esters and subsequent catalytic hydrogenation of **9a**, **9b** and **10a** – synthesis of compounds **15a**, **15b**, **16a***

To a solution of starting compound (**9a**, **9b**, **10a**) in methanol/water (1/1) (0.05 M) lithium hydroxide (1.5 eq.) was added and the reaction mixture was stirred at room temperature. The progress of the reaction was monitored by TLC. After completion methanol was removed under reduced pressure. Water phase was cooled to 0 °C and acidified (pH = 1–2) with addition of conc. hydrochloric acid. Precipitated crystals were separated by filtration and subjected to conditions of General procedure for catalytic hydrogenation of 1,2,4-oxadiazol-5(4*H*)-one (4.1.25.).

*4.1.29. (S)-1-(4-(5-((4-Carbamimidoylbenzamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylic acid (**15a**)*

The crude product (not completely soluble in hot methanol) was recrystallized from methanol to obtain beige crystals, yield: 286 mg (69%), mp > 300 °C. $[\alpha]_D^{20}$ −51.2 (c 0.23, 0.5% CF_3COOH in DMSO). ^{1}H NMR (DMSO-d_6) δ 1.94–2.00 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 2.13–2.17 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 2.63–2.71 (m, 1H, $CH_2C\underline{H}CH_2$), 3.51 (t, 2H, J = 11.0 Hz, $C\underline{H}_2NC\underline{H}_2$), 3.59–3.62 (m, 2H, $C\underline{H}_2NC\underline{H}_2$), 3.68–3.70 (m, 2H, $CONHC\underline{H}_2$), 3.92 (dd, 1H, J = 9.0 Hz, 6.1 Hz, $ArNC\underline{H}_2CH$), 4.23 (t, 1H, J = 9.0 Hz, $ArNC\underline{H}_2CH$), 4.89–4.95 (m, 1H, $ArNCH_2C\underline{H}$), 7.61 (d, 2H, J = 9.0 Hz, Ar–H^2,H^6), 7.69 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.91 (d, 2H, J = 8.4 Hz, Ar–H$^{2'}$,H$^{6'}$), 8.04 (d, 2H, J = 8.4 Hz, Ar–H$^{3'}$,H$^{5'}$), 9.14 (t, 1H, J = 5.6 Hz, $CONHCH_2$), *CNH(NH$_2$) and COOH peaks not seen*. ^{13}C NMR (DMSO-d_6) δ 25.81, 36.90, 42.31, 47.38, 54.46, 71.46, 110.72, 113.59, 116.45, 118.91, 119.32, 121.80, 127.66, 128.25, 138.30, 154.06, 164.89, 165.69, 174.49. MS (ESI) (%) = 466.2 (M$^+$, 25), 233.6 (100). HRMS (ESI) calcd for $C_{24}H_{28}N_5O_5$ 466.2090, found 466.2084. IR (ATR) 2943, 1736, 1658, 1544, 1513, 1484, 1407, 1381, 1285, 1230, 1206, 1140, 1108, 1041 cm^{-1}. HPLC: 98.3%, t_r = 5.9 min.

*4.1.30. (S)-1-(4-(5-(((4-Carbamimidoylbenzyl)amino)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylic acid (**16a**)*

The crude product was recrystallized from ether/methanol to obtain beige crystals, yield: 30 mg (58%), mp 192–195 °C. $[\alpha]_D^{20}$ −43.6 (c 0.19, 0.5% CF_3COOH in DMSO). ^{1}H NMR (DMSO-d_6) δ 1.59–1.69 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 1.88–1.92 (m, 2H, $C\underline{H}_2CHC\underline{H}_2$), 2.34–2.42 (m, 3H, $CH_2C\underline{H}CH_2$, $ArC\underline{H}_2NH$), 2.66–2.71 (m, 2H, $C\underline{H}_2NC\underline{H}_2$), 3.20–3.22 (m, 2H, $NHC\underline{H}_2CH$), 3.54 (td, 2H, J = 12.5 Hz, 3.5 Hz, $C\underline{H}_2NC\underline{H}_2$), 3.75 (dd, 1H, J = 9.2 Hz, 6.7 Hz, $ArNC\underline{H}_2CH$), 4.10 (t, 1H, J = 9.1 Hz, $ArNC\underline{H}_2CH$), 4.83–4.90 (m, 1H, $ArNCH_2C\underline{H}$), 6.93 (d, 2H, J = 9.3 Hz, Ar–H^2,H^6), 7.33 (d, 2H, J = 9.2 Hz, Ar–H^3,H^5), 7.36 (d, 2H, J = 8.2 Hz, Ar–H$^{2'}$,H$^{6'}$), 7.68 (d, 2H, J = 8.2 Hz, Ar–H$^{3'}$,H$^{5'}$), *CNH(NH$_2$), COOH and NH peaks not seen*. ^{13}C NMR (DMSO-d_6) δ 20.93, 25.95, 37.16, 41.53, 47.05, 54.00, 69.61, 110.78, 113.65, 116.51, 119.13, 119.38, 121.42, 128.02, 129.45, 144.42, 153.51, 165.20, 171.93, 174.62. IR (ATR) 3254, 2948, 1741, 1703, 1612, 1560, 1515, 1410, 1328, 1306, 1285, 1222, 1192, 1169, 1135, 1042, 1005 cm^{-1}. HPLC: 100%, t_r = 6.0 min.

*4.1.31. (S)-1-(4-(5-((3-Amino-4-chlorobenzamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylic acid (**18a**)*

Synthesized according to the alkaline hydrolysis part of the General procedure for alkaline hydrolysis (4.1.8.). The crude

product was recrystallized from ether/methanol to obtain beige crystals, yield: 149 mg (53%), mp 164–168 °C. $[\alpha]_D^{20}$ −21.7 (c 0.24, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.55–1.66 (m, 2H, C$\underline{H}_2$CHC$\underline{H}_2$), 1.81 (d, 2H, J = 11.3 Hz, C$\underline{H}_2$CHC$\underline{H}_2$), 1.90–1.95 (m, 1H, CH$_2$C$\underline{H}$CH$_2$), 2.58 (t, 2H, J = 11.2 Hz, C$\underline{H}_2$NC$\underline{H}_2$), 3.48–3.66 (m, 4H, CONHC$\underline{H}_2$, C$\underline{H}_2$NC$\underline{H}_2$), 3.88 (dd, 1H, J = 8.4 Hz, 5.9 Hz, ArNC$\underline{H}_2$CH), 4.10 (t, 1H, J = 8.9 Hz, ArNC$\underline{H}_2$CH), 4.74–4.80 (m, 1H, ArNCH$_2$C$\underline{H}$), 5.58 (brs, 2H, N$\underline{H}_2$), 6.92 (d, 2H, J = 8.9 Hz, Ar–H^2,H^6), 7.04 (d, 1H, J = 8.2 Hz, Ar–H$^{6'}$), 7.25 (d, 2H, J = 8.1 Hz, Ar–H$^{5'}$), 7.34 (d, 1H, J = 9.1 Hz, Ar–H^3,H^5), 7.47 (s, 1H, Ar–H$^{2'}$), 9.02 (t, 1H, J = 5.4 Hz, CON$\underline{H}$CH$_2$), *COOH peak not seen*. ^{13}C NMR (DMSO-d_6) δ 29.12, 42.28, 43.55, 47.87, 49.24, 70.78, 114.72, 115.25, 115.99, 119.65, 128.70, 129.56, 133.58, 144.66, 148.29, 154.29, 166.68, 178.40, *one alkyl C-atom behind solvent peak*. MS (ESI) (%) = 473.2 (M$^+$, 100) for ^{35}Cl, 475.2 (M$^+$, 30) for ^{37}Cl. HRMS (ESI) calcd for $C_{23}H_{26}N_4O_5Cl$ 473.1592, found 473.1594. IR (ATR) 3310, 1740, 1649, 1538, 1514, 1488, 1409, 1385, 1315, 1274, 1208, 1139, 1112, 1087 cm^{-1}. HPLC: 100%, t_r = 17.2 min.

4.1.32. (S)-1-(4-(5-((4-Chloro-3-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzamido)methyl)-2-oxooxazolidin-3-yl)phenyl)piperidine-4-carboxylic acid (**20**)

Synthesized according to the alkaline hydrolysis part of the General procedure for alkaline hydrolysis (4.1.8.). The crude product (not completely soluble in hot methanol) was recrystallized from methanol to obtain white crystals, yield: 168 mg (66%), mp 221–225 °C. $[\alpha]_D^{20}$ −38.9 (c 0.23, DMSO). ^{1}H NMR (DMSO-d_6) δ 1.64 (ddd, 2H, J = 24.2 Hz, 11.4 Hz, 3.8 Hz, C$\underline{H}_2$CHC$\underline{H}_2$), 1.90 (dd, 2H, J = 13.2 Hz, 3.1 Hz, C$\underline{H}_2$CHC$\underline{H}_2$), 2.34–2.42 (m, 1H, CH$_2$C$\underline{H}$CH$_2$), 2.72 (dt, 2H, J = 11.9 Hz, 2.5 Hz, C$\underline{H}_2$NC$\underline{H}_2$), 3.58 (td, 2H, J = 12.3 Hz, 3.2 Hz, C$\underline{H}_2$NC$\underline{H}_2$), 3.65 (t, 2H, J = 5.5 Hz, CONHC$\underline{H}_2$), 3.82 (dd, 1H, J = 9.1 Hz, 6.1 Hz, ArNC$\underline{H}_2$CH), 4.14 (t, 1H, J = 9.1 Hz, ArNC$\underline{H}_2$CH), 4.79–4.86 (m, 1H, ArNCH$_2$C$\underline{H}$), 6.96 (d, 2H, J = 9.2 Hz, Ar–H^2,H^6), 7.36 (d, 2H, J = 9.1 Hz, Ar–H^3,H^5), 7.85 (d, 1H, J = 8.5 Hz, Ar–H$^{5'}$), 8.10 (dd, 1H, J = 8.5 Hz, 2.2 Hz, Ar–H$^{6'}$), 8.20 (d, 1H, J = 2.1 Hz, Ar–H$^{2'}$), 9.09 (t, 1H, J = 5.8 Hz, CON$\underline{H}$CH$_2$), 12.24 (brs, 1H, N$\underline{H}$COO), 13.00 (brs, 1H, COO$\underline{H}$), *COOH peak not seen*. ^{13}C NMR (DMSO-d_6) δ 27.51, 42.53, 47.78, 48.50, 70.99, 116.31, 119.57, 123.00, 130.08, 130.40, 130.70, 131.97, 133.29, 135.02, 147.61, 154.23, 156.05, 159.43, 164.85, 175.93. MS (ESI) (%) = 540.1 (M$^+$, 100) for ^{35}Cl, 542.1 (M$^+$, 35) for ^{37}Cl. HRMS (ESI) calcd for $C_{25}H_{23}N_5O_7Cl$ 540.1286, found 540.1277. IR (ATR) 3428, 3144, 1782, 1752, 1701, 1632, 1546, 1516, 1480, 1428, 1409, 1388, 1306, 1288, 1264, 1223, 1197, 1172, 1148, 1121, 1089, 1042, 1026 cm^{-1}. HPLC: 100%, t_r = 18.1 min.

4.2. Biochemical evaluation

4.2.1. 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 [29,30]. 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 (K_m = 2.6 μM) with the substrate S-2238 (Chromogenix) at 20 and 40 μM final concentration, and fXa at the final concentration of 1 mBAEE E/mL (K_m = 164 μM) 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 mM) 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 was then added. The 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 [29], and the final result was given as the average value. Rivaroxaban (factor Xa, K_i = 0.66 ± 0.09 nM) and dabigatran (thrombin, K_i = 6.3 ± 1.1 nM) were used as controls.

4.2.2. Inhibition of in vitro binding of fibrinogen to isolated GPIIb/IIIa receptors

Binding affinities to GPIIb/IIIa receptor were measured by a solid-phase competitive displacement assay [31]. Human fibrinogen (100 mg) was dissolved in aqueous NaCl (0.3 M, 5 mL) at 30 °C and then diluted with 0.1 M aqueous $NaHCO_3$ 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%) was added to give the stock solution. Human integrin (10 μL of GPIIb/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 was measured with a BioTek Synergy H4 multimode research reader three times over 10 min. Positive controls received no inhibitors, whereas negative controls received no receptor. Tirofiban (IC_{50} (GPIIb/IIIa) = 0.37 ± 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 were calculated from the dose–response curve (OriginPro, OriginLab, version 7.5).

4.3. Docking studies

The binding modes for the ligands to fXa and GPIIb/IIIa were studied by CDOCKER [28], a docking tool based on the CHARMm force field, which is incorporated into Discovery Studio 3.1 (Accelrys Software Inc.). In CDOCKER, random ligand conformations were generated through molecular dynamics, and a variable number of translations/rotations were applied to each conformation to generate low-energy orientations of the ligand within the active site of rigid receptor. The orientations were further refined by grid-

based simulated annealing (in our example 2000 heating steps, target temperature 700 K, 5000 cooling steps, target temperature 300 K), which was followed by final minimization using full potential. Final ligand conformations were sorted by CHARMm energy (interaction energy plus ligand strain). The crystal structures of fXa (PDB entry code: 2W26, resolution 2.08 Å) [23] and GPIIb/IIIa (PDB entry code: 2VDM, resolution 1.23 Å) [24] were extracted from the Brookhaven Protein Database. All ligands were docked in all possible stereoisomeric forms in an active site located sphere with 8.62303 Å for fXa and 11.72740 Å radius for GPIIb/IIIa, which was generated with the CreateSphere function around the subsequently removed crystal structure ligand. A total of 100 dockings for each ligand were performed, and the conformers with the lowest CHARMm energy were chosen for interpreting the docking results.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.056.

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