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Cite this: *Med. Chem. Commun.*, 2014, 5, 197

Transformation of a selective factor Xa inhibitor rivaroxaban into a dual factor Xa/thrombin inhibitor by modification of the morpholin-3-one moiety†

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Received 3rd September 2013
Accepted 29th November 2013

DOI: 10.1039/c3md00250k

www.rsc.org/medchemcomm

Replacement of the P_4 morpholin-3-one moiety in a selective factor Xa inhibitor rivaroxaban by 2-ethoxycarbonylpiperidine resulted in a dual factor Xa/thrombin inhibitor 24, possessing a K_i of 62 ± 18 nM for factor Xa and a K_i of 353 ± 75 nM for thrombin. Presented rationalization of dual activity provides a good starting point for "designing in" thrombin inhibitory activity to potent factor Xa inhibitor rivaroxaban.

Introduction

Factor Xa (fXa) and thrombin are key enzymes at the junction of the intrinsic and extrinsic coagulation pathways and are the most attractive pharmacological targets for the development of novel anticoagulants, with wider therapeutic windows obviating the need for regular blood monitoring.[1,2] In the last ten years a major breakthrough in the field of anticoagulant agents has been achieved by the introduction of the selective and orally available direct thrombin inhibitor dabigatran[3] and the direct factor Xa inhibitors rivaroxaban[4] and apixaban.[5] These drugs are safer alternatives to warfarin, currently the most frequently applied oral anticoagulant. Although the recent search for effective small molecule oral anticoagulants has been focused on selective thrombin and fXa inhibitors, dual fXa/thrombin inhibitors could offer certain advantages over inhibitors selective for one of the coagulation enzymes.[2,6] Dual inhibition of coagulation enzymes is well known in haematophagous animals such as *Ornithodoros moubata* and *Hirudo medicinalis*, in which multiple inhibition of coagulation enzymes and platelet activation is often combined for prevention of blood clotting.[6,7] Heparin, a widely used parenteral anticoagulant, also acts as an indirect inhibitor of both fXa and thrombin.[8] Some studies show that concurrent direct inhibition of thrombin and factor Xa, while producing not only an additive but also synergistic antithrombotic effects, can lead to fewer bleeding complications.[9,10] These observations stimulated the search for small molecule dual inhibitors of thrombin and factor Xa and support the direct dual inhibition of both targets by the same compound as a viable concept in the search for novel anticoagulants.

As a logical consequence of the structural similarity of the fXa and thrombin active sites,[2,6,11] several non-selective fXa and thrombin inhibitors have been identified in medicinal chemistry programs, leading to the discovery of rivaroxaban, dabigatran and other selective fXa and thrombin inhibitors.[12–16] On the other side, some groups have succeeded in the rational design of dual fXa/thrombin inhibitors, meeting the structural constraints of both targets. Nar *et al.*[6] described 4-(1-methylbenzimidazole-2-yl)-methylaminobenzamidine as a suitable scaffold that fits equally well into the S_1 and S_2 pockets of both thrombin and fXa, although in slightly different conformations. By exploring distal substituents on the inhibitor central core, fitting into the S_4 subsites of both enzymes, they obtained BIBM1015, a well-balanced dual fXa/thrombin inhibitor with low nanomolar inhibition constants (Fig. 1).

A dual fXa/thrombin inhibitor RWJ-445167 possessing a less basic oxyguanidine group as a replacement for the P_1 benzamidine moiety[17] (Fig. 1), when used in an animal vascular injury model, showed better efficacy in comparison to selective thrombin inhibitor argatroban, again suggesting that there is a cooperative antithrombotic effect *in vivo* when both thrombin and factor Xa are inhibited simultaneously.[10]

Replacement of a strongly basic P_1 benzamidine group in thrombin and factor Xa inhibitors, which facilitates binding to Asp189 in the S_1 subsite, with non-basic residues such as aryl chlorides as a prerequisite for bioavailability after oral dosing, has been an important aim in the development of factor Xa and thrombin inhibitors. Interaction between aryl chloride and Tyr228 in the S_1 subsite of fXa was first explored in connection with development of rivaroxaban and later also proved relevant for incorporation into thrombin inhibitors. It was successfully exploited also in dual fXa/thrombin inhibitors either by "designing in"[18] thrombin inhibitory activity to selective non-basic fXa inhibitors[19,20] (Fig. 2, top) or by "designing in" fXa inhibitory activity to selective non-basic

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† Electronic supplementary information (ESI) available: Characterization data of compounds, experimental procedures for synthesis and biological evaluations. See DOI: 10.1039/c3md00250k

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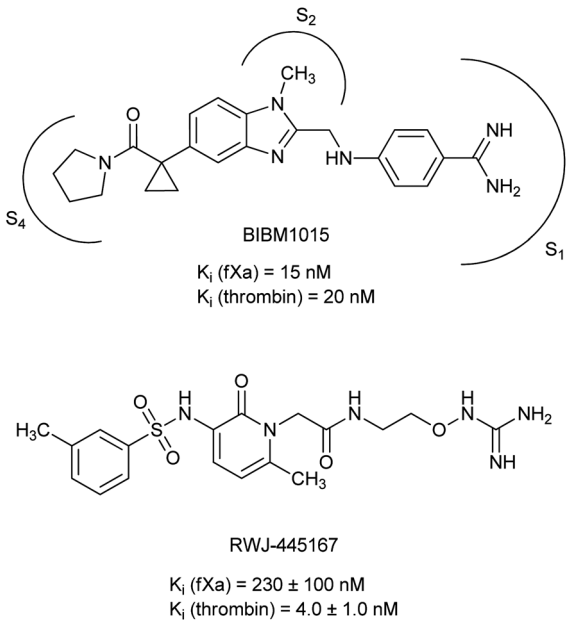

Fig. 1 Dual factor Xa/thrombin inhibitors BIBM1015 and RWJ-445167.

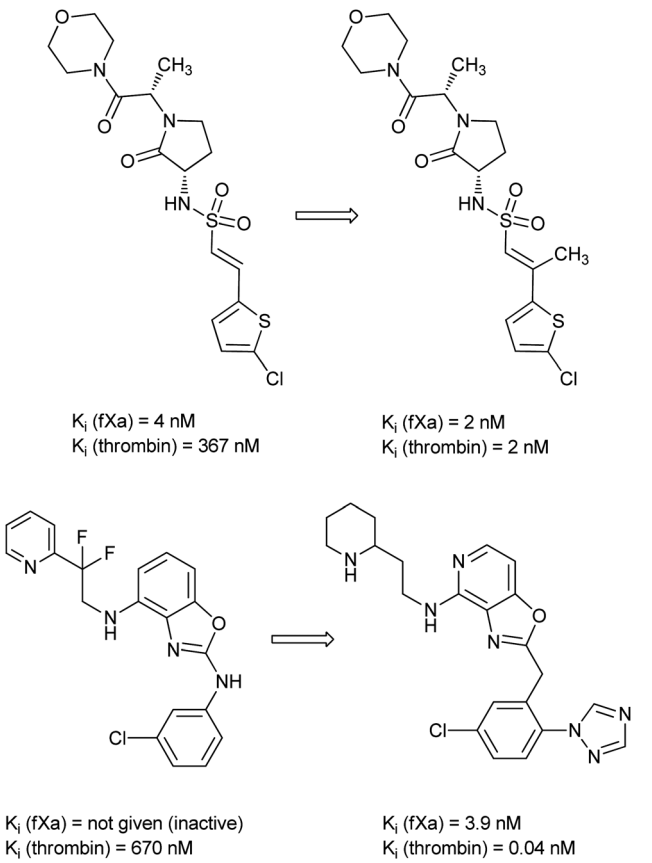

Fig. 2 Examples of "designing in" thrombin inhibitory activity to a selective fXa inhibitor (top) and "designing in" fXa inhibitory activity to a selective thrombin inhibitor (bottom), in order to obtain a dual fXa/thrombin inhibitor.

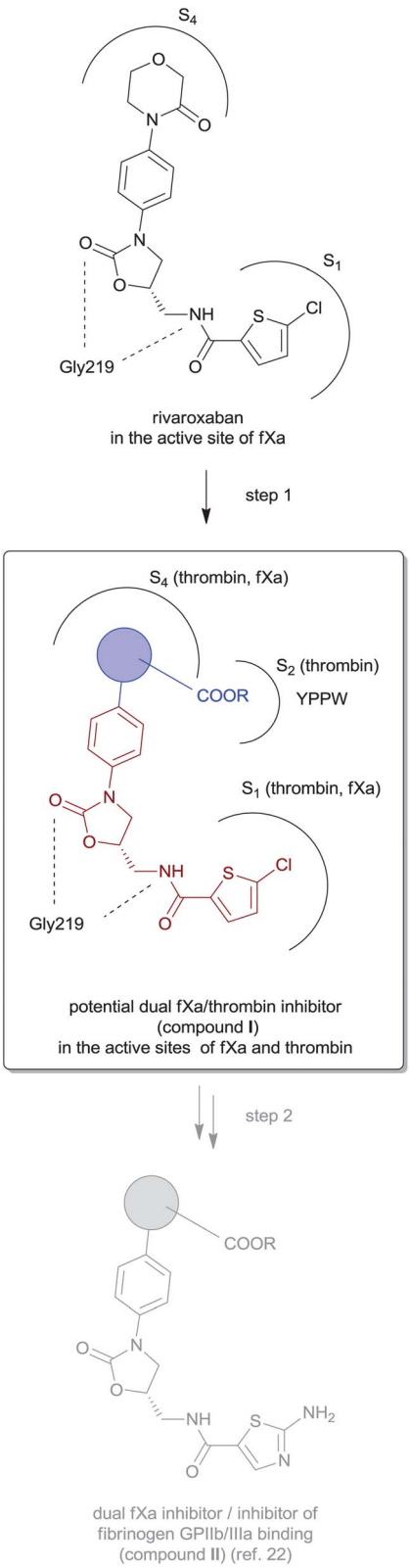

Fig. 3 Evolution of potential dual fXa/thrombin inhibitors (frame) by modification of the P_4 morpholin-3-one moiety of rivaroxaban, on the way to dual inhibitors of factor Xa and fibrinogen binding to GPIIb/IIIa.

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thrombin inhibitors containing a P_1 aryl chloride moiety[21] (Fig. 2, bottom).

Results

Design

We have recently described the first low molecular weight dual inhibitors of factor Xa and fibrinogen binding to GPIIb/IIIa with a general structure **II** (Fig. 3), incorporating highly overlapped pharmacophores of a selective factor Xa inhibitor rivaroxaban and GPIIb/IIIa antagonists,[22] obtained by modification of rivaroxaban at the P_4 morpholin-3-one and P_1 5-chlorothiophene moieties. In connection with these studies we report herein an interesting transformation of a selective factor Xa inhibitor rivaroxaban into dual fXa/thrombin inhibitor **24** by stepwise modification of a rivaroxaban P_4 morpholin-3-one core (Fig. 3) and try to provide rationalization of the observed dual activity.

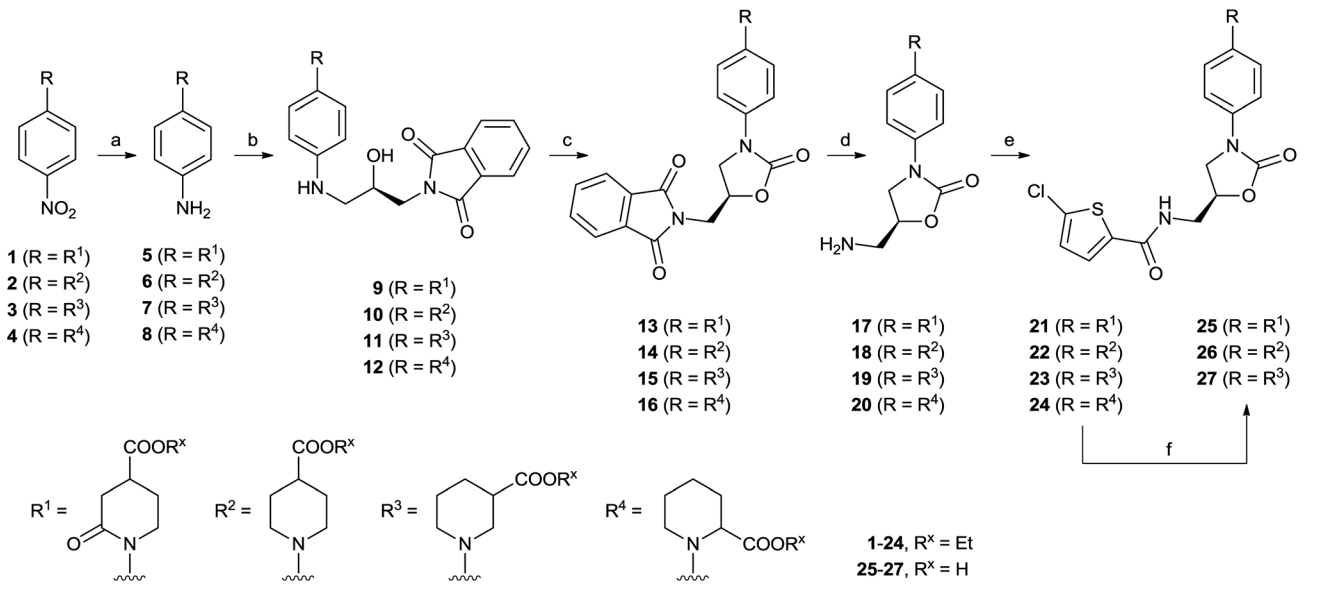
The first step of rivaroxaban modification towards potential dual antithrombotic compounds **II**[22] comprised introducing P_4 morpholin-3-one surrogates bearing a carboxylate group at positions 2, 3 or 4 to give compounds **I** (R = H, Et), followed by replacing the 5-chlorothiophene group by the 2-aminothiazole moiety in the second step (Fig. 3). Interaction between the 5-chlorothiophene moiety in rivaroxaban[4] and various other aryl chlorides[23–25] with Tyr228 in the S_1 subsites of both fXa and thrombin had been successfully exploited in selective fXa inhibitors,[4] selective thrombin inhibitors[26,27] and dual fXa/thrombin inhibitors.[19] Therefore, we assumed that, besides inhibition of factor Xa, 2-chlorothiophene compounds **I** bearing appropriate P_4 substituents could also inhibit thrombin. Docking of compounds **I** to factor Xa and thrombin suggested that the 5-chlorothiophene moiety would occupy the S_1 subsites of both enzymes, while a novel P_4 moiety could locate in the S_4 subsites and, when appropriately substituted, extend to the S_2 subsite of thrombin and interact with the YPPW loop (Fig. 3, middle). Based on these docking results and the accessible synthetic options, piperidine and 2-oxopiperidine, each bearing an optionally positioned carboxyl group or its ethyl ester counterpart, were selected as the most convenient moieties to replace the P_4 morpholin-3-one of rivaroxaban in order to "design in" thrombin inhibitory activity.

Chemistry

The synthesis of target dual fXa/thrombin inhibitors **21–27** under non-racemizing conditions[4] is depicted in Scheme 1. The key intermediates **17–20** were prepared by hydrazinolysis of (*S*)-2-((2-oxo-3-phenyloxazolidin-5-yl)methyl)isoindoline-1,3-dione derivatives **13–16**, which were synthesized in three steps from 4-nitrophenyl derivatives **1–4** by catalytic hydrogenation, opening of (*S*)–*N*-(2,3-epoxypropyl)phthalimide (99% e.e.) by resulting anilines **5–8** and subsequent cyclization of amino alcohols **9–12** to oxazolidin-2-ones **13–16**. Intermediates **17–20** were then coupled with 5-chlorothiophene-2-carbonyl chloride to give esters **21–24** which, after alkaline hydrolysis, yielded compounds **25–27**. The carboxylic acid counterpart of compound **24** was prepared in the same way, but could not be isolated in sufficient quantity from the reaction mixture. Compounds **22** and **26**, bearing only one chiral center, were obtained as *S* enantiomers, whereas **21**, **23–25** and **27** were prepared as 1 : 1 mixtures of two diastereomers, as the synthesized compounds are stereochemically stable under the applied experimental conditions.[4,28,29]

Biological evaluation

The target potential dual fXa/thrombin inhibitors **21–27** were evaluated for inhibitory activity on factor Xa, thrombin and



Scheme 1 *Reagents and conditions*: (a) H_2/Pd–C, EtOH, rt, 10–15 h; (b) (*S*)–*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) 5-chlorothiophene-2-carbonyl chloride, Et_3N, DCM, 0 °C → rt, 15 h; (f) LiOH, MeOH/H_2O = 1 : 1, rt, 15 h.

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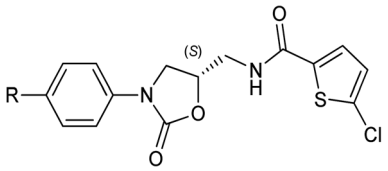
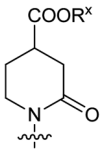
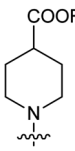
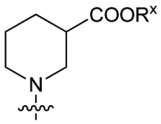
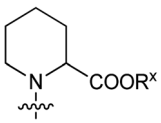
Table 1 Inhibitory activity of (*S*)-5-chloro-*N*-((2-oxo-3-(4-(piperidin-1-yl)phenyl)oxazolidin-5-yl)methyl)-thiophene-2-carboxamides on the serine proteases factor Xa, thrombin and trypsin



			K_i (μM)		
Comp.	R	R^x	fXa	Thrombin	Trypsin
21		Et	0.0219 ± 0.0049	>100	>100
25		H	0.337 ± 0.073	69.6 ± 9.7	>100
22		Et	0.769 ± 0.085	>100	>100
26		H	0.0887 ± 0.0224	16.6 ± 6.2	>100
23		Et	0.0624 ± 0.0085	>100	>100
27		H	1.22 ± 0.18	17.6 ± 1.5	>100
24		Et	0.0622 ± 0.0179	0.353 ± 0.075	>100

trypsin, using the standard enzyme amidolytic assay for inhibition of serine proteases[30] (Table 1). Since all the compounds differ from rivaroxaban only in the P_4 moiety, they showed, as expected, good fXa inhibitory activities ranging from $K_i = 21.9 \pm 4.9$ nM for ethyl 2-oxopiperidine-4-carboxylate analogue **21** to K_i of 1.22 ± 0.18 μM for piperidine-3-carboxylic acid analogue **27**. Comparison of the potencies of compounds **21** and **22** demonstrates that introduction of an oxo group to the piperidine-4-carboxylate moiety improved fXa inhibitory potency 35-fold, while the opposite, though less pronounced, effect was observed for the corresponding carboxylic acids **25** and **26**, in which introduction of the oxo group caused a 3.8-fold reduction of fXa inhibitory potency.

Repositioning the ethoxycarbonyl group on the piperidine ring resulted in the same fXa inhibitory activity for the 3-substituted piperidine analogue **23** ($K_i = 62.4 \pm 8.5$ nM) and the 2-substituted piperidine analogue **24** ($K_i = 62.2 \pm 1.79$ nM), both with a 12.4-fold increase of binding affinity in contrast to the 4-subsituted piperidine analogue **22** ($K_i = 769 \pm 8.5$ nM). Comparison of the fXa inhibitory potencies of carboxylic acid derivatives **26** ($K_i = 88.7 \pm 22.4$ nM) and **27** ($K_i = 1.22 \pm 0.18$ μM) however shows a reduction in inhibitory potency when the carboxyl group is moved from position 4 to position 3 on the piperidine ring. Thus, factor Xa inhibition was strengthened by moving the ester group from position 4 to position 2 or 3 on the piperidine ring, while the opposite was true for repositioning the carboxylic group.

The 2-aminothiazole counterparts of compounds **21–27** (Fig. 3, bottom) displayed factor Xa inhibition constants in the range of 28.7 to 111 μM and were devoid of thrombin inhibitory activity ($K_i > 100$ μM).[22] Rivaroxaban, a potent fXa inhibitor with a K_i of 0.7 nM also failed to inhibit thrombin at concentrations up to 20 μM, demonstrating a more than 10 000-fold selectivity for fXa.[4] In a series of prepared potential dual inhibitors **21-27**, thrombin inhibition was absent in 4- and 3-ethoxycarbonyl derivatives **21**, **22** and **23** ($K_i > 100$ μM) and very weak in their respective carboxylic acid counterparts **25**, **26** and **27** (K_i between 16.6 ± 6.2 and 69.6 ± 9.7 μM). To our satisfaction, the 2-ethoxycarbonyl derivative **24** displayed thrombin inhibition, with K_i of 353 ± 75 nM which, together with its potent factor Xa inhibition ($K_i = 62.2 \pm 17.9$ nM) and excellent selectivity against trypsin, makes **24** a promising dual fXa/thrombin inhibitor for which action of both diastereomers remains to be established. In a preliminary experiment, on incubation of compounds **24** and **22** with human plasma at 37 °C, 100 percent of **24** and 95 percent of **22** were present after 4 hours, indicating their increased stability in comparison to primary carboxylic acid ethyl ester dabigatran etexilate which was used as a control. However, in-depth *in vitro* metabolic stability studies (*e.g.*, microsomes) would be necessary to elucidate the *in vitro* stability profile of compounds **21–27**. A literature survey revealed mention of compound **24** in a patent dealing with preparation of rivaroxaban,[31] however neither its preparation and characterization nor its fXa/thrombin inhibitory properties and, most importantly, indication of its dual activity were described.

Rationalization of dual activity

Docking of both diastereomers of **24** into the active sites of factor Xa and thrombin suggested plausible binding orientations that could explain the observed dual activity of compound **24** (we used CDOCKER, a CHARMM-based docking program[32] that systematically poses the flexible ligands within a static active site and conducts low-level energy calculations for each pose). The best-posed conformation of (*R*)-ethyl 1-(4-((*S*)-5-((5-chlorothiophene-2-carboxamido)methyl)-2-oxooxazolidin-3-yl)-phenyl)piperidine-2-carboxylate isomer of compound **24** [(2*R*,5″*S*)-**24**] possessed lower predicted binding energy to both targets than (2*S*,5″*S*)-**24**. As shown in Fig. 4, (2*R*,5″*S*)-**24** binds into the active site of factor Xa similarly to rivaroxaban (green).[4] The 5-chlorothiophene moiety fits almost identically into the S_1 subsite, while the carbonyl oxygen of the oxazolidinone core and the NH group of the chlorothiophenecarboxamide form hydrogen bonds (2.0 Å and 2.3 Å) with Gly219. The phenyl ring extends across the face of Trp215, while the ethoxycarbonyl 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.[4] Analysis of the binding mode of (2*R*,5″*S*)-**24** in the

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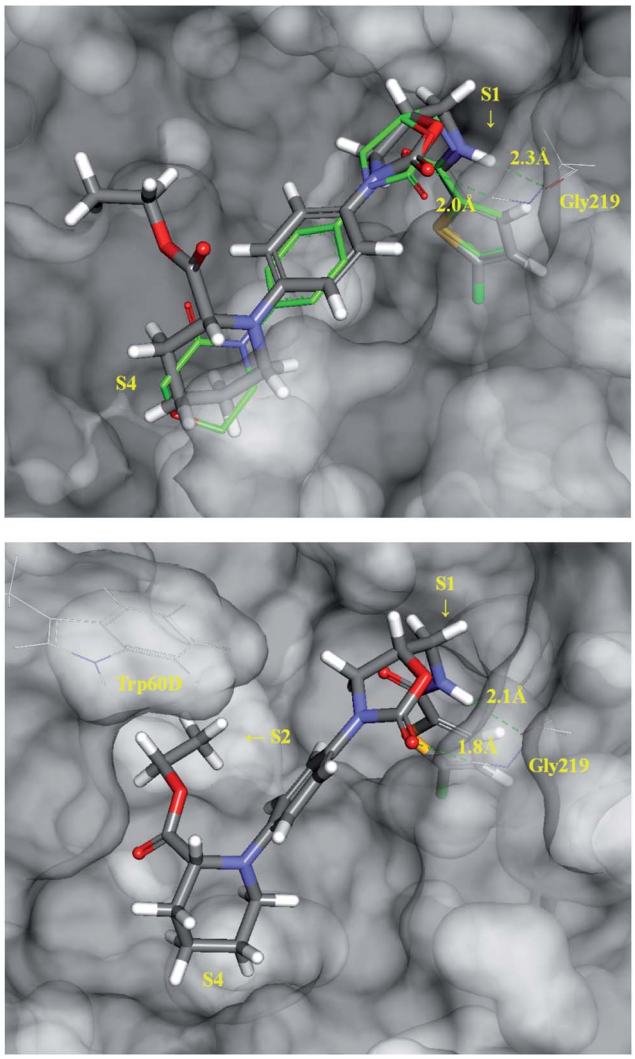

Fig. 4 (2*R*,5″*S*)-**24** docked into the fXa active site[4] (top) and thrombin active site[33] (bottom). The conformation of rivaroxaban in complex with fXa (from the X-ray crystal structure) is indicated in green (top) (the figures were prepared by Discovery Studio 3.1).

active site of thrombin shows that it assumes a conformation similar to that in the active site of fXa, with an RMSD value of 1.54 Å for the heavy atoms. The chlorine atom of the 5-chlorothiophene moiety, that occupies the S_1 subsite, interacts with the aromatic ring of Tyr228 at the bottom of the specificity pocket. As in factor Xa, in the thrombin active site the carbonyl oxygen of the oxazolidinone core and the chlorothiophenecarboxamide NH group also form hydrogen bonds (1.8 Å and 2.1 Å) with Gly219. Shorter H-bonds in the range of 1.8–2.0 Å most certainly indicate a limitation of the docking method used, which treats the enzyme as a rigid entity. The 2-ethoxycarbonyl group, which affects the perpendicular arrangement of the two rings in the ethyl phenylpiperidine-2-carboxylate moiety, extends to the S_2 pocket (in contrast to 3-COOEt and 4-COOEt isomers) and interacts with the YPPW loop of the thrombin active site, thus contributing to binding to thrombin and conferring dual fXa/thrombin inhibitory activity on compound **24**.

Conclusions

In conclusion, replacement of a morpholine-3-one core in rivaroxaban by the 2-ethoxycarbonylpiperidine moiety transformed a selective factor Xa inhibitor rivaroxaban into a balanced dual factor Xa/thrombin inhibitor **24**, each with a nanomolar inhibition constant. Rationalization of the inhibition of both enzymes has provided a good starting point for "designing in" thrombin inhibitory activity to the potent factor Xa inhibitor rivaroxaban by exploration of SAR regarding the moiety bound at position 2 of the piperidine ring and stereochemistry of the piperidine moiety.

Acknowledgements

This work was supported by the Slovenian Research Agency (Grant P1-0208). The authors thank Professor Roger Pain for critical reading of the manuscript.

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