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Allosteric Inhibition of Factor XIa. Sulfated Non-Saccharide Glycosaminoglycan Mimetics as Promising Anticoagulants

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Abstract

Recent development of sulfated non-saccharide glycosaminoglycan mimetics, especially sulfated pentagalloyl glucopyranoside (SPGG), as potent inhibitors of factor XIa (FXIa) (*J. Med. Chem.* 2013; 56:867–878 and *J. Med. Chem.* 2014; 57:4805–4818) has led to a strong possibility of developing a new line of factor XIa-based anticoagulants. In fact, SPGG represents the first synthetic, small molecule inhibitor that appears to bind in site remote from the active site. Considering that allosteric inhibition of FXIa is a new mechanism for developing a distinct line of anticoagulants, we have studied SPGG's interaction with FXIa with a goal of evaluating its pre-clinical relevance. Comparative inhibition studies with several glycosaminoglycans revealed the importance of SPGG's non-saccharide backbone. SPGG did not affect the activity of plasma kallikrein, activated protein C and factor XIIIa suggesting that SPGG-based anticoagulation is unlikely to affect other pathways connected with coagulation factors. SPGG's effect on APTT of citrated human plasma was also not dependent on antithrombin or heparin cofactor II. Interestingly, SPGG's anticoagulant potential was diminished by serum albumin as well as factor XI, while it could be reversed by protamine or polybrene, which implies possible avenues for developing antidote strategy. Studies with FXIa mutants indicated that SPGG engages Lys529, Arg530 and Arg532, but not Arg250, Lys252, Lys253 and Lys255. Finally, SPGG competes with unfractionated heparin, but not with polyphosphates and/or glycoprotein Iba, for binding to FXIa. These studies enhance understanding on the first allosteric inhibitor of FXIa and highlight its value as a promising anticoagulant.

Graphical abstract

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Author Contributions

RAAH performed experiments, analyzed data and contributed to writing the manuscript; DG contributed FXIa mutants and analyzed data; URD conceived and supervised the project, analyzed data and wrote the manuscript.

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SPGG2 :: R = -OH or -OSO₃Na
SPGG2-OH :: R = -H

Introduction

Current clinically approved anticoagulants including heparins, coumarins, hirudins, and synthetic peptidomimetics primarily target only two factors (thrombin and/or factor Xa) of the coagulation cascade [1, 2]. Despite their clinical success, each agent is associated with multiple adverse effects, especially major and/or minor bleeding [3, 4]. Other less desirable effects include osteoporosis, thrombocytopenia, inconsistent patient response, drug-drug and drug-food interactions, or lack of easy reversibility [5]. Of these, bleeding and, to a lesser extent, lack of an effective antidote to reverse excessive anticoagulation continue to challenge recently introduced target-specific anticoagulants such as fondaparinux, dabigatran, rivaroxaban, and apixaban, despite their overall improved safety profile in comparison to the older anticoagulants.

Several lines of evidence have suggested that factor XIa (FXIa) is a promising target to develop a new line of anticoagulants with potentially minimal risk of bleeding [6, 7]. For example, hemophilia C patients with congenital FXI deficiency are known to suffer minimal bleeding and severe deficiency of FXI has been resolved through the use of FXI concentrates [8–10]. Epidemiologic studies show that FXI-deficient patients are relatively less susceptible to venous thrombosis [11] and ischemic stroke [12], while on the flip side, excessive levels of FXIa enhanced risk for cardiovascular diseases in women [13]. Supporting these studies in humans, animal models of arterial, venous, and cerebral thrombosis have validated FXIa as an attractive drug target [14]. In fact, FXI-null mice do not develop clots in FeCl₃-induced carotid artery model while showing no effect on bleeding time [15]. Finally, FXIa inhibition appears to only interfere with the pathologic coagulation process (thrombosis) but not the physiologic process (hemostasis) [16, 17]. Finally, a recent publication on the efficacy and safety of a second-generation antisense oligonucleotide shows that the targeting FXI levels is at least noninferior, and most probably superior, to enoxaparin in patients undergoing total knee arthroplasty [18]. To develop a new line of anticoagulants, we recently introduced a group of non-saccharide glycosaminoglycan

mimetics (NSGMs) that bind to coagulation factors and induce inhibition. A novel NSGM discovered in the process includes sulfated *pentagalloyl* β -D-glucopyranoside (SPGG, Figure 1), which potently inhibited FXIa [6,7]. Structurally, SPGG is a unique NSGM containing multiple sulfate groups on an aromatic scaffold. The fundamental principle used in the design of SPGG was that an appropriate anionic scaffold will target the highly electropositive heparin-binding site(s) of FXIa resulting in allosteric dysfunction of its catalytic function. Screening a small library of sulfated heparin mimetics led to the discovery of SPGG, the first synthetic, small allosteric inhibitor of human FXIa with a promising plasma and whole blood anticoagulant potential [6, 7]. No small molecule anticoagulant used in the clinic today is an allosteric inhibitor of coagulation, which implies that not much is known about this mechanism. Thus, it is important to understand the molecular interaction of SPGG with FXIa and other plasma proteins as well as evaluate the nature of allosteric inhibition by SPGG. Hence, we sought to answer some key questions: Is the aromatic scaffold of SPGG important for FXIa targeting? Can FXIa inhibition by SPGG be reversed? Which FXIa residues are engaged by SPGG? Does SPGG modulate FXIa interaction with known endogenous ligands? In this work, we have addressed these important questions to improve our understanding of the novel allosteric mechanism of FXIa inhibition by SPGG. Our results advance the promise of SPGG as a relatively specific and potentially safe anticoagulant. The studies also aid the design of effective strategies to be evaluated in animal models of bleeding arising from excessive anticoagulation.

Experimental Procedures

Chemicals and proteins

Two versions of SPGG, SPGG2 and SPGG2-OH (Figure 1), were chemically synthesized and purified as reported earlier [6,7]. The structural characterization of SPGG2 and SPGG2-OH was performed using NMR and reverse phase ion-pairing UPLC-MS. Bovine unfractionated heparin (UFH), chondroitin sulfate A (CSA), B (dermatan sulfate (DS)), and chondroitin sulfate C (CSC) were purchased from Sigma-Aldrich (St. Louis, MO). LMWH (enoxaparin sodium) was from Sandoz Inc., whereas fondaparinux was from GlaxoSmithKline. Sucrose octasulfate (SOS) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyprene, protamine sulfate, and bovine serum albumin (BSA) were from Sigma and used as received. *N,N*-dimethylcasein, dansyl-cadaverine, dithiothreitol (DTT), polyphosphate-45 (polyP45) were from Sigma-Aldrich (St. Louis, MO). Polyphosphate-700 (polyP700) was a gift from Dr. James Morrissey of University of Illinois. Glycoprotein Iba (GPIba) was from R&D systems (Minneapolis, MN).

Human FXIa, FXI, FXIIIa and APC were obtained from Haematologic Technologies (Essex Junction, VT). Human plasma kallikrein was purchased from Sigma (St. Louis, MO). Stock solutions of FXIa and FXI were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 0.1% PEG8000, and 0.02% Tween80; that of FXIIIa was prepared in 50 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, 1 mM CaCl₂, and 2 mg/mL *N,N*-dimethyl-casein; that of APC was prepared in 50 mM Tris-HCl buffer, pH 8.0, containing 125 mM NaCl and 10 mM CaCl₂; and that of kallikrein was prepared in 20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 2 mM CaCl₂ and 0.4% BSA [19–21]. FXIa chromogenic substrate (S-2366, L-

PyroGlu-Pro-Arg-*p*-NA) was obtained from Diapharma (West Chester, OH). Spectrozyme PK (*H*-D-Pro-hexahydrotyrosyl-Arg-*p*-NA) and Spectrozyme PCa (*H*-D-(γ -carboxybenzoxyl)-Lys-Pro-Arg-*p*-NA) were purchased from American Diagnostica (Greenwich, CT). FXIa catalytic domain (rFXIa-CD_{WT}), and its recombinant mutants rFXIa-CD_{K529A}, rFXIa-CD_{R530A}, and rFXIa-CD_{R532A} were gifts from Dr. Alireza Rezaie of Saint Louis University and recombinant FXIa wild type (rFXIa-WT) and its anion-binding site (ABS) mutants (rFXIa_{ABS1} and rFXIa_{ABS2}, respectively) were prepared in the Gailani laboratory (Vanderbilt University), as reported previously [22].

Pooled normal human plasma for coagulation assays was purchased from Valley Biomedical (Winchester, VA). Thromboplastin-D and activated partial thromboplastin time reagent containing ellagic acid (APTT-LS) were obtained from Fisher Diagnostics (Middletown, VA). AT/HC II deficient plasma was from Affinity Biologicals Inc. (Ancaster, ON), whereas FXI deficient plasma was from Haematologic Technologies (Essex Junction, VT).

Direct inhibition of human plasma FXIa and recombinant FXIa mutants

Direct inhibition of FXIa was measured using chromogenic substrate hydrolysis assay at 37 °C, as reported earlier [6, 7]. In this assay, each well of a 96-well microplate generally contained 85 μ L pH 7.4 buffer to which 5 μ L potential inhibitor (or reference) and 5 μ L plasma or recombinant FXIa (~0.765 nM final concentration) were sequentially added. After incubation, 5 μ L FXIa substrate (345 μ M final concentration) was rapidly added and the residual FXIa activity was measured from the initial rate of increase in absorbance at 405 nm. The relative residual enzyme activity at each concentration of the inhibitor was calculated from the ratio of enzyme activity in the presence and absence of the inhibitor. Logistic Eq. 1 was used to fit the dose-dependence of residual protease activity to obtain the potency (IC_{50}) and efficacy (Y) of inhibition. In this equation, Y is the ratio of residual enzyme activity in the presence of inhibitor to that in its absence (fractional residual activity), Y_M and Y_0 are the maximum and minimum possible values of the fractional residual protease activity, IC_{50} is the concentration of the inhibitor that results in 50% inhibition of enzyme activity, and HS is the Hill slope. Nonlinear curve fitting resulted in Y_M , Y_0 , IC_{50} and HS values.

$$Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(\log[I]_0 - \log IC_{50})(HS)}} \quad (1)$$

Direct inhibition of plasma kallikrein, APC and FXIlla

Direct inhibition of plasma kallikrein and APC was studied using the substrate hydrolysis assay in a 96-well microplate format adapted from literature [19]. In these assays, 5 μ L of SPGG2 was diluted with 85 μ L of an appropriate buffer at 37 °C followed by addition of 5 μ L of the protease solution and incubation for 5 min. The activity of the protease was evaluated by addition of 5 μ L of a chromogenic substrate and measuring the initial rate of increase in absorbance at 405 nm. The buffers used in these studies included 20 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, and 0.1% PEG8000, pH 7.5, at 37 °C for kallikrein and 50 mM Tris-HCl, 125 mM NaCl, and 10 mM CaCl₂, pH 8.0, at 37 °C for APC. The enzyme

concentrations were 0.005 U/mL (2.3 nM) for kallikrein and 7 nM for APC. The chromogenic substrates concentrations were 100 μ M Spectrozyme PK for kallikrein and 200 μ M Spectrozyme PCa for APC. For FXIIIa assay, a fluorescence-based trans-glutaminase assay was performed, as reported in literature [20]. Briefly, 1 μ L of SPGG2 was diluted with 87 μ L of pH 7.4 buffer (50 mM Tris-HCl, 1mM CaCl₂, 100 mM NaCl, and 2 mg/mL *N,N*-dimethylcasein) and 5 μ L DTT (20 mM) at 37 °C followed by addition of 2 μ L of FXIIIa (0.3 μ M) and incubation for 10 min. The activity of FXIIIa was evaluated by addition of 5 μ L of dansylcadaverine (2 mM) and measuring the initial rate of increase in fluorescence emission ($\lambda_{\text{Ex.}}$ = 360 nm and $\lambda_{\text{Em.}}$ = 490 nm). The dose dependence of the fractional residual enzyme activity at each concentration of SPGG2 was analyzed using Eq. 1 to obtain the apparent concentration of SPGG2 required to reduce enzyme activity to 50% of its initial value. Same assays were used to evaluate the specificity of SPGG2-OH. The inhibitor-to-enzyme ratio in these assays was greater than 14,000:1 (14,577:1 for SPGG2 and 75,947:1 for SPGG2-OH).

PT and APTT studies

To confirm the major contribution of FXIa inhibition by SPGG2 to their overall anticoagulant activity in human plasma and to assess whether AT and/or HC II-mediated inhibition of serine proteases of coagulation cascade contributes to this activity; its effect on clotting was assessed as reported earlier [6,7]. Clotting time was measured in a standard one-stage recalcification assay with a BBL Fibrosystem fibrometer (Becton-Dickinson, Sparks, MD). For prothrombin time (PT) assays, thromboplastin-D was reconstituted according to the manufacturer's directions and warmed to 37 °C. A 10 μ L of SPGG2 aqueous solution, to give the desired concentration, was brought up to 100 μ L with citrated human plasma, incubated for 30 s at 37°C followed by addition of 200 μ L of prewarmed thromboplastin-D and time to clot was measured. For the activated partial thromboplastin time (APTT) assay, a 10 μ L of SPGG2 aqueous solution was mixed with 90 μ L of citrated human plasma and 100 μ L of prewarmed APTT reagent (0.2% ellagic acid). After incubation for 240 s at 37 °C, clotting was initiated by adding 100 μ L of prewarmed 25 mM CaCl₂ and time to clot was determined. The data were fit to a quadratic trend line, which was used to determine the concentration of SPGG2 necessary to double the clotting time. Effect of SPGG2 on APTT using FXI-deficient human plasma or AT/HC II-deficient human plasma was studied in a similar fashion. Clotting time in the absence of an anticoagulant was determined in a similar fashion using 10 μ L of deionized water and was found to be 18.3 s (CI: 13.2–23.3) for PT and 63 s (CI: 58.6–68.2) for APTT in case of normal human plasma, 60.2 s for APTT using AT/HC II deficient plasma, and 180.8 s for APTT using FXI-deficient plasma.

Reversibility studies

To assess the *in vitro* reversibility of inhibitory effects of SPGG2, the FXIa restored activity profiles were established using an increasing concentration of polyprene, protamine sulfate, or SOS in the presence of two concentrations of SPGG2 (5 and 250 μ g/mL), which inhibit FXIa approximately 100%. The measurements were performed by a chromogenic substrate hydrolysis assay using a microplate reader (FlexStation III, Molecular Devices) at physiological conditions of pH 7.4 and 37 °C. Generally, each well of the 96-well

microplate contained 80 μL of 50 mM Tris-HCl buffer of pH 7.4 containing 150 mM NaCl, 0.02% Tween80 and 0.1% PEG8000 to which 5 μL of SPGG2 (or reference), 5 μL of FXIa (0.765 nM), and 5 μL of potential reversing agent were sequentially added. After a 10 min incubation, 5 μL of FXIa substrate S-2366 (345 μM) was rapidly added and the restored FXIa activity was measured from the initial rate of increase in absorbance at 405 nm. Stocks of potential reversing agents were serially diluted to give 12 different aliquots in the wells. SOS was used in the range of 0–4.6 mg/mL (stock was 0–92 mg/mL) whereas 0–1 mg/mL (stock was 0–20 mg/mL) concentrations were exploited for protamine sulfate or polybrene. Relative restored FXIa activity at each concentration of the reversing agent was calculated from the ratio of FXIa activity in the presence and absence of the reversing agent. Logistic Eq. 2 was used to fit the dose dependence of restored protease activity to obtain the effective concentration of reversing agent required to restore 50% of enzyme activity at specific inhibitor concentration (EC_{50}) and the efficacy (Y) of reversing process.

$$Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(\log[R]_0 - \log EC_{50})(HS)}} \quad (2)$$

Studies with Serum Albumin and Zymogen FXI

The SPGG2-mediated inhibition of FXIa was also assessed in the presence of different concentrations of BSA (0, 10, 20, and 40 mg/mL) as well as FXI (0, 113, 225, and 1125 nM). The inhibition profiles of FXIa by varying levels of SPGG2 in the presence of different concentrations of either BSA or FXI were determined as described above in the inhibition study and the corresponding IC_{50} values were calculated using logistic Eq.1.

Competitive inhibition studies

Competitive studies were performed essentially as described above, except for the presence of polyphosphate-45 (polyP45), polyphosphate-700 (polyP700), or glycoprotein Iba (GPIba). A 5 μL solution of SPGG2 (0–1.5 mg/mL) and 5 μL of FXIa (0.765 nM) with 5 μL of polyphosphate (0–100 μM) or GPIba (0–0.50 μM) in 80 μL of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, and 0.1% PEG8000 was incubated at 37 °C. Following incubation, 5 μL of FXIa substrate (345 μM) was added and the initial change in absorbance at 405 nm was measured. The dose dependence of the fractional residual FXIa activity at each concentration of the potential competitor was fitted using Eq. 1 to obtain the apparent concentration of SPGG2 required to reduce FXIa activity to 50% of its initial value.

Results

The aromatic scaffold of SPGG is essential for potent and selective inhibition of FXIa

We synthesized SPGG2, a pentagalloyl glucopyranoside preparation predominantly made up of decasulfated SPGG species, as described in our earlier work [6, 7]. This analog contains five pentagalloyl moieties, each of which possesses on an average two sulfate groups, located at the *meta*-positions of the galloyl moiety (Figure 1). Our earlier thermodynamic studies indicated that the anionic sulfate groups are essential for potent inhibition of FXIa. Yet, nearly 89% of binding energy of SPGG2 arose from non-ionic forces [7]. To clarify whether

other sulfated scaffolds can exhibit FXIa inhibition, we studied sulfated glycosaminoglycans (GAGs) including UFH, enoxaparin, fondaparinux, sucrose octasulfate (SOS), chondroitin sulfate A (CSA), dermatan sulfate (DS), and chondroitin sulfate C (CSC). Each of these is highly sulfated molecules containing a non-aromatic, saccharide backbone. This contrasts with SPGG's highly sulfated, aromatic ring-containing saccharide backbone. The library of GAG analogs represents a considerable diversity in the chain length (di- to poly-saccharide), molecular weight (600 – 15000 Da), sulfation level (2 to 8 sulfates per repeating unit) and the overall saccharide scaffold. In addition, we studied a SPGG2 analog, named SPGG2-OH here (Figure 1), in which the *para*-phenolic group was replaced with a hydrogen atom.

A chromogenic substrate hydrolysis assay was used to evaluate the inhibition of FXIa under physiologically relevant conditions (pH 7.4, 150 mM NaCl, and 37 °C), as reported earlier [6, 7]. Table 1 shows the results obtained in inhibition studies. None of the GAGs inhibited FXIa at the highest concentrations tested (>50 μ M). In contrast, SPGG2 and SPGG2-OH inhibited FXIa with IC_{50} s of 0.50 ± 0.02 and 1.4 ± 0.1 μ M, respectively. This suggests that although the two SPGGs contain a core saccharide (glucose), the aromatic units are essential for FXIa inhibition.

The selectivity of SPGG2 for FXIa inhibition was studied earlier and found to be greater than 200-fold over a broad spectrum of serine proteases including factors IIa, VIIa/tissue factor, IXa, Xa, and XIIa [6]. Yet, to better assess its clinical promise, it is important to study its effect on plasma proteases that play other key roles of relevance to coagulation. FXIIIa, a transglutaminase, catalyzes the covalent crosslinking of fibrin polymers and contributes to the stability of a growing thrombus [23]. Excessive inhibition of FXIIIa is likely to manifest as bleeding [24], which is not desirable in an agent targeting FXIa. Likewise, APC, a key serine protease of the protein C pathway, inactivates factor VIIIa and factor Va [25]. Thus, APC inhibition can depress the procoagulation signal, which can also be detrimental for FXIa inhibitors attempting to maintain the normal hemostasis potential. Finally, plasma kallikrein, a coagulation cascade protease, is also a key component of kinin – kallikrein system that regulates inflammation and bradykinin formation [26]. Thus, we studied the two SPGGs' activity against plasma kallikrein, APC, and FXIIIa to expand the characterization of its selectivity for FXIa. A fluorescence-based conjugation assay was used to evaluate the activity of SPGG2 and SPGG2-OH against FXIIIa, while UV-based chromogenic substrate hydrolysis assays were used to study its activity against plasma kallikrein and APC. Figure 2 shows the comparative profiles, which demonstrate that the two SPGGs were at least 200–300-fold selective against human FXIa in comparison to plasma kallikrein, APC, and FXIIIa. Thus, SPGGs most probably will not affect fibrin cross-coupling, reduce APC-driven clotting, or modify plasma kallikrein activity.

Finally, selectivity studies were also performed to assess whether the presence of two anticoagulant serpins, including AT and HCII, which are known to possess heparin-binding sites [27, 28] and may competitively affect the potency of SPGGs. To assess this, we resorted to human plasma clotting time assays (APTT and PT) in the presence and absence of the two serpins. The concentrations of SPGG2 required to double APTT of normal pooled plasma was measured to be 45 ± 3 μ M (Table 2). For human plasmas deficient in both AT

and HCII, a 2-fold increase in APTT required $40 \pm 6 \mu\text{M}$ SPGG2 (Table 2) suggesting the anticoagulant potency of SPGG2 remains essentially unaffected by the absence of two key serpins. By comparison, a 3-fold increase in SPGG2 level to $114 \pm 8 \mu\text{M}$ is necessary to induce anticoagulation in plasma deficient of FXI (Table 2). The observation that SPGG2 is active despite the deficiency of FXI suggests that at high enough concentrations, it can target other coagulation factors, as shown earlier [6]. With regard to selectivity of factors being targeted, we found that $373 \pm 52 \mu\text{M}$ SPGG2 is required to double the PT of normal pooled human plasma (Table 2), a value nearly 8 times higher than that for APTT. Thus, the plasma clotting time studies indicate that the anticoagulant activity of SPGG2 arises primarily from inhibition of the intrinsic coagulation pathway and does not involve two key heparin-binding serpins. These results further support the selectivity profile of this class of novel allosteric inhibitors against FXIa.

FXIa inhibition by SPGG can be reversed

A key aspect for developing new agents is reversibility of anticoagulation. To assess whether FXIa inhibition by SPGG2 can be reversed, we studied protamine, polybrene and SOS. Protamine is a clinically used arginine-rich polypeptide that counteracts the anticoagulant activity of UFH and LMWHs [29], whereas polybrene is a quaternary amine-containing hydrophobic polymer, which has been explored as a probe of electrostatic interactions [30]. SOS is an FDA approved antacid that is known to bind in heparin-binding sites on proteins such as thrombin [31]. FXIa was first treated with saturating concentrations of SPGG2 (5 and $250 \mu\text{g/mL}$) and the recovery of FXIa activity by the potential antidote studied spectrophotometrically through hydrolysis of FXIa substrate (S-2366) at pH 7.4 and 37°C (Figure 3). The efficacy and the effective concentration of the potential antidote to restore 50% of enzyme activity (EC_{50}) were calculated and are reported in Table 3.

At the highest concentration of SOS tested (4.6 mg/mL), SPGG2 inhibition of FXIa remained unaffected. By contrast, SPGG2 ($5.0 \mu\text{g/mL}$) inhibition of FXIa could be reversed ~30% by protamine sulfate ($0\text{--}1.0 \text{ mg/mL}$) and ~90% by polybrene ($0\text{--}1.0 \text{ mg/mL}$). The EC_{50} was calculated to be $16 \mu\text{g/mL}$ for protamine and $4.0 \mu\text{g/mL}$ for polybrene (Table 3). Essentially similar results were obtained for reversing FXIa inhibition at a higher level of SPGG2 ($250 \mu\text{g/mL}$). In this case, FXIa activity recovered was ~40% ($EC_{50} = 107 \mu\text{g/mL}$) and 76% ($EC_{50} = 15 \mu\text{g/mL}$) in presence of protamine and polybrene, respectively.

We also considered two important proteins that can potentially affect SPGG2 inhibition of FXIa, serum albumin and zymogen FXI. Serum albumin is a major component of plasma and is well known to nonspecifically bind to a wide variety of drugs, including negatively charged molecules such as the heparin. In fact, a specific site on bovine serum albumin (BSA), which is rich in arginine and lysine, has been identified as the heparin-binding site [32]. Thus, we studied BSA as a surrogate for human serum albumin in reversing SPGG2 action. The effect of BSA on SPGG2 inhibition of FXIa was studied by measuring the IC_{50} at three different concentrations of the antidote using the corresponding chromogenic substrate hydrolysis assay (data not shown). Table 4 displays the change in inhibition parameters at varying BSA concentrations. The IC_{50} of FXIa inhibition increased from $1.0 \pm 0.1 \mu\text{g/mL}$ to $1684 \pm 390 \mu\text{g/mL}$ as the BSA concentration increased from 0 to 40 mg/mL .

This indicates that the potency of SPGG2 decreased significantly in the presence of BSA, which most probably sequesters a substantial fraction of the allosteric inhibitor. In an alternative experiment, we incubated 1.5 mg/mL SPGG2 with varying levels of BSA and studied recovery of FXIa activity. The residual FXIa activity was found to increase from ~0% in the absence of BSA to 33, 44, and 62% as the concentration of BSA was raised to 10, 20, and 40 mg/mL, respectively (Figure 4). This suggested that SPGG2 inhibition of FXIa is reduced by levels of BSA typically found in bovine plasma. Equivalent results are expected for human serum albumin. It is important to note that binding to serum albumin should not be assumed as problematic. Binding to serum albumin may also extend the duration of action of a drug by serving as a repository of the molecule from which the free and active form of the anticoagulant becomes available at a defined rate. Thus, serum albumin binding of SPGG2 may turn out to be an advantage, although these studies remain to be performed.

We considered the zymogen FXI because our earlier fluorescence studies have shown that SPGG2 binds to FXI with an affinity almost equal to that of FXIs [7]. To assess the impact of this interaction, we measured the IC_{50} of SPGG2 against FXIa in presence of increasing concentrations of FXI. Figure 5 shows the change in dose–response profiles of SPGG2 inhibiting FXIa in the presence of 0, 113, 225, and 1125 nM of FXI at pH 7.4 and 37 °C. The IC_{50} of FXIa inhibition increased about 300-fold from 0.9 μ g/mL to 273 μ g/mL as FXI concentration increased from 0 to 1125 nM and the inhibition efficacy decreased from 98 to 75 (Table 4). Thus, SPGG2 inhibition of FXIa could also be diminished by FXI.

SPGG2 interacts with Lys529, Arg530 and Arg532 residues of the catalytic domain of FXIa

SPGG was originally designed as a sulfated NSGM and therefore, predicted to bind either in the A3 domain containing Arg250, Lys252, Lys253 and Lys255, and/or in the catalytic domain containing Lys529, Arg530 and Arg532 [33–36]. To identify whether SPGG2 engages either or both of these heparin-binding sites, we studied inhibition of several FXIa mutants, which were readily available from laboratories working on FXIa. These included three recombinant FXIa catalytic domains (CDs) containing Lys529, Arg530 or Arg532 replaced with Ala and two recombinant, full-length FXIa containing block replacement of all basic residues of the A3 domain (Arg250, Lys252, Lys253 and Lys255) or the catalytic domain (Lys529, Arg530 and Arg532) with Ala. Corresponding recombinant FXIa catalytic domain (rFXIa–CD) and recombinant, full-length FXIa (rFXIa–FL) were also studied as reference proteases.

Table 5 lists the IC_{50} s of SPGG2 toward the four CD variants measured using the chromogenic substrate hydrolysis assay. rFXIa–CD was inhibited by SPGG2 with an IC_{50} of 1.2 μ g/mL similar to that of purified, full-length FXIa alluding to idea that most probably the site of binding of SPGG2 is in the catalytic domain. Single point mutations at Lys529, Arg530 or Arg532 in the CD resulted in IC_{50} s of 1.7, 2.3, and 1.5 μ g/mL, respectively, without any effect on HS or Y. This represents a 1.5–2.5-fold decrease in the potency of SPGG2 against each single point mutant relative to the wild type CD.

Although the individual defects are small suggesting less likelihood of engaging these residues, we predicted that SPGG2 may actually be simultaneously engaging more than one

residue, which prevents expression of a larger defect. This was tested by studying block mutants. In comparison to the corresponding full-length, recombinant FXIa (9.9 µg/mL), the rFXIa-ABS⁻² containing all three basic CD residues (Lys529Ala, Arg530Ala, and Arg532Ala) was inhibited by SPGG2 with an IC_{50} of 69 µg/mL (Figure 6, Table 5). This implies a significant >7-fold decrease in the potency of SPGG2. Additionally, rFXIa-ABS⁻² displayed an inhibition of only 48 %, which almost half of that for recombinant, full-length protease rFXIa-FL. In contrast, rFXIa-ABS⁻¹, in which the heparin-binding site present on the A3 domain was block mutated (Arg250, Lys252, Lys253, and Lys255), SPGG2 actually displayed a more potent inhibition with an IC_{50} of 4.5 µg/mL. The 2-fold increase in the SPGG2 potency for this variant most probably arises from non-specific interaction of SPGG2 with the hydrophobic patch generated by substituting 4 positively charged residues with 4 alanines in the ABS1 site of the A3 domain. Thus overall, the results indicate that the primary binding site of SPGG2 is the ABS2 of the catalytic domain of human FXIa.

Polyphosphates or GPIIb do not compete with SPGG2 binding to FXIa

As described above, SPGG2 engages basic residues on the catalytic domain [6, 7] and does not interact with the residues of A3 domain. Likewise, SPGG2 also binds to the zymogen FXI with an affinity similar to FXIa. Most probably the site of binding on FXI is likely to be identical to that on FXIa. The heparin-binding sites on both FXIa and FXI play important roles in biology. FXI is activated to FXIa by FXIIa [37], thrombin [38–41], or by pre-formed FXIa [41] in reactions that are enhanced by inorganic polyphosphates (polyP) secreted by platelets [38, 42–44]. Recently, Geng *et al.* revealed that ABSs in both the A3 domain and the CD are required for the procoagulant activity of polyP during FXI activation [22]. Likewise, FXI and FXIa bind to GPIIb on the surface of activated platelets [45–49]. FXI recognizes GPIIb through its A3 domain [45–48], whereas FXIa appears to bind GPIIb by a site near the ABS2 of its catalytic domain [49]. Considering the importance of polyP and GPIIb to FXI/FXIa biochemistry, we studied the effect of SPGG2 on FXIa in the presence of polyP or GPIIb. Competition with such macromolecules may confound the therapeutic application of a promising agent such as SPGG2 and it is important to understand the molecular interactions, if any.

We studied SPGG2 inhibition of human plasma FXIa in presence of GPIIb, polyP45 and polyP700. The latter two polyphosphates are low and high molecular weight variants of the polymer, respectively. Considering the known binding constants for polyP45 and polyP700 to FXIa under similar conditions [45, 50], we used two 5 (polyP45) and 100 µM (polyP45, polyP700) concentrations of the two species, and three concentrations of GPIIb (0.05, 0.25, and 0.50 µM). For comparison, competition with UFH was also studied. The IC_{50} 's were measured using chromogenic substrate hydrolysis assay under physiologically relevant conditions (Table 6).

As the concentration of UFH increased from 0 to 180 µM, the IC_{50} increased from 1.0 to 34 µg/mL suggesting substantial competition, which is consistent with the observation that SPGG2 binds to or in the vicinity of the ABS2 in the CD. In contrast, the IC_{50} s of SPGG2 inhibition of plasma FXIa in the presence of the two variants of polyP was virtually invariant at 1.1–1.3 µg/mL and not significantly different from that observed in their absence

(0.9 µg/mL). This suggested that SPGG2 does not interfere with polyP binding to either ABS1 or ABS2 of FXIa. This implies that SPGG2 is not expected to affect the polyP-mediated FXI activation. For GPIIb α competition, the IC_{50} of FXIa inhibition marginally increased from 1.0 to 2.8 µg/mL as the concentration of the competitor increased from 0 to 0.5 µM. This suggested a weak competition between SPGG2 and GPIIb α . It has been suggested that GPIIb α recognizes a site near the ABS2 on the CD of FXIa, which lend support to the observation of weak competition. However, the two molecules do not appear to be competing for the same residues on the CD. Yet, the impact of such weak competition requires further studies. Overall, these competition studies support the proposed binding site of SPGG2 in the ABS2 of FXI catalytic domain and show essentially no effect of SPGG2 on the polyP-mediated FXI zymogen activation process.

Discussion

This work shows that SPGGs, especially SPGG2 and SPGG2-OH, represent a promising class of direct FXIa inhibitors with excellent potency and selectivity. SPGGs are also interesting because of their allosteric mechanism of action. Although allosteric inhibition has not really been studied with regard to anticoagulation, nature tends to exploit allosterism to confer specific regulation and recognition [51]. Allosterism typically offers two advantages: 1) a higher level of specificity and 2) regulation of intrinsic efficiency (k_{CAT}/K_M) [52–54]. With respect to catalytic efficiency, allosterism relies on energetic coupling between the site of binding and the catalytic site. Theoretically, such coupling may not be most optimal resulting in variable modulation of catalytic efficiency. In contrast, orthosterism, or competitive inhibition, does not involve modulation of catalytic efficiency. Thus, whereas dose is the only parameter for modulation of physiologic/pathologic response for orthosteric molecules, dose and catalytic efficiency are two parameters available for modulation of response by allosteric molecules. SPGGs display catalytic efficiency of 100%, which limits its modulation characteristics, but these first-of-a-kind molecules may yield novel allosteric anticoagulants that present variable catalytic efficiencies in the future.

With respect to selectivity, orthosterism, such as that demonstrated by all direct and indirect anticoagulants (except warfarin), is typically beset with similar catalytic sites, e.g., trypsin-like proteases. In contrast, allosterism is likely to possess higher selectivity of action because allosteric sites are less conserved [52–54]. It appears that SPGG2's allosteric mechanism of action is the reason for its selectivity observed against thrombin, factor Xa, factor VIIa/tissue factor, factor IXa, factor XIIa, trypsin, chymotrypsin, plasma kallikrein, APC, and FXIIIa. In addition, AT and HCII also do not appear to affect its potency.

In this study, we have identified that the ABS2 present on FXIa's catalytic domain is the most likely binding site for SPGG2. Although we have not performed an extensive mutagenesis studies, especially with regard to other domains (A1, A2, and A4), yet our previous studies, using full-length wild type FXIa and recombinant FXIa catalytic domain [6,7], had shown that SPGG most probably targets the heparin-binding site present on the catalytic domain of FXIa. Current results with multi-site recombinant FXIa mutants confirm this prediction and identify few residues including Lys529, Arg530 and Arg532 to

contribute to SPGG2 recognition. This site is known to be involved in heparin's allosteric modulation of the FXIa's function [33–36].

This heparin-binding site has also been implicated in two other important interactions that play important roles in FXIa's biochemistry. 1) Inorganic polyphosphates function as physiologic pro-coagulants through their interaction with ABSs of FXIa, which facilitates activation of the zymogen FXI [22, 38, 41–43]. 2) Likewise, GPIIb α of the activated platelets binds to a site on the CD of FXIa resulting in localization of FXIa on platelet surface [49]. Yet, competition studies revealed that SPGG2 either does not compete or marginally competes with polyPs and GPIIb α , respectively. This implies that subtle differences exist between the interactions of SPGG2 and polyP or GPIIb α with ABS2 of the CD. Considering that it is not firmly established that polyPs occupies only ABS2 of the CD, this result is not unusual. However, the result suggests that SPGG2 is not likely to interfere with the zymogen FXI activation by thrombin and/or pre-formed FXIa [38, 41]. Likewise, the results also suggest that SPGG2 is less likely to change FXIa localization on activated platelets, which could impact hemostasis.

The study of FXIa inhibition by common GAGs led to a rather unexpected result. Despite the presence of numerous sulfate groups, no GAG was found to directly inhibit FXIa at the highest concentration tested. This result adds to the growing body of evidence that aromatic NSGMs possess an intrinsic ability to bind proteins with higher non-ionic binding energy [55, 56], which contributes to the observed higher specificity of interaction. Another result of considerable clinical value is the discovery that SPGG2 inhibition can be reversed or diminished using protamine or polybrene. These antidote alternatives may enhance the value of pursuing this line of allosteric anticoagulants. The lack of availability of such antidotes is a continuing issue with current target specific oral anticoagulants used in the clinic [57, 58].

In conclusion, we have defined several important features of SPGG that govern selectivity, allosterism and reversible inhibition of FXIa. We have also found that SPGG2 binds well to bovine serum albumin, a surrogate of the human protein, which implies that future pharmacokinetics and pharmacodynamics studies will have to take into consideration this interaction. The binding site of SPGG2 was delineated through site-directed mutant analysis, which will likely guide the design of next generation of SPGG-like allosteric FXIa inhibitors. Overall, SPGG2 exhibits considerable pre-clinical promise as the first synthetic, small molecule allosteric inhibitor of FXIa.

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Abbreviations

ABS	anion binding site
APC	activated protein C

APTT	activated partial thromboplastin time
AT	antithrombin
BSA	Bovine serum albumin
CS	chondroitin sulfate
DS	dermatan sulfate
DTT	dithiothreitol
FXIa	factor XIa
FXI	factor XI
FXIa-CD	factor XIa catalytic domain
FXIa-FL	factor XIa full length
FXIIIa	factor XIIIa
GAGs	glycosaminoglycans
GPIIb/IIIa	glycoprotein IIb/IIIa
LMWHs	low molecular weight heparins
PolyPs	polyphosphates
PT	prothrombin time
rhFXIa	recombinant human factor XIa
SOS	sucrose octasulfate
SPGG	pentagalloyl glucopyranoside
UFH	unfractionated heparin

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Highlights

- Sulfated pentagalloyl glucopyranoside (SPGG) inhibits human factor XIa
- SPGG is the first synthetic small molecule that allosterically inhibits FXIa
- SPGG inhibition of FXIa can be reduced by cationic polymers or serum albumin
- SPGG inhibition of FXIa is unaffected by polyphosphates or GPIIb/IIIa
- SPGG binds to Lys529, Arg530 and Arg532 of the catalytic domain of human FXIa

SPGG2 :: R = -OH or -OSO₃Na
SPGG2-OH :: R = -H

Figure 1.
 Chemical structures of the hydrophobic, nonsaccharide, aromatic, GAG mimetics of pentagalloyl glucopyranosides (SPGGs) e.g. SPGG2 and SPGG2-OH which were used in this study.

Figure 2.

Proteolytic activity of human factor XIa (FXIa), plasma kallikrein (PK), activated protein C (APC), and factor XIIIa (FXIIIa) in the presence of SPGG2 (200 $\mu\text{g/mL}$) and SPGG2-OH (1000 $\mu\text{g/mL}$) using the corresponding assays. The assays were performed using substrates appropriate for the enzymes being studied under conditions closest to the physiological condition. The ratio of the proteolytic activity of an enzyme in the presence of SPGG2 or SPGG2-OH to that in their absence was used to determine percent activity (%). See Experimental procedures for details.

Figure 3.

Reversibility of SPGG2 interaction with FXIa by potential reversing agents. Shown is the *in vitro* restored FXIa activity (%) (inhibited by 5 μ g/mL of SPGG2) in the presence of increasing concentration of polybrene (●), protamine sulfate (○), and sucrose octasulfate (○). The restored activity profiles were determined spectrophotometrically at pH 7.4 and 37 °C. Solid lines represent fits by the dose-response Eq. 2 to obtain the EC_{50} as described in Experimental Procedures.

Figure 4.

Effect of bovine serum albumin (BSA) on the inhibitory profile of SPGG2 toward FXIa. The inhibition of FXIa by SPGG2 in the presence of BSA was determined spectrophotometrically at pH 7.4 and 37 °C. Shown is a bars graph of the residual FXIa activity (%) upon inhibition by a dose of 1.5 mg/mL of SPGG2 in the presence of 0, 10, 20, and 40 mg/mL of BSA. See Experimental procedures.

Figure 5.

Effect of FXI on the inhibitory profile of SPGG2 toward FXIa. Shown is the inhibition of FXIa by SPGG2 in the presence of FXI which was determined spectrophotometrically at pH 7.4 and 37 °C. Solid lines represent fits by the dose-response Eq. 1 to obtain the IC_{50} as described in Experimental Procedures. The concentrations of FXI selected for the study were 0 (●), 113 (◇), 225 (■), and 1125 nM (△).

Figure 6.

Direct inhibition of purified and recombinant FXIa (rFXIa) by SPGG2 in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 0.1 % PEG8000, and 0.02% Tween80. Shown is the inhibition of purified FXIa (○), rFXIa-ABS⁻¹ (◆), rFXIa-FL (△), and rFXIa-ABS⁻² (■). Solid lines represent dose-response fits of Eq. 1 to the data to derive the IC₅₀, HS, and Y values. See text for details.

Figure 7. FXIa catalytic domain (PDB ID: 1ZHM) generated with PyMole showing positions of Lys and Arg residues (blue) (K529, R530, R532) involved in heparin binding and positions of Ser, His, and Asp of active site (orange). The heparin binding site in FXIa catalytic domain is the putative binding site of SPGG2.

Table 1Direct inhibition of FXIa by SPGG2 and other glycosaminoglycans.^a

GAG or GAG mimetic	FXIa IC_{50} (μ M)
UFH	>250
Enoxaparin	>1100
H5	>360
SOS	>4000
CSA	>50
DS	>50
CSC	>50
SPGG2-OH	1.4 ± 0.1^b
SPGG2	0.50 ± 0.02

^aThe IC_{50} values were obtained at ~pH 7.4 and 37 °C using the corresponding chromogenic substrate hydrolysis assays. See details under Experimental Procedures.

^bErrors represent $\pm$ 1 S.E.

Table 2Concentrations of SPGG2 that double plasma clotting times.^a

Plasma	Test ^b	Concentration	
		($\mu\text{g/mL}$)	(μM)
Normal	APTT	89 ± 6^c	45 ± 3
	PT	746 ± 52	373 ± 26
AT/HC II deficient	APTT	79 ± 11	40 ± 6
FXI deficient	APTT	227 ± 15	114 ± 8

^a Prolongation of clotting time as a function of concentration of SPGG2 in either the activated partial thromboplastin time assay (APTT) or the prothrombin time assay (PT). Clotting assays were performed in duplicate as described in the Experimental Procedures.

^b For APTT and PT values in the absence of SPGG2, the 95% confidence interval was 13.2–23.3 and 58.6–68.2, respectively.

^c Errors represent ± 1 S.E.

Table 3*In vitro* reversibility of SPGG2 action by protamine sulfate, polybrene, or SOS.^a

[SPGG2]($\mu\text{g/mL}$)	Reversing Agent	EC_{50} ($\mu\text{g/mL}$)	Y^b
5	Protamine sulfate	16 ± 4^c	30 ± 4
	Polybrene	4.0 ± 0.2	92 ± 2
	SOS	>4600	ND^d
250	Protamin sulfate	107 ± 24	42 ± 2
	Polybrene	15 ± 2	76 ± 9
	SOS	>4600	ND

^aThe EC_{50} and Y values were obtained following non-linear regression analysis (Eq. 2). Reversing direct inhibition of human factor XIa activity was performed in 50 mM TrisHCl buffer, pH 7.4, containing 150 mM NaCl, 0.1% PEG8000, and 0.02% Tween80 at 37 °C. Inhibition was monitored by spectrophotometric measurement of residual factor XIa activity. See details under Experimental Procedures.

^b Y corresponds to the difference between the minimum and maximum efficacy.

^c Errors represent ± 1 S.E.

^d Not determined.

Table 4*In vitro* reversibility of SPGG2 action by BSA and FXI.^a

Reversing Agent	FXIa IC ₅₀ (μg/mL)	HS ^b	Y ^c
[BSA] (mg/mL)			
0	1.0 ± 0.1 ^d	1.5 ± 0.3	99 ± 6
10	772 ± 46	1.6 ± 0.4	103 ± 3
20	1206 ± 60	1.4 ± 0.3	107 ± 5
40	1684 ± 390	2.0 ± 0.8	95 ± 41
[FXI] (nM)			
0	0.9 ± 0.1	1.4 ± 0.1	98 ± 3
113	30 ± 1	1.3 ± 0.2	93 ± 4
225	63 ± 6	1.6 ± 0.2	89 ± 4
1125	273 ± 26	1.5 ± 0.4	75 ± 8

^a The IC₅₀, HS, and Y values were obtained following non-linear regression analysis (Eq.1) of direct inhibition of human factor XIa in 50 mM TrisHCl buffer, pH 7.4, containing 150 mM NaCl, 0.1% PEG8000, and 0.02% Tween80 at 37 °C. Inhibition was monitored by spectrophotometric measurement of residual factor XIa activity. See details under Experimental Procedures.

^b HS is Hill slope.

^c Y corresponds to the difference between the minimum and maximum efficacy.

^d Errors represent ± 1 S.E.

Table 5SPGG2-mediated inhibition of different FXIa variants.^a

FXIa variant	IC ₅₀ (μg/mL)	HS	Y
Purified FXIa	1.0 ± 0.1 ^b	1.5 ± 0.3	99 ± 6
rFXIa – CD _{WT}	1.2 ± 0.1	1.8 ± 0.4	106 ± 6
rFXIa – CD _{K529A}	1.7 ± 0.1	1.5 ± 0.3	84 ± 5
rFXIa – CD _{R530A}	2.3 ± 0.2	1.4 ± 0.3	96 ± 5
rFXIa – CD _{R532A}	1.5 ± 0.2	1.7 ± 0.7	103 ± 6
rFXIa – FL	9.9 ± 0.7	1.1 ± 0.2	85 ± 4
rFXIa – ABS ⁻¹	4.5 ± 0.5	1.6 ± 0.5	76 ± 7
rFXIa – ABS ⁻²	69 ± 10	1.0 ± 0.3	48 ± 7

^aThe IC₅₀, HS, and Y values were obtained following non-linear regression analysis (Eq.1) of direct inhibition of human factor XIa in 50 mM TrisHCl buffer, pH 7.4, containing 150 mM NaCl, 0.1% PEG8000, and 0.02% Tween80 at 37 °C. Inhibition was monitored by spectrophotometric measurement of residual factor XIa activity (n = 2). See details under Experimental Procedures.

^bErrors represent ± 1 S.E.

Table 6Competitive inhibition of FXIa by SPGG2 in the presence of UFH, polyP45, polyP700, and GPIIb α .^a

Competitor	Conc. (μ M)	IC_{50} (μ g/mL)	HS	Y
UFH^b	0	1.0 ± 0.1^c	1.6 ± 0.2	98 ± 3
	5	1.1 ± 0.1	1.7 ± 0.2	98 ± 3
	100	12 ± 3	1.3 ± 0.4	100 ± 6
	180	34 ± 5	2.3 ± 0.5	93 ± 3
PolyP45	0	0.9 ± 0.1	1.8 ± 0.2	98 ± 3
	5	0.9 ± 0.1	1.3 ± 0.4	108 ± 11
	100	1.3 ± 0.2	1.4 ± 0.4	101 ± 11
PolyP700	0	1.0 ± 0.1	1.5 ± 0.3	99 ± 6
	100	1.1 ± 0.1	2.4 ± 0.7	91 ± 7
GPIIbα	0	1.0 ± 0.1	1.3 ± 0.3	100 ± 5
	0.05	1.0 ± 0.1	1.5 ± 0.1	100 ± 2
	0.25	2.0 ± 0.2	1.0 ± 0.2	97 ± 7
	0.50	2.8 ± 0.1	1.2 ± 0.1	98 ± 2

^aThe IC_{50} , HS, and Y values were obtained at ~pH 7.4 and 37 °C using the corresponding chromogenic substrate hydrolysis assays. See details under Experimental Procedures.

^bTaken from reference (7).

^cErrors represent ± 1 S.E.