DOI: 10.1002/cmdc.201200224

β-D-Glucosyl Conjugates of Highly Potent Inhibitors of Blood Coagulation Factor Xa Bearing 2-Chorothiophene as a P1 Motif

Gianfranco Lopopolo,[a] Modesto de Candia,[a] Luigi Panza,[b] Maria Rosaria Romano,[c] Marcello Diego Lograno,[c] Francesco Campagna,[a] and Cosimo Altomare*[a]

We synthesized a novel *O*-glucoside of the recently reported potent factor Xa (fXa) inhibitor **1**, which bears a 5-chlorothien-2-yl moiety and 1-isopropylpiperidine as fragments that bind the S1 and S4 enzyme pockets, respectively. A β-D-glucosyl unit was conjugated through an ether-linked C3-alkyl spacer to the central phenyl ring of **1**. The synthesized β-D-glucose-based compound **16** achieved picomolar inhibitory potency against human fXa ($K_i = 60$ pM) and high selectivity over thrombin and other serine proteases. In addition to the chlorothienyl S1 binder, a large gain in ΔG resulted from the addition of protonated 1-isopropylpiperidine ($\Delta\Delta G = 29.7$–30.5 kJ mol^{-1}), which should bind to the aromatic S4 pocket through efficient cation–π and C–H···π interactions. Instead, the C3-alkyl-linked glucose fragment, which is likely directed toward the solvent outside the enzyme binding site, improves ΔG by an average of 2.9–3.8 kJ mol^{-1}. Compound **16** showed sub-micromolar in vitro anticoagulant activity, as assessed by prothrombin time (PT) and activated thromboplastin time (aPTT) clotting assays in pooled human plasma (PT_2 and $aPTT_2$ equal to 0.135 and 0.389 μM, respectively). Although compound **16** was 1.4-fold less active than parent compound **1** in the ex vivo anticoagulant assay in mice, it showed a significant (1.6-fold) prolongation of PT relative to controls ($P < 0.05$) 60 min after oral dosing (75 mg kg^{-1}).

Introduction

The serine protease factor Xa (fXa), located at the confluence of the intrinsic and extrinsic pathways of the blood coagulation cascade, catalyzes the cleavage of prothrombin to form thrombin, thus initiating the common pathway that leads to the formation of fibrin clots. Among the newly developed anticoagulant drugs with better safety profiles than those currently used, thus allowing fixed oral dosing and not requiring time- and resource-intensive monitoring, fXa inhibitors have emerged as attractive options for venous thromboembolism (VTE) treatment and stroke prevention in patients with atrial fibrillation (AF).[1] As fXa inhibitors should prevent the generation of new thrombin without affecting the basal thrombin level that ensures primary hemostasis,[2] they should have a lower risk of bleeding than current antithrombotic therapy (heparins and warfarin),[3] and an even higher therapeutic ratio than direct thrombin inhibitors (DTIs)[4] such as dabigatran etexilate.[5]

Intensive efforts are underway toward the pharmaceutical development of direct fXa inhibitors as new antithrombotic agents, as evidenced by the growing number of publications and patents filed in the last decade.[6] Large clinical trial programs have established the effectiveness of fXa inhibitors, such as apixaban and rivaroxaban, in patients experiencing (or at risk of) VTE in orthopedic surgery,[1] and these drugs are predicted to have a great impact on the anticoagulant market.[7]

The rational design of orally bioavailable small-molecule fXa inhibitors, achieving good enzyme potency and selectivity, has been considerably supported by the X-ray co-crystal structures of protein–ligand complexes, sophisticated molecular modeling techniques, and 3D QSAR studies.[8] FXa contains a serine protease domain in a trypsin-like closed β-barrel fold, encompassing the catalytic triad Ser 195-His 57-Asp 102 and two proximal binding pockets, namely S1 and S4. Potent fXa inhibitors are usually L-shaped, with a preorganized scaffold orienting two nearly orthogonal molecular fragments toward the proximal S1 and S4 pockets in the enzyme binding site. Due to the high shape retention of the active site and the well-defined subpockets, fXa is a good model for molecular recognition studies.[9,10]

We recently reported *N*-(2-hydroxyphenyl)acetamide-based inhibitors of fXa bearing 5-chlorothien-2-yl and 1-isopropylpiperidin-4-yl moieties as P1 and P4 fragments, respectively.[11] The most potent compound **1** (Figure 1) inhibited fXa with a K_i value of 0.3 nM and very high selectivity over thrombin and other serine proteases, achieving in vitro anticoagulant activity in the low micromolar range, as assessed by the prothrombin time clotting assay ($PT_2 = 3.30$ μM). Based on these data, com-

[a] Dr. G. Lopopolo, Dr. M. de Candia, Prof. F. Campagna, Prof. C. Altomare
Dipartimento Farmaco Chimico
University of Bari A. Moro, Via E. Orabona 4, 70125 Bari (Italy)
E-mail: altomare@farmchim.uniba.it

[b] Prof. L. Panza
Dipartimento di Scienze del Farmaco
University of Piemonte Orientale A. Avogadro
Via Bovio 6, 28100 Novara (Italy)

[c] Dr. M. R. Romano, Prof. M. D. Lograno
Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche
University of Bari A. Moro, Via E. Orabona 4, 70125 Bari (Italy)

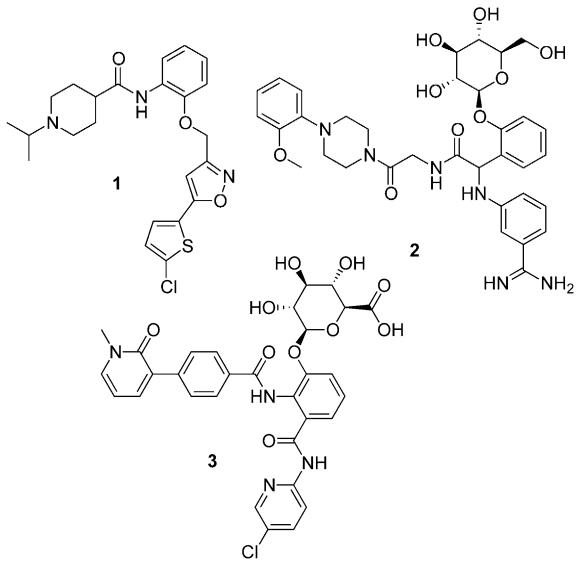


Figure 1. Structures of factor Xa inhibitors. Compound **1** was recently described by us as a potent and selective fXa inhibitor.[11] Compounds **2** (MCM09)[16,17] and **3**[18] are two patented glycan conjugates of phenylglycinamide- and anthranilamide-based fXa inhibitors, respectively.

pound **1** could be considered a promising lead for further physicochemical optimization and pharmacological studies.

As predicted by our docking calculations (Figure 2), and in good agreement with the X-ray crystallographic structures of fXa–inhibitor complexes,[12,13] compound **1** preferably directs

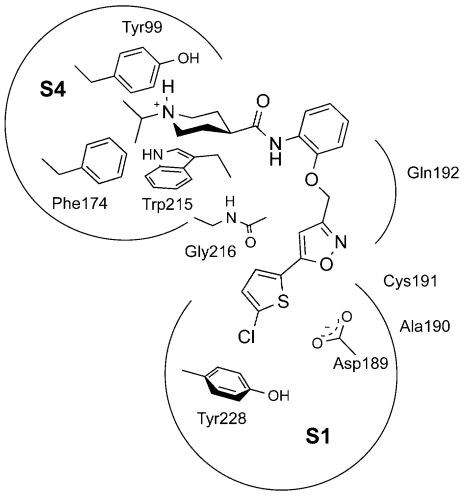


Figure 2. Schematic representation of the binding mode of compound **1** into the binding site of human fXa, according to our docking calculations.[11] Key residues in the S1 and S4 binding sites are highlighted.

the neutral 2-chlorothiophene group into the S1 pocket, with the chlorine atom projecting toward the center of the Tyr 228 phenyl ring. The protonated N^1-isopropyl residue (the tertiary ammonium head) resides at the center of the S4 aromatic box, where it undergoes efficient cation–π interactions and additional C–H···π interactions with the isopropyl group as well with the side chains of Phe 174, Tyr 99, and Trp 215.

An optimal oral anticoagulant should have a predictable pharmacokinetic (PK) profile which allows fixed oral dosing and a relatively wide therapeutic index. From a PK viewpoint, it should be characterized by low plasma protein binding, low volume of distribution, low systemic clearance for decreasing drug–drug interactions, rapid onset of action, and ability to bind clot-bound coagulation factors.[14] As a trend, small-molecule inhibitors of fXa and thrombin possessing similar enzyme binding affinities show better PK profiles and in vivo anticoagulant activity when their log *P* values are less than 4.[15] The high lipophilicity of compound **1** ($\log P > 4$), for example, may unfavorably affect its plasma protein binding, distribution volume, and systemic clearance. This prompted us to decrease its lipophilicity through glucosidation, which is a molecular optimization strategy that allows improvement of both drug solubility and targeting via glucose transporters.

An examination of the preferred docking pose of **1** into the fXa binding site (Figure 2) suggested that the position *ortho* to the amide moiety on the central phenyl ring could be a suitable site for functionalization, likely allowing the hydrophilic sugar moiety to be oriented toward the solvent and avoiding any clash with the S1 and S4 enzyme pockets.

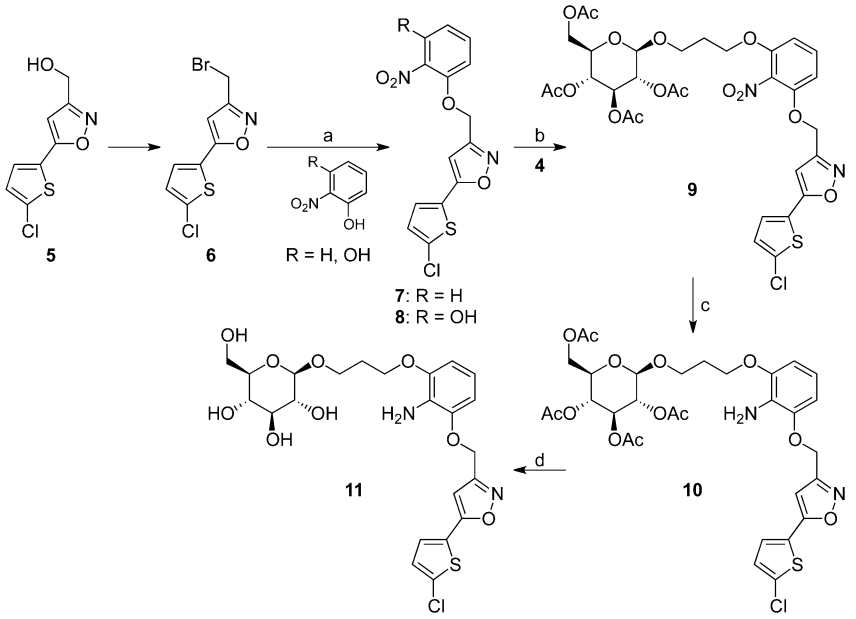
As a sugar unit, we chose β-D-glucose which, in addition to enhancing solubility, may be recognized by glucose transporters (GLUTs) localized in the gastrointestinal (GI) tract. Our approach was also supported by recent patents disclosing two glycan-bearing fXa inhibitors (Figure 1). Researchers at Morphochem Chemie[16,17] reported a number of 3-benzamidino derivatives of phenylglycine (PhGly), including MCM09 (**2**) which has a β-D-glucose unit at the *ortho* position of the phenyl side chain, whereas Astellas Pharma scientists disclosed the glucuronic acid derivative (**3**) of an anthranilamide-based fXa inhibitor.[18] In particular, compound **2** inhibited human fXa with an in vitro IC_{50} value of 2.4 nM and also showed good ex vivo anticoagulant properties in mice. Interestingly, **2** inhibited melanoma lung cancer colonies in mice in a dose-dependent manner.[19] The mechanism underlying this anticancer effect has not yet been well-elucidated; however, it is known that tumor cells express several pro-coagulant activities which may be involved in hemostasis disturbances and metastasis, mediated by the action of serine proteases on the proteinase-activated receptors (PARs), suggesting that inhibitors of coagulation factors could interfere with cellular proliferation in cancer cell lines.[20] Moreover, it is known that GLUTs are overexpressed in malignant cells,[21–24] such as colon, breast, and lung carcinomas, not only contributing to glucose uptake but also allowing the targeted delivery of glycan conjugates of anticancer agents.[25]

In this study, we modified compound **1** by connecting a β-D-glucose unit to the central phenyl ring (**16**) through a propyl spacer. Herein we report the synthesis, inhibition of blood coagulation factors and related serine proteases, and in vitro and ex vivo anticoagulant activity of glycan **16** and related derivatives.

Results and Discussion

Synthesis

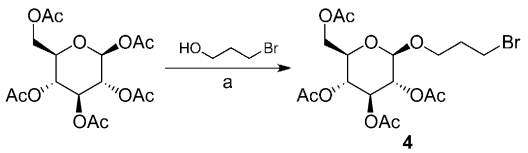
β-D-Glucose was conjugated at the *ortho* position of the amide group on the central phenyl ring of compound **1** through an ether C3-alkyl linker. This minimum spacer length was preferred in order to avoid as many unfavorable intramolecular and intermolecular interactions as possible that might arise from directly linking to the glucose moiety and/or from using shorter spacers, such as a steric clashes between the sugar moiety and the enzyme binding sites, as well as to avoid possible formation of β-elimination side products during synthesis. The peracetyl-protected glucose was used as the starting material, taking into account the compatibility of the acetyl ester cleavage conditions for preserving the other functional groups present in the target molecule. Moreover, due to the anchimeric assistance of the C2′ oxycarbonyl group, the 2′-OAc group preferentially affords 1,2-*trans*-glycosidation, in our case, β-anomer formation.



Scheme 2. Compounds **5**, **6**, and **7** were reported previously.[11] *Reagents and conditions*: a) compound **8**, K_2CO_3, dry DMF, RT, overnight; b) K_2CO_3, dry DMF, RT, overnight; c) $SnCl_2{\cdot}2H_2O$, EtOAc, reflux, 48 h; d) MeONa, MeOH, RT, 3 h.

The synthesis pathways for the target glucosyl conjugate **16** and related compounds are shown in Schemes 1–3. Compound **4** was prepared in 53% yield by direct glycosidation of 3-bromo-1-propanol with 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose, using $BF_3{\cdot}Et_2O$ as a promoter (Scheme 1).

3-Bromopropyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside **4**, the bromomethyl derivative **6** (prepared according to previously reported procedures[11]), and 2-nitroresorcinol were used as starting materials for the synthesis of compound **10** (Scheme 2). Monoalkylation of a large excess of 2-nitroresorcinol with 3-(bromomethyl)-5-(2-chlorothiophen-5-yl)isoxazole (**6**) afforded compound **8** (58% yield), which subsequently reacted with **4**, allowing nitrophenylether **9** to be obtained in almost quantitative yield. Reduction of the nitro to an amino group in compound **10** was accomplished using $SnCl_2{\cdot}2H_2O$ in EtOAc at reflux, which proved the best conditions among those explored (N_2H_4/Ni-Raney in MeOH at reflux, Fe/NH_4Cl in EtOH at reflux, H_2/Pt), to obtain the desired compound (52% yield), overcoming side reactions and preserving as much of the integrity of the entire molecule as possible. Cleavage of the acetyl-protecting groups was achieved with MeONa/MeOH at room temperature for 3 h to give **11** in almost quantitative yield.



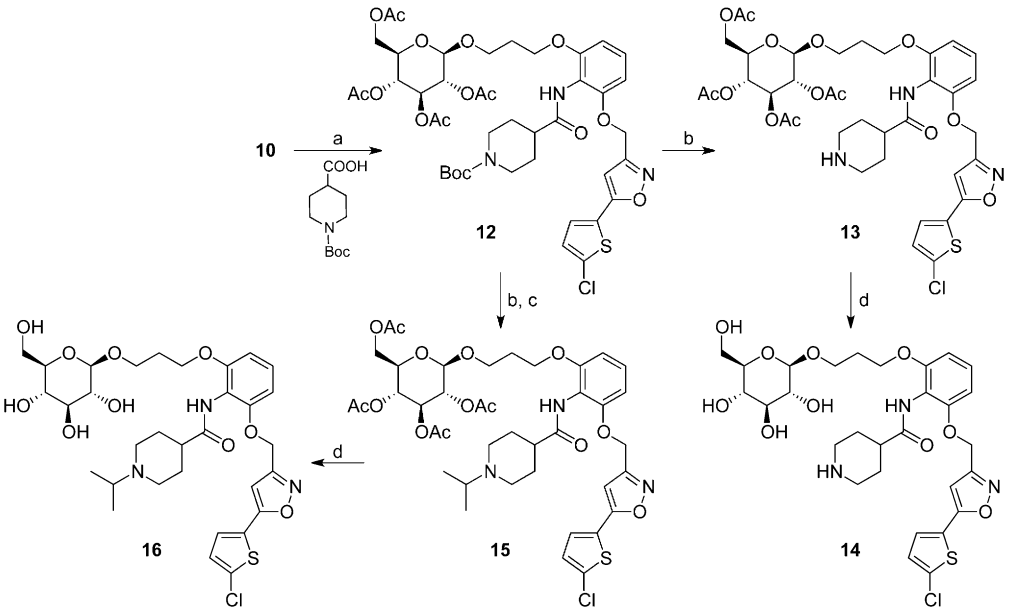
Scheme 1. *Reagents and conditions*: a) $BF_3{\cdot}Et_2O$, MS 4 Å, dry CH_2Cl_2, 0 °C→RT, overnight.

Compound **10** was coupled with N-Boc-isonipecotic acid in DCC/HOBt/THF (**12**, 40% yield), followed by removal of the Boc-protecting group to yield **13** (Scheme 3). In a one-pot procedure, Boc-deprotection and reductive amination of **12** with acetone afforded *N*-isopropyl derivative **15** (86% yield). Deacetylation of **13** and **15** under basic conditions (MeONa/MeOH) at room temperature provided compounds **14** and **16**, respectively, in greater than 90% yields.

Inhibition of factor Xa and related enzymes

To explore fragment-growing effects, the newly synthesized C3-alkyl-linked glucosyl conjugates **14**–**16**, along with compounds **5**, **7**, **8**, and **11**, were evaluated in vitro for inhibition of fXa. The half maximal inhibitory concentration (IC_{50}) was determined for each compound using fixed amounts of hfXa (2 nM) and the chromogenic substrate *Z*-D-Arg-Gly-Arg-*p*-NA (40 μM) and varying the concentrations of the inhibitor. Each IC_{50} was determined in at least triplicate, and the values were averaged. The IC_{50} values were then used to calculate the inhibition constants (K_i values; Table 1) using the Cheng–Prusoff equation.[26]

Target compound **16** achieved sub-nanomolar fXa inhibition activity (K_i = 0.06 nM), displaying a fivefold increase in potency relative to parent compound **1**. Lineweaver–Burk plots were generated for **16** using a fixed amount of hfXa (2 nM) and varying concentrations of substrate (25–200 μM) in the absence or presence of inhibitor at four concentrations (0.075–0.6 nM), which displayed a competitive inhibition mechanism.



Scheme 3. *Reagents and conditions*: a) DCC, HOBt, dry THF, RT, 48 h; b) CF_3COOH, CH_2Cl_2, RT, 3 h; c) acetone, $Na(CN)BH_3$, MeOH, RT, 24 h; d) MeONa, MeOH, RT, overnight.

Table 1. Evaluation of fragment-growing effects on fXa inhibition.

Compd	K_i [nM][a]
1	0.3[b]
5	93000
7	45000
8	70000
11	14700
14	37[c]
15	0.2
16	0.06

[a] Inhibition constant values are means of three duplicate determinations (SEM < 5% of the mean). [b] Value taken from previous study.[11] [c] K_i value for thrombin is 17.6 μM.

Re-plotting the slopes of the above double reciprocal plots against inhibitor concentration gave a K_i value (0.11 ± 0.06 nM) which was not significantly different from that calculated by the Cheng–Prusoff equation.

The peracetylated glycoside derivative **15**, assayed for fXa inhibition, proved to be almost equipotent with **1** (K_i = 0.2 nM) and three times less potent than **16**. Despite its lower inhibitory activity in vitro, it is possible that peracetylated compound **15** could have a cell permeability (and likely GI absorption) better than that of compound **16**, as demonstrated for peracetylated xylosides endowed with antiproliferative activity.[27] Compound **14**, the des-isopropyl analogue of **16**, showed much lower fXa affinity (K_i = 37 nM) and selectivity; the comparison between the respective fXa/thrombin selectivity ratios showed that **16** is 30-fold more selective than **14** toward fXa.

The data in Table 1 allowed us to quantify the contributions of single fragments to the measured free energy of binding of compound **16**. We took into account the binding mode of the parent compound **1**, supported by our docking calculations,[11] which were in full agreement with X-ray co-crystal structures of fXa in complex with structurally similar inhibitors,[12,13] assuming the 5-(5-chlorothien-2-yl)isoxazol-3-yl group as the S1 binding fragment (**5**) to assess the stepwise free energy gain ($\Delta\Delta G$) of the linker (2-aminophenyl), S4 binder (1-isopropylpiperidine), and hydrophilic carrier (C3-alkyl-linked glucose).

The 5-chlorothien-2-yl-bearing compound **5** showed a K_i value of 93 μM, corresponding to a ΔG value of −23.0 kJ mol^{-1}; this fragment has 13 heavy atoms and a ligand efficiency (LE, namely the ratio of ΔG to the number of non-hydrogen atoms) of 0.42, which is the same LE as the highly potent fXa inhibitor **1** (ΔG = −54.4 kJ mol^{-1}). Adding a 2-aminophenyl linker to fragment **5** resulted in **7**, with a small ΔG gain ($\Delta\Delta G_{5\rightarrow7}$ = −1.7 kJ mol^{-1}). The binding affinity further increases upon connection of **7** with a propyloxy-glucose fragment at the position *ortho* to NH_2, improving ΔG for **11** by 2.9 kJ mol^{-1}. A slightly higher ΔG gain, due to the C3-alkyl-linked glucose fragment, was calculated by comparing **16** with the parent compound **1** ($\Delta\Delta G_{1\rightarrow16}$ = −3.8 kJ mol^{-1}).

The largest increase in affinity relative to **7** resulted from addition of the piperidine fragments (S4 binders). The piperidine moiety was linked to the aromatic NH_2 in **11** through an amide bond, with the secondary amino group fully protonated under the conditions of the enzyme assay (pH 8), improving ΔG of **14** by 14.6 kJ mol^{-1}. Alkylation of the piperidine nitrogen in **14** with an isopropyl group improves the affinity of **16** still further ($\Delta\Delta G_{14\rightarrow16}$ = −15.9 kJ mol^{-1}). Globally, the measured contribution of the protonated 1-isopropylpiperidine ($\Delta\Delta G_{11\rightarrow16}$) equals −30.5 kJ mol^{-1}, which is a ΔG gain similar to that calculated when comparing compounds **1** and **7** ($\Delta\Delta G_{7\rightarrow1}$ = −29.7 kJ mol^{-1}). With the exception of the deep S1 pocket, the active site of fXa, including the S4 aromatic binding pocket, is located on the surface of the enzyme. The crucial role in fXa molecular recognition of the cation–π interactions in the S4

binding site has been well-established by a series of high-affinity ligands with excellent inhibitory potencies.[13] The inhibitors examined herein should bind to the fXa active site, with the protonated piperidine N^1-isopropyl residue (i.e., the tertiary ammonium head) at the center of the S4 aromatic box, where the isopropyl group can undergo cation–π interactions and additional C–H···π interactions. Our data indicate that the gain in the binding ΔG amounts to increments of 14.6 and 15.9 kJ mol^{-1}, corresponding to efficient N–H···π interactions (**11**→**14**) and C–H···π interactions of the isopropyl group (**14**→**16**) with the side chains of Phe 174, Tyr 99, and Trp 215. The 2-aminophenyl linker effect was estimated to be only 1.7 kJ mol^{-1}, and the fragment deconstruction of sugar-bearing compounds **11** and **16** resulted in a rather small ΔG gain (2.9–3.8 kJ mol^{-1}) for the C3-alkyloxy-linked glucose, which is unlikely to arise from favorable interactions of the glucose moiety with specific binding sites. Most likely, the hydrophilic sugar moiety is directed out of the fXa binding site into the solvent environment, as shown in literature for other hydrophilic moieties installed on the central linker connecting S1 and S4 binding groups in fXa inhibitors.[28]

The most potent glycan fXa inhibitors, **15** and **16**, were assayed for their inhibitory activity against blood coagulation factors, namely thrombin and factor VIIa (fVIIa), and other serine proteases, such as α-chymotrypsin, trypsin, and leukocyte elastase, with the results compared with those obtained with the reference compound **1** (Table 2). Both compounds **15** and **16** displayed higher anti-thrombin activity than that of parent compound **1**, with K_i values in the nanomolar range. Additionally, their fXa/thrombin selectivity ratios remained high (775 and 1500 for **15** and **16**, respectively). Compound **16** demonstrated excellent selectivity (> 1.7×10^6-fold) against fVIIa of the coagulation cascade and the three other serine proteases tested.

Table 2. Inhibition data against blood coagulation factors and other serine proteases, and in vitro anticoagulant activities of compounds **1**, **15**, and **16**.

Enzyme K_i values[a] and clotting assay data[b]	Compd 1	15	16
FXa [nM]	0.3[c]	0.2	0.06
Thrombin [nM]	11 000[c]	155	90
FVIIa [μM]	>100	NT	>100
α-Chymotrypsin [μM]	60[c]	>100	>100
Trypsin [μM]	>100	NT	>100
Leukocyte elastase [μM]	>100	NT	>100
PT_2 [μM]	3.30[c]	0.495	0.135
$aPTT_2$ [μM]	5.03	0.840	0.389

[a] Inhibition constants against human activated factor Xa, bovine thrombin, human recombinant activated fVIIa, bovine α-chymotrypsin and trypsin, and human leukocyte elastase; maximum concentration tested was 100 μM. Data are means of three duplicate determinations (SEM < 5% of the mean); NT = not tested. [b] Concentrations of test compounds required to double the prothrombin time (PT) and activated partial thromboplastin time (aPTT) of the uninhibited clotting times in human plasma. [c] Data taken from previous study.[11]

In vitro and ex vivo anticoagulant activity

Compounds **15** and **16** were characterized in vitro using clotting assays in human plasma, namely, the measurements of prothrombin time (PT) and activated partial thromboplastin time (aPTT). in vitro anticoagulant activity was expressed as the concentrations of test compounds required to double PT and aPTT of the uninhibited clotting times. Both glycans showed in vitro anticoagulant activity in the nanomolar range, that is, one order of magnitude higher than that of parent compound **1**. In particular, **16** showed PT_2 and $aPTT_2$ values of 0.135 and 0.389 μM, respectively. The observed anticoagulant activity was higher than that expected from inhibition constant values of the purified fXa enzyme. In fact, compound **16** displayed a fivefold increase in fXa inhibitory potency relative to **1**, whereas the corresponding anticoagulant activity increased by 24- and 13-fold in PT and aPTT clotting assays, respectively. This difference in anti-clotting potencies may result from a substantial decrease in lipophilicity for **16** (Clog $P = 2.05$, as calculated by Bio-Loom software, Biobyte, Claremont, CA, USA) relative to **1** (Clog $P = 4.02$), with a consequent lowering of nonspecific interactions of **16** with plasma proteins and other plasma components and, ultimately, an increase in the concentration available to bind the target coagulation factors (fXa, thrombin). The in vitro PT_2 and $aPTT_2$ values for **15** were determined to be 0.495 and 0.840 μM, respectively. Although less potent in vitro than **16**, the peracetylated derivative **15** may undergo in vivo hydrolysis, catalyzed by nonspecific esterases able to cleave the acetyl groups and release the more active compound **16**.[27]

For an early assessment of their ability as orally active anticoagulants, we examined the anticoagulant potency of compounds **1** and **16** after oral dosing in mice. Preliminary results showed no significant effects of peracetylated derivative **15** (data not shown). Figure 3 depicts the PT prolongation effects of **1** and **16**, administered via oral gavage at equimolar doses (50 and 75 mg kg^{-1}, respectively), in blood samples withdrawn at the 1 h time point.

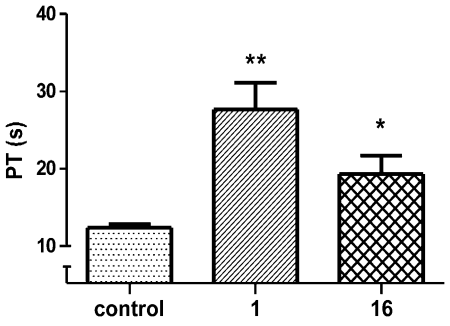


Figure 3. Ex vivo anticoagulant activity of compounds **1** (50 mg kg^{-1}) and **16** (75 mg kg^{-1}) after oral dosing in mice. Animals were dosed via oral gavage with either vehicle (control) or test compounds at equimolar doses (0.11 mmol kg^{-1}), and prothrombin time (PT) was measured 60 min after administration. PT values are expressed as means ± SEM of duplicate determinations for three mice per group (**$P < 0.005$, *$P < 0.05$ using Dunnett's test).

Compound **1** (50 mg kg^{-1}) significantly prolonged plasma PT 60 min after treatment (27.7 ± 3.5 s) versus controls (12.4 ± 0.5 s) ($P < 0.005$ with Dunnett's test). The mean PT value of the group receiving compound **16** (75 mg kg^{-1}) was also significantly higher than that of the controls (19.4 ± 2.3 s; $P < 0.05$), but its ex vivo effect in mice was 1.4-fold lower than that observed for the group receiving compound **1**. In contrast with the results from clotting assays in vitro, compounds **1** and **16** showed 2.2- and 1.6-fold PT prolongations, respectively, compared with controls, evaluated 1 h after oral administration of one high single dose (0.11 mmol kg^{-1}). To better understand the origin of this apparent contrast, further ex vivo anticoagulant activity experiments (PT measurements at different time points and doses), and pharmacokinetic studies (oral bioavailability) are needed.

Conclusions

In this study, glucose-based derivatives of recently reported highly potent fXa inhibitors were synthesized, in which a D-glucose unit was linked, through a β-*O*-glycoside bond, to the central phenyl group (linker) of the inhibitor using an ether C3-alkyl spacer. The O-glucoside **16**, which bears 2-chlorothiophene and 1-isopropylpiperidine as fragments binding the S1 and S4 enzyme pockets, respectively, achieved in vitro excellent inhibition potency against human fXa, with a K_i value in the picomolar range (60 pM) and high selectivity over thrombin, fVIIa, and other serine proteases of the trypsin family. Assuming the 2-chlorothiophene S1 binder was the minimal fragment, we deconstructed β-D-glucose-bearing compound **16** and analyzed fragment-growing effects on its affinity to fXa. Our analysis revealed that the largest ΔG gain results from addition of the protonated 1-isopropylpiperidine ($\Delta\Delta G = 29.7$–30.5 kJ mol^{-1}), which should undergo efficient cation–π and C–H···π interactions within the aromatic S4 pocket by the piperidine N–H and the isopropyl group, respectively. The C3-alkyl-linked glucose improves ΔG by 2.9–3.8 kJ mol^{-1}, indicating that the hydrophilic sugar moiety most likely extends outside of the enzyme binding site toward the solvent.

Compound **16** showed good in vitro anticoagulant activity, as assessed by the clotting assays in pooled human plasma (PT_2 and $aPTT_2$ values of 0.135 and 0.389 μM, respectively). In a preliminary ex vivo anticoagulant assay in mice, compound **16** showed a 1.6-fold PT prolongation compared with controls ($P < 0.05$) 60 min after oral dosing (75 mg kg^{-1}), although it was 1.4-fold less active than the parent compound **1** at equimolar doses (2.2 PT prolongation; $P < 0.005$). Unlike **1**, compound **16** does not fully comply with the Lipinski's guidelines for an oral drug with regard to molecular weight (696 Da) and number of hydrogen bond acceptors (12 nitrogen and oxygen atoms). Nevertheless, compound **16**, in addition to being a lead structure for further optimization of highly potent fXa inhibitors as orally bioavailable or parenteral compounds for use in intensive care medicine, may have potential for improving the delivery of anticoagulants to malignant cells overexpressing GLUTs, ultimately strengthening its possible role in cancer therapy.[19,29–31]

Experimental Section

Chemistry

Melting points were determined using the capillary method on a Stuart Scientific SMP3 electrothermal apparatus and are uncorrected. IR spectra were recorded using KBr disks on a PerkinElmer Spectrum One FT-IR spectrophotometer (PerkinElmer, Buckinghamshire, UK), and the most significant absorption bands are expressed in cm^{-1}. ^{1}H NMR spectra were recorded at 300 MHz on a JEOL ECP 300 instrument. Chemical shift values are expressed in δ and the coupling constants J in hertz. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet, dd, doublet of doublets; m, multiplet; br, broad. Signals due to NH and OH protons were located by deuterium exchange with D_2O. Mass spectra were recorded on an Agilent GC–MS 689-973. Elemental analyses (C, H, N) were performed on an Euro EA3000 analyzer (Eurovector, Milan, Italy) using the Analytical Laboratory Service of the Dipartimento Farmaco Chimico of the University of Bari, and the results were within 0.40% agreement of theoretical values. HPLC analyses were carried out using a Symmetry 5 μm C18 column (3.9 × 150 mm) from Waters (Milford, MA, USA), eluted at different mobile phase compositions of MeOH/H_2O mixtures. All HPLC measurements were carried out at 25 ± 1 °C with a flow rate of 1.0 mL min^{-1} at both 254 and 320 nm on a Waters HPLC 1525 multisolvent delivery system, equipped with a Waters 2487 variable wavelength UV detector. Chromatographic separations were performed on silica gel 60 for column chromatography (Merck 70–230 mesh or, alternatively, 15–40 mesh for flash chromatography). Several compounds were synthesized according to known procedures with slight modifications; their melting points and spectral data were in full agreement with those reported in literature, and no effort was made at this stage to optimize the yields. Unless otherwise stated, all chemicals and solvents were purchased from Sigma–Aldrich.

3-Bromopropyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (4): A solution of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (2.50 g, 6.40 mmol) and 3-bromo-1-propanol (0.87 mL, 9.61 mmol) in dry CH_2Cl_2 (32 mL) containing 4 Å molecular sieves (250 mg) was cooled to 0 °C, and $BF_3{\cdot}Et_2O$ (4.10 mL, 32.0 mmol) was added over 2 min. The mixture was stirred overnight, neutralized with NEt_3, and filtered. The resulting solution was washed with saturated $NaHCO_3$, H_2O, and brine, then dried (Na_2SO_4) and concentrated under reduced pressure to provide a residue which was purified by silica gel flash chromatography (EtOAc/hexane, 1:1 *v/v*), providing compound **4** as a white foam (1.58 g, 53%). Spectral data were in full agreement with those reported:[32] ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.21$ (t, $J = 9.5$ Hz, 1H), 5.07 (t, $J = 9.8$ Hz, 1H), 4.97 (dd, $J = 9.8$, 8.0 Hz, 1H), 4.51 (d, $J = 8.0$ Hz, 1H), 4.26 (dd, $J = 12.2$, 4.9 Hz, 1H), 4.14 (dd, $J = 12.2$, 2.4 Hz, 1H), 4.01–3.94 (m, 1H), 3.73–3.65 (m, 2H), 3.45 (t, $J = 5.2$ Hz, 2H), 2.15 (m, 2H), 2.09 (s, 3H), 2.06 (s, 3H), 2.01(s, 3H), 1.99 ppm (s, 3H).

3-{[5-(5-Chlorothiophen-2-yl)isoxazol-3-yl]methoxy}-2-nitrophenol (8): K_2CO_3 (196 mg, 1.42 mmol) was added to a stirred solution of 2-nitroresorcinol (2.00 g, 12.9 mmol) in dry DMF (14 mL). After 30 min, a solution of 3-(bromomethyl)-5-(5-chlorothiophen-2-yl)isoxazole **5** (333 mg, 1.29 mmol), prepared as previously reported,[11] in dry DMF (3 mL) was added dropwise in 1 h. The reaction mixture was stirred overnight at room temperature, diluted with H_2O, and extracted with EtOAc. The combined organic layers were sequentially washed with saturated Na_2CO_3 and brine, dried (Na_2SO_4), and concentrated under reduced pressure to afford a residue, which was purified by silica gel chromatography (EtOAc/cyclohexane, 1:1 *v/v*) to provide compound **8** as yellow solid (266 mg, 58%); mp:

152–154 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.12 (s, 1H), 7.41 (t, J = 8.3 Hz, 1H), 7.30 (d, J = 3.7 Hz, 1H), 6.95 (d, J = 3.9 Hz, 1H), 6.77 (dd, J = 8.3, 1.2 Hz, 1H), 6.64 (dd, J = 8.6, 1.2 Hz, 1H), 6.58 (s, 1H), 5.27 ppm (s, 2H); IR (KBr): $\tilde{\nu}$ = 3391, 3146, 3110, 1613, 1593, 1530, 1482, 1468, 1361, 1192, 1164, 1101, 797, 787 cm^{-1}; Anal. calcd for $C_{14}H_9ClN_2O_5S$: C 47.6, H 2.57, N 7.94, found: C 48.04, H 2.82, N 7.83.

3-(3-{[5-(5-Chlorothiophen-2-yl)isoxazol-3-yl]methoxy}-2-nitrophenoxy)propyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (9): K_2CO_3 (821 mg, 5.94 mmol) was added to a stirred solution of **8** (1.40 g, 2.83 mmol) in dry DMF (20 mL). After 30 min, a solution of 3-bromopropyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside **4** (1.46 g, 3.12 mmol) in dry DMF (10 mL) was added dropwise. The reaction mixture was stirred overnight at room temperature, diluted with H_2O, and extracted with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to provide a residue which was purified by silica gel flash chromatography (EtOAc/hexane, 1:1 *v/v*), to provide **9** as a white solid (2.03 g, 97%): mp: 84–86 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.34–7.28 (m, 2H), 6.95 (d, J = 4.0 Hz, 1H), 6.73 (d, J = 8.3 Hz, 1H), 6.67 (d, J = 8.3 Hz, 1H), 6.44 (s, 1H), 5.25 (s, 2H), 5.19 (t, J = 9.5 Hz, 1H), 5.06 (t, J = 9.5 Hz, 1H), 4.96 (dd, J = 9.5, 8.0 Hz, 1H), 4.49 (d, J = 8.0 Hz, 1H), 4.24 (dd, J = 12.2, 4.6 Hz, 1H), 4.15–4.05 (m, 3H), 4.00–3.93 (m, 1H), 3.72–3.64 (m, 2H), 2.10–1.87 (m, 2H), 2.06 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.75 ppm (s, 3H); IR (KBr): $\tilde{\nu}$ = 1754, 1535, 1479, 1370, 1268, 1228, 114, 1046, 807, 792 cm^{-1}; Anal. calcd for $C_{31}H_{33}ClN_2O_5S \times H_2O$: C 49.05, H 4.65, N 3.69, found: C 49.36, H 4.60, N 3.61.

3-(2-Amino-3-{[5-(5-chlorothiophen-2-yl)isoxazol-3-yl]methoxy}phenoxy)propyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (10): $SnCl_2 \cdot 2H_2O$ (1.01 g, 4.49 mmol) was added to a stirred solution of **9** (665 mg, 0.90 mmol) in EtOAc (15 mL). The mixture was stirred at reflux for 48 h, diluted with EtOAc, and washed four times with cold 1 N NaOH. The organic phase was washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by silica gel flash chromatography (CH_2Cl_2/EtOAc/cyclohexane, 7:2:1 *v/v/v*) provided **10** as a colorless amorphous solid (347 mg, 52%), which was used in the next step without further purification: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.26 (d, J = 4.0 Hz, 1H), 6.92 (d, J = 4.0 Hz, 1H) 6.66–6.49 (m, 3H), 6.46 (s, 1H), 5.18 (t, J = 9.2 Hz, 1H), 5.17 (s, 2H), 5.06 (t, J = 9.8 Hz, 1H), 4.98 (dd, J = 9.5, 8.0 Hz, 1H), 4.52 (d, J = 8.0 Hz, 1H), 4.24 (dd, J = 12.2, 4.6 Hz, 1H), 4.14–4.01 (m, 6H), 3.75–3.63 (m, 2H), 2.08–1.95 (m, 2H), 2.05 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H), 1.92 ppm (s, 3H).

3-(2-Amino-3-{[5-(5-chlorothien-2-yl)isoxazol-3-yl]methoxy}phenoxy)propyl β-D-glucopyranoside (11): MeONa (35 mg, 0.60 mmol) was added to a 0 °C solution of **10** (200 mg, 0.28 mmol) in MeOH (10 mL), and the reaction mixture was stirred overnight at room temperature. After concentration under reduced pressure, the obtained residue was purified by silica gel flash chromatography (CH_2Cl_2/MeOH, 9:1 *v/v*), providing compound **11** in good purity (> 95% by HPLC) as a pale yellow solid (130 mg, 85%): mp: 100–102 °C; ^{1}H NMR (300 MHz, D_2O): δ = 7.26 (d, J = 4.0 Hz, 1H), 6.92 (d, J = 4.0 Hz, 1H) 6.66–6.49 (m, 3H), 6.46 (s, 1H), 5.18 (t, J = 9.2 Hz, 1H), 5.17 (s, 2H), 5.06 (t, J = 9.8 Hz, 1H), 4.98 (dd, J = 9.5, 8.0 Hz, 1H), 4.52 (d, J = 8.0 Hz, 1H), 4.24 (dd, J = 12.2, 4.6 Hz, 1H), 4.14–4.01 (m, 6H), 3.75–3.63 (m, 2H), 2.08–1.95 ppm (m, 2H); IR (KBr): $\tilde{\nu}$ = 3300, 1601, 1225, 1118, 1060, 790 cm^{-1}.

3-(3-[1-(*tert*-Butoxycarbonyl)piperidine-4-carboxamido]-2-{[5-(5-chlorothiophen-2-yl)isoxazol-3-yl]methoxy}phenoxy)propyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (12): *N*-hydroxybenzotriazole hydrate (HOBt, 66 mg, 0.49 mmol) and *N,N'*-dicyclohexylcarbodiimide (DCC, 101 mg, 0.49 mmol) were added to a 0 °C solution of 1-(*tert*-butoxycarbonyl)piperidine-4-carboxylic acid (123 mg, 0.54 mmol) in THF (5 mL). Compound **10** (347 mg, 0.49 mmol) was added after 30 min of stirring. The reaction mixture was stirred for 48 h at room temperature, then the DCU was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was dissolved in EtOAc and sequentially washed with saturated 5% $NaHCO_3$, 1 N HCl, and brine. The organic phase was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Purification by silica gel flash chromatography (EtOAc/cyclohexane, 8:2 *v/v*) provided **12** as a white solid (180 mg, 40%), which was used in the next reaction without further purification: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.26 (d, J = 4.0 Hz, 1H), 7.14 (t, J = 8.6 Hz, 1H), 6.93 (d, J = 4.0 Hz, 1H), 6.82 (br, 1H), 6.63 (d, J = 8.3 Hz, 1H), 6.59 (d, J = 8.3 Hz, 1H), 6.51 (s, 1H), 5.18 (t, J = 9.2 Hz, 1H), 5.15 (s, 2H), 5.06 (t, J = 9.8 Hz, 1H), 4.97 (dd, J = 9.5, 8.0 Hz, 1H), 4.52 (d, J = 8.0 Hz, 1H), 4.23 (dd, J = 12.2, 4.3 Hz, 1H), 4.14–3.95 (m, 6H), 3.71–3.65 (m, 2H), 2.89–2.72 (m, 2H), 2.52–2.40 (m, 1H), 2.09–1.98 (m, 2H), 2.04 (s, 3H), 2.00 (s, 3H), 1.97 (s, 3H), 1.89 (s, 3H), 1.81–1.62 (m, 4H), 1.44 ppm (s, 9H); IR (KBr): $\tilde{\nu}$ = 1756, 1693, 1618, 1232, 1168, 1111, 1040, 903, 798 cm^{-1}.

3-(3-{[5-(5-Chlorothiophen-2-yl)isoxazol-3-yl]methoxy}-2-(piperidine-4-carboxamido)phenoxy)propyl β-D-glucopyranoside (14): Redistilled TFA (0.15 mL, 1.95 mmol) was added to a 0 °C solution of **12** (180 mg, 0.20 mmol) in CH_2Cl_2 (5 mL), and the mixture was stirred at room temperature for 3 h. After concentration under reduced pressure, the residue was partitioned between EtOAc and 1 N NaOH, and the aqueous layer was extracted twice with EtOAc. The combined organic layers were dried (Na_2SO_4), filtered, and concentrated under reduced pressure to provide the crude product **13** (150 mg, 0.18 mmol), which was cooled to 0 °C in MeOH (10 mL). MeONa (25 mg, 0.46 mmol) was added, and the mixture was stirred overnight at room temperature. After concentration under reduced pressure, the resulting residue was purified by silica gel flash chromatography (CH_2Cl_2/MeOH, 8:2 *v/v*), providing **14** as a white solid (100 mg, 83%): mp: 165–167 °C; ^{1}H NMR (300 MHz, D_2O): δ = 7.25 (d, J = 6.0 Hz, 1H), 7.10 (t, J = 8.3 Hz, 1H), 6.95 (d, J = 6.0 Hz, 1H), 6.80 (s, 1H), 6.76 (d, J = 8.3 Hz, 1H), 6.60 (d, J = 8.6 Hz, 1H), 5.10 (s, 2H), 4.85 (m, 1H), 4.07 (d, J = 7 Hz, 1H), 4.01 (t, J = 5.5 Hz, 2H), 3.92–3.85 (m, 4H), 3.64 (d, J = 11.3 Hz, 1H), 3.62–3.55 (m, 1H), 3.43 (dd, J = 11.3, 4.6 Hz, 1H), 3.14–2.99 (m, 1H), 2.92 (t, J = 7.5 Hz, 1H), 2.85–2.70 (m, 2H), 2.65–2.55 (m, 1H), 2.30–2.19 (m, 1H), 2.10–2.02 (m, 1H), 1.95–1.80 (m, 2H), 1.76–1.45 ppm (m, 2H); IR (KBr): $\tilde{\nu}$ = 3405, 3210, 1660, 1260, 1110, 1030, 790 cm^{-1}; MS (ESI+): *m/z*: 654 $[M+H]^+$; Anal. calcd for $C_{29}H_{38}ClN_3O_{10}S \times H_2O$: C 51.67, H 5.98, N 6.23, found: C 51.77, H 5.84, N 6.39.

3-(2-{[5-(5-Chlorothiophen-2-yl)isoxazol-3-yl]methoxy}-3-[1-(isopropyl)piperidine-4-carboxamido]phenoxy)propyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (15): Redistilled TFA (0.15 mL, 1.95 mmol) was added to a 0 °C solution of **12** (180 mg, 0.20 mmol) in CH_2Cl_2 (5 mL), and the mixture was stirred at room temperature for 3 h. After concentration under reduced pressure, the residue was dissolved in MeOH (5 mL) at 0 °C, and acetone (1 mL) and $Na(CN)BH_3$ (15 mg, 0.23 mmol) were added. The mixture was stirred at room temperature for 24 h, then concentrated under reduced pressure. The residue was partitioned between EtOAc and 1 N NaOH, and the aqueous layer was extracted twice with EtOAc. The combined organic phases were dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Purification by silica gel flash chromatography (EtOAc/MeOH, 9:1 *v/v*), provided **15** as a white solid (149 mg, 86%): mp: 154–156 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.25 (d, J = 3.7 Hz, 1H), 7.10 (t, J = 8.3 Hz, 1H), 6.90 (d,

J=3.7 Hz, 1H), 6.80 (br, 1H), 6.60 (d, J=8.6 Hz, 1H), 6.56 (d, J=8.6 Hz, 1H), 6.52 (s, 1H), 5.16 (t, J=9.8 Hz, 1H), 5.12 (s, 2H), 5.04 (t, J=9.5 Hz, 1H), 4.95 (dd, J=9.5, 8.0 Hz, 1H), 4.50 (d, J=8.0 Hz, 1H), 4.22 (dd, J=12.2, 4.3 Hz, 1H), 4.12–3.90 (m, 4H), 3.71–3.62 (m, 2H), 2.90–2.88 (m, 2H), 2.79–2.62 (m, 1H), 2.38–2.11 (m, 3H), 2.09–1.72 (m, 6H), 2.03 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.88 (s, 3H), 1.02 ppm (d, J=6.7 Hz, 6H); ^{13}C (300 MHz, [D$_6$]DMSO): δ=20.0 (2C), 22.3, 22.4 (2C), 22.5, 31.2 (2C), 50.1 (3C), 56.4, 63.6, 64.2, 66.6, 67.9, 70.2, 73.0, 73.5, 74.6, 100.8, 102.6, 107.5, 107.7, 116.9, 128.1, 129.0 (2C), 129.3 (2C), 129.4, 135.1, 155.8 (2C), 156.6, 165.2, 171.2 (2C), 171.9, 172.4 ppm; IR (KBr): $\tilde{\nu}$=2995, 1745, 1669, 1462, 1383, 1227, 1116, 1045, 790 cm^{-1}; MS (ESI+): m/z: 864 $[M+H]^+$; Anal. calcd for $C_{40}H_{50}ClN_3O_{14}S$: C 55.58, H 5.83, N 4.86, found: C 55.84, H 5.84, N 5.08.

3-(2-{[5-(5-Chlorothiophen-2-yl)isoxazol-3-yl]methoxy}-3-[(1-isopropyl)piperidine-4-carboxamido]phenoxy)propyl β-D-glucopyranoside (16): MeONa (20 mg, 0.37 mmol) was added to a 0 °C solution of **15** (149 mg, 0.172 mmol) in MeOH (5 mL), and the mixture was stirred overnight at room temperature. After concentration under reduced pressure, the resulting residue was purified by silica gel flash chromatography (CH_2Cl_2/MeOH, 9:1 *v/v*), providing **16** as a white solid (115 mg, 96%): mp: 164–166 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=8.72 (br, 1H), 7.55 (d, J=4.0 Hz, 1H), 7.30 (d, J=4.0 Hz, 1H), 7.16 (t, J=8.3 Hz, 1H), 6.86 (s, 1H), 6.76 (d, J=8.3 Hz, 1H), 6.70 (d, J=8.6 Hz, 1H), 5.14 (s, 2H), 4.93 (br, 4H), 4.10 (d, J=7.7 Hz, 1H), 4.01 (t, J=5.5 Hz, 2H), 3.91–3.84 (m, 1H), 3.64 (d, J=11.3 Hz, 1H), 3.62–3.55 (m, 1H), 3.43 (dd, J=11.3, 4.6 Hz, 1H), 3.14–2.99 (m, 3H), 2.92 (t, J=8.0 Hz, 1H), 2.79–2.70 (m, 2H), 2.65–2.57 (m, 1H), 2.38–2.19 (m, 1H), 2.09–2.02 (m, 2H), 1.96–1.85 (m, 2H), 1.76–1.49 (m, 4H), 0.92 ppm (d, J=6.4 Hz, 6H); ^{13}C (300 MHz, $CDCl_3$): δ=20.2 (2C), 31.3 (2C), 31.6, 50.1 (2C), 56.1, 63.3, 64.2, 67.5, 67.6, 72.2, 75.7, 78.9, 79.1, 102.2, 105.3, 108.3, 108.7, 118.2, 129.6 (2C), 129.7, 129.9, 130.8 (2C), 135.1, 156.8, 157.6 (2C), 165.9 ppm; IR (KBr): $\tilde{\nu}$=3405, 3210, 1661, 1601, 1262, 1109, 1029, 794 cm^{-1}; MS (ESI+): m/z: 696 $[M+H]^+$; Anal. calcd for $C_{32}H_{42}ClN_3O_{10}S\times0.5\ H_2O$: C 54.50, H 6.14, N 5.96, found: C 54.28, H 6.10, N 6.03.

Biological assays

Inhibition assays for factor Xa, thrombin, and other serine proteases: The test compounds were assayed in vitro for their inhibitory activity toward fXa, thrombin, and other serine proteases, determining the hydrolysis rates of the synthetic chromogenic substrates monitored at 405 nm. Enzymes and substrates were used as follows (final concentrations): 2 nM human factor Xa and 0.04 mM S-2765 (*Z*-D-Arg-Gly-Arg-*p*-NA) from Chromogenix AB-Instrumentation Laboratories (Milan, Italy); 0.41 U mL^{-1} bovine thrombin from Sigma–Aldrich (Milan, Italy) and 0.05 mM S-2238 (D-Phe-Pip-Arg-*p*-NA) from Chromogenix ABInstrumentation Laboratories; 30 nM human recombinant factor VIIa (reconstituted with 0.2 mL of deionized water) and 1 mM Spectrozyme fVIIa from American Diagnostics (Stamford, CT, USA); 0.4 μg mL^{-1} bovine α-chymotrypsin and 0.185 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-NA from Sigma–Aldrich; 2 nM bovine pancreas trypsin from Calbiochem (Darmstadt, Germany) and 4 mM S-2238; 5 nM human neutrophil leukocyte elastase and 0.66 mM *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val- *p*-NA from Sigma–Aldrich. All buffer salts were purchased from Sigma–Aldrich.

Enzyme solutions were incubated with DMSO solutions of the test inhibitors (DMSO did not exceed 1%) in various concentrations (0.1–100 nM or 0.1–100 μM), before the respective chromogenic substrates were added to initiate the enzyme kinetics. Kinetic studies were performed at pH 8 for fXa and thrombin, and at pH 7.5 for α-chymotrypsin, as previously reported.[33] Kinetics for recombinant human fVIIa, pancreas bovine trypsin, and human leukocyte elastase (200 mM Tris buffer,150 mM NaCl, and 0.1% PEG6000, pH 7.5), were performed by monitoring the absorbance increase at 405 nm and 25 °C with a microplate BioRad spectrophotometer. The enzyme solution (50 μL) and buffer (50 μL) were mixed with 2 μL of DMSO solution containing the test compound or DMSO alone as the control, and incubated (15 min for fVIIa, 30 min for trypsin, and 10 min for elastase). Reactions were initiated by adding 100 μL of substrate solutions, and the increase in absorbance was monitored for 5 min. Initial velocities were determined, and the concentrations of the inhibitors required to diminish the control velocity by 50% (IC_{50}) were calculated by nonlinear (sigmoidal) regression. At least three independent IC_{50} values were determined to calculate inhibition constants (K_i) using the Cheng–Prusoff equation.[26]

In vitro plasma clotting time assays: Clotting time(s) of the test compounds, namely prothrombin time (PT) and activated partial thromboplastin time (aPTT), were measured using a coagulometer (Behnk Electronic, Norderstedt, Germany) and compared with those from human control plasma. Pooled lyophilized human plasma (100 μL; Futura Systems, Formello, Rome, Italy) was incubated for 3 min at 37 °C with test compound solution (10 μL) or solvent (DMSO maximum of 1%), followed by the addition of PT reagent (200 μL), or aPTT reagent (100 μL) and 0.025 M $CaCl_2$ (100 μL; Futura Systems), to trigger clot formation. Each measurement was performed in triplicate, and the concentrations of test compound which caused twofold prolongation of the basal clotting times (PT_2 and $aPTT_2$) were calculated from each individual concentration–response curve.

Ex vivo anticoagulant assays in mice: All animal experiments were performed in agreement with the Italian law on animal care No. 116/1992 and the EEC/609/86, and all efforts were made to minimize the number of animals used. CD1 male mice (Harlan Laboratories) weighing 25–35 g were used. The mice were housed under standard laboratory conditions with open access to standard food and tap water. After overnight fasting, mice were anesthetized by intraperitoneal injection with urethane (1.2 g kg^{-1}), and test compounds **1** and **16**, suspended in a 0.5% methylcellulose solution, were administered orally at equimolar concentrations (0.11 mmol kg^{-1}) using a gastric tube (FTP-20-30, InstechLabs). At 1 h after inhibitor administration, blood (0.2 mL) was collected from the inferior vena cava into syringes containing sodium citrate (3.8% *v/v*). Platelet poor plasma (PPP) was then prepared by centrifugation for 20 min at 1200 *g* to measure PT. Data (mean ± SEM) were compared with those of the vehicle group. One way ANOVA and multiple comparison tests (Dunnett's test) were performed according to a standard statistical package. *P* values less than 0.05 were considered statistically significant.

Acknowledgements

C.A. and M.d.C. thank Prof. Raimondo De Cristofaro (Institute of Internal Medicine and Geriatrics, and Hemostasis Research Center, Catholic University School of Medicine, Rome, Italy) for use of laboratory devices and helpful discussions on enzymatic assay data pertaining to blood coagulation factors. C.A. acknowledges financial support from the Italian Ministry for Education Universities and Research (MIUR, Rome, Italy; PRIN 2007, grant no. 2007T9HTFB 003).

Keywords: factor Xa · glycosides · inhibitors · thrombin · thromboembolism

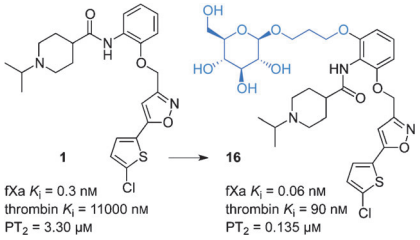
[1] D. J. P. Pinto, J. M. Smallheer, D. L. Cheney, R. M. Knabb, R. R. Wexler, *J. Med. Chem.* **2010**, *53*, 6243 – 6274.
[2] R. J. Leadley, Jr., *Curr. Top. Med. Chem.* **2001**, *1*, 151 – 159.
[3] L.-A. Linkins, *Annu. Rev. Med.* **2005**, *56*, 63 – 77.
[4] P. C. Wong, E. J. Crain, C. A. Watson, B. Xin, *J. Thromb. Haemostasis* **2009**, *7*, 1313 – 1320.
[5] N. H. Hauel, H. Nar, H. Priepke, U. Ries, J.-M. Stassen, W. Wienen, *J. Med. Chem.* **2002**, *45*, 1757 – 1766.
[6] M. de Candia, G. Lopopolo, C. Altomare, *Expert Opin. Ther. Pat.* **2009**, *19*, 1535 – 1580.
[7] I. Melnikova, *Nat. Rev. Drug Discovery* **2009**, *8*, 353 – 354.
[8] S. Maignan, V. Mikol, *Curr. Top. Med. Chem.* **2001**, *1*, 161 – 174.
[9] M. Nazaré, H. Matter, D. W. Will, M. Wagner, M. Urmann, J. Czech, H. Schreuder, A. Bauer, K. Ritter, V. Wehner, *Angew. Chem.* **2012**, *124*, 929 – 935; *Angew. Chem. Int. Ed.* **2012**, *51*, 905 – 911.
[10] L. M. Salonen, M. C. Holland, P. S. J. Kaib, W. Haap, J. Benz, J. L. Mary, O. Kuster, W. B. Schweizer, D. W. Banner, F. Diederich, *Chem. Eur. J.* **2012**, *18*, 213 – 222.
[11] G. Lopopolo, F. Fiorella, M. de Candia, O. Nicolotti, S. Martel, P. A. Carrupt, C. Altomare, *Eur. J. Pharm. Sci.* **2011**, *42*, 180 – 191.
[12] M. Nazaré, D. W. Will, H. Matter, H. Schreuder, K. Ritter, M. Urmann, M. Essrich, A. Bauer, M. Wagner, J. Czech, M. Lorenz, V. Laux, V. Wehner, *J. Med. Chem.* **2005**, *48*, 4511 – 4525.
[13] L. M. Salonen, C. Bucher, D. W. Banner, W. Haap, J.-L. Mary, J. Benz, O. Kuster, P. Seiler, W. B. Schweizer, F. Diederich, *Angew. Chem.* **2009**, *121*, 825 – 828; *Angew. Chem. Int. Ed.* **2009**, *48*, 811 – 814.
[14] S. Haas, *Vasa* **2009**, *38*, 13 – 29.
[15] M. Remko, *J. Mol. Struct.: THEOCHEM* **2009**, *916*, 76 – 85.
[16] Morphochem Chemie, Munich (Germany), US2008051688, **2008**.
[17] T. Fuchs, R. Eckl (Morphochem Chemie, Munich, Germany), WO/2006/008141, **2006**.
[18] F. Hirayama, J. Fujiyasu, D. Kaga, K. Negoro, D. Sasuga, N. Seki, K. Suzuki, (Astellas Pharma Inc., Tokyo, Japan), WO2007055183, **2007**.
[19] C. Rossi, S. Hess, R. W. Eckl, A. Di Lena, A. Bruno, O. Thomas, A. Poggi, *J. Thromb. Haemostasis* **2006**, *4*, 608 – 613.
[20] M. Wojtukiewicz, E. Sierko, W. Kisiel, *Semin. Thromb. Hemostasis* **2007**, *33*, 621 – 642.
[21] M. M. Abouzied, E. S. Crawford, H. A. Nabi, *J. Nucl. Med. Technol.* **2005**, *33*, 145 – 155.
[22] X. C. Nguyen, W. W. Lee, J. H. Chung, S. Y. Park, S. W. Sung, Y. K. Kim, Y. So, D. S. Lee, J. K. Chung, M. C. Lee, S. E. Kim, *Eur. J. Radiol.* **2007**, *62*, 214 – 219.
[23] J. Reinhard, W. E. Hull, C. W. von der Lieth, U. Eichhorn, H. C. Kliem, B. Kaina, M. Wiessler, *J. Med. Chem.* **2001**, *44*, 4050 – 4061.
[24] C. D. Young, A. S. Lewis, M. C. Rudolph, M. D. Ruehle, M. R. Jackman, U. J. Yun, O. Ilkun, R. Pereira, E. D. Abel, S. M. Anderson, *PLOS One* **2011**, *6*, e23205.
[25] Y.-S. Lin, R. Tungpradit, S. Sinchaikul, F. M. An, D. Z. Liu, S. Phutrakul, S. T. Chen, *J. Med. Chem.* **2008**, *51*, 7428 – 7441.
[26] Y. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099 – 3108.
[27] F. Cheng, R. Johnsson, J. Nilsson, L. Å. Fransson, U. Ellervik, K. Mani, *Cancer Lett.* **2009**, *273*, 148.
[28] T. Ishihara, N. Seki, F. Hirayama, M. Orita, H. Koshio, Y. Taniuchi, Y. Sakai-Moritani, Y. Iwatsuki, S. Kaku, T. Kawasaki, Y. Matsumoto, S. Tsukamoto, *Bioorg. Med. Chem.* **2007**, *15*, 4175 – 4192.
[29] L. R. Zacharski, *Cancer Lett.* **2002**, *186*, 1 – 9.
[30] J. T. Loynes, L. R. Zacharski, *Expert Opin. Ther. Targets* **2003**, *7*, 399 – 404.
[31] A. K. Kakkar, *Cancer Treat. Rev.* **2003**, *29*, 23 – 26.
[32] M. E. Jung, E. C. Yang, B. T. Vu, M. Kiankarimi, E. Spyrou, J. Kau, *J. Med. Chem.* **1999**, *42*, 3899 – 3909.
[33] M. de Candia, F. Liantonio, A. Carotti, R. De Cristofaro, C. Altomare, *J. Med. Chem.* **2009**, *52*, 1018 – 1028.

Received: April 30, 2012
Revised: July 11, 2012
Published online on ■■ ■■, 0000

*G. Lopopolo, M. de Candia, L. Panza, M. R. Romano, M. D. Lograno, F. Campagna, C. Altomare**

■■ – ■■

β-D-Glucosyl Conjugates of Highly Potent Inhibitors of Blood Coagulation Factor Xa Bearing 2-Chorothiophene as a P1 Motif



***O*-Glucosides of fXa inhibitors:** β-D-Glucose was conjugated through an ether-linked C3-alkyl spacer to the central phenyl ring of compound **1**, providing **16**, which showed picomolar inhibitory potency (K_i = 60 pM) against factor Xa (fXa), good selectivity over thrombin, sub-micromolar in vitro activity in the prothrombin time (PT) clotting assay, and a significant (1.6-fold) prolongation of the basal PT in an ex vivo assay in mice.

