

Discovery of Benzyl Tetraphosphonate Derivative as Inhibitor of Human Factor Xla

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The inhibition of factor Xla (FXla) is a trending paradigm for the development of new generations of anticoagulants without a substantial risk of bleeding. In this report, we present the discovery of a benzyl tetra-phosphonate derivative as a potent and selective inhibitor of human FXla. Biochemical screening of four phosphonate/phosphate derivatives has led to the identification of the molecule that inhibited human FXla with an IC_{50} value of $\sim 7.4 \mu\text{M}$ and a submaximal efficacy of $\sim 68\%$. The inhibitor was at least 14-fold more selective to FXla over thrombin, factor IXa, factor Xa, and factor XIIIa. It also inhibited FXla-mediated activation of factor IX and prolonged the activated partial thromboplastin time of human plasma. In Michaelis-Menten kinetics experiment, inhibitor 1 reduced the V_{MAX} of FXla hydrolysis of a chromogenic substrate without

significantly affecting its K_{M} suggesting an allosteric mechanism of inhibition. The inhibitor also disrupted the formation of FXla – antithrombin complex and inhibited thrombin-mediated and factor XIIIa-mediated formation of FXla from its zymogen factor XI. Inhibitor 1 has been proposed to bind to or near the heparin/polyphosphate-binding site in the catalytic domain of FXla. Overall, inhibitor 1 is the first benzyl tetraphosphonate small molecule that allosterically inhibits human FXla, blocks its physiological function, and prevents its zymogen activation by other clotting factors under *in vitro* conditions. Thus, we put forward benzyl tetra-phosphonate 1 as a novel lead inhibitor of human FXla to guide future efforts in the development of allosteric anticoagulants.

1. Introduction

Thrombosis is a condition in which the blood unnecessarily and/or excessively clots in blood vessels and/or heart chambers leading to life-threatening pathologies. Thrombosis can initiate in a vein-driven manner as it is in deep vein thrombosis and pulmonary embolism.^[1–3] It can also be of arterial origin as in ischemic heart disease and stroke.^[1–3] Thrombosis has also been linked to a host of other chronic and serious diseases including inflammation,^[4] cancer,^[5] neurodegenerative diseases,^[6] and microbial infections.^[7] Importantly, micro- and macro-vascular as well as venous and arterial thrombotic conditions have been implicated in the ongoing pandemic of coronavirus disease of 2019 (COVID-19).^[8–10] In fact, COVID-19-associated thrombotic events appear to often lead to poor clinical outcomes of hospitalization, ICU admission and mechanical ventilation, and death.^[11] In this arena, important components that variably contribute to thromboembolic diseases are the platelets and the procoagulant factors of the coagulation system. Therefore,

drugs that are clinically used to treat thrombosis either target platelet proteins i.e. antiplatelets or inhibit the procoagulant factors of the coagulation system i.e. anticoagulants. Considering the origin of the pathological clots, antiplatelets are generally used in arterial thrombosis^[12] whereas anticoagulants are more frequently used in treating and/or preventing venous thromboembolism.^[13,14] Combinations of the two classes of antithrombotics are also used.^[15,16]

On the anticoagulants front, clinically available anticoagulants include the indirect anticoagulants of warfarin and heparins as well as the direct anticoagulants of thrombin inhibitors (argatroban, dabigatran, and bivalirudin) and factor Xa inhibitors (rivaroxaban, apixaban, edoxaban, and betrixaban). Despite their structural diversity, all available anticoagulants inhibit thrombin and/or factor Xa (FXa), two serine proteases in the common coagulation pathway (Figure 1).^[13–15] This leads to a very efficient inhibition of the pathological formation of the blood clot, yet it also disrupts hemostasis. Thus, all currently used anticoagulants are associated with serious bleeding events^[17–22] which complicate their effective and safe use in several patient populations such as those with atrial fibrillation^[23–25] or chronic kidney diseases.^[25–27] Therefore, the development of anticoagulants that do not cause bleeding is the main goal of contemporary drug discovery programs in the field.^[28–30] In this direction, several other procoagulant factors including factors VIIa (FVIIa),^[31] IXa (FIXa),^[32] XIa (FXIa),^[28–30,33] XIIa (FXIIa),^[33] and XIIIa (FXIIIa)^[34] have been considered to design and develop new effective anticoagulants with limited-to-none bleeding risk. In particular, FXla appears to be gaining momentum owing to several epidemiological, animal, and human observations which collectively indicate that FXla activity contributes to thrombosis but not to hemostasis.^[28–30,33]

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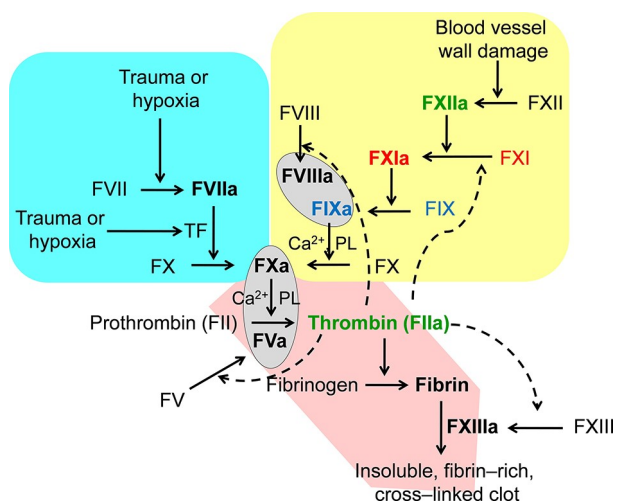


Figure 1. The coagulation process is depicted. Among the most important factors are FVIIa of the extrinsic coagulation pathway, FXIIa/FXIIa/FIXa of the intrinsic/contact activation pathway, and thrombin and FXa of the common coagulation pathway. Thrombin and FXa are the molecular targets of all currently available anticoagulants. FXIa is the molecular target in this study. Targeting human FXIa is expected to yield effective anticoagulants without the risk of bleeding because FXIa contributes to thrombosis, but not hemostasis. Physiologically, FXIa activates FIX to FIXa which subsequently forms the intrinsic tenase complex that further activates FXa, and then, thrombin. FXIa is produced by inorganic polyphosphate-mediated activation of FXI via the action of thrombin and FXIIa. FXIa can also be produced by autoactivation.

In fact, given the promise of FXI(a) as a drug target for safer anticoagulants, several FXI(a)-targeting agents are under development and these include small molecules,^[35–41] monoclonal antibodies,^[42–45] antisense oligonucleotides,^[46,47] and aptamers.^[48,49] Many of those inhibitors are active site inhibitors and few are allosteric inhibitors.^[30]

Biochemically, FXIa is a serine protease homodimer that belongs to the intrinsic pathway of coagulation (Figure 1). Physiologically, FXIa activates factor IX to FIXa so as to eventually amplify thrombin generation.^[50] Importantly, the zymogen form of FXIa i.e. factor XI (FXI) is activated by thrombin or FXIIa in the presence of negatively charged macromolecules such as heparin, inorganic polyphosphates,

and dextran sulfate via a template-mediated mechanism.^[51] The negatively charged macromolecules binds to anion-binding sites on the activating enzymes as well as on FXI. Interestingly, while the negatively charged heparin facilitates the activation of the zymogen FXI, heparin also directly and allosterically inhibits the active enzyme FXIa.^[52] Furthermore, the inorganic polyphosphates have also been found to bind to the same anion binding sites of FXI and acts as cofactors for its autoactivation and for its activation by thrombin and FXIIa.^[53]

Accordingly, to identify a new line of FXI(a)-targeting anticoagulants, we have considered the aromatic mimetics of inorganic polyphosphates so as to allosterically inhibit the function of human FXIa by targeting its anion-binding sites. In this arena, we tested four phosphonate/phosphate derivatives (1–4) (Figure 2) to evaluate their potential to inhibit human FXIa. As a result, we have identified the benzyl tetraphosphonate derivative (1) as the first aromatic mimetic of inorganic polyphosphates that allosterically inhibits human FXIa, as determined in the corresponding *in vitro* experiments of chromogenic substrate hydrolysis assay and Michaelis-Menten kinetics. The benzyl tetraphosphonate 1 inhibited human FXIa with an IC_{50} value of $\sim 7.4 \mu\text{M}$ and a submaximal efficacy of $\sim 68\%$. The molecule has demonstrated at least 14-fold selectivity toward FXIa over other procoagulant factors of thrombin, FIXa, FXa, and FXIIIa. The inhibitor also selectively prolonged the activated partial thromboplastin time (APTT) of human plasma. Interestingly, inhibitor 1 concentration-dependently inhibited the physiological function of FXIa i.e. FIX activation and inhibited thrombin-mediated and FXIIa-mediated activation of FXI. The inhibitor disrupted the formation of FXIa – antithrombin complex in the presence of heparin, suggesting that it may compete with heparin for binding to or near the anion-binding site(s) of FXIa. Overall, we put forward benzyl tetraphosphonate derivative 1, the first potent, selective, and partial allosteric inhibitor of FXIa, to be considered in the development of effective anticoagulants with a limited risk of bleeding complications.

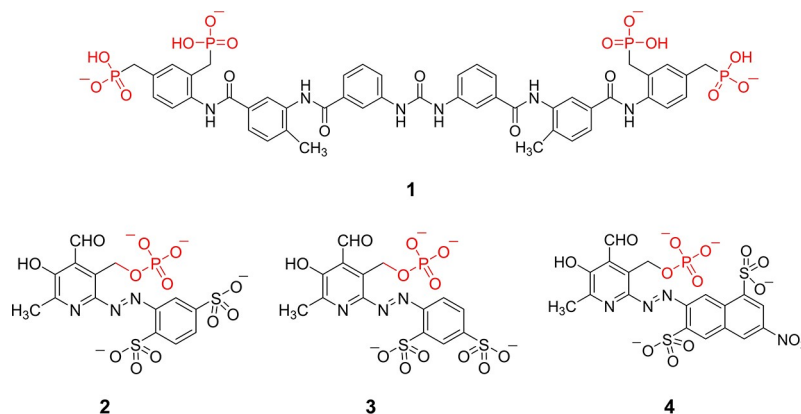


Figure 2. Chemical structures of phosphonate and phosphate derivatives (1–4) that were screened against human FXIa in this study.

2. Results

2.1. Rationale for Screening Phosphonate/Phosphate Derivatives (1–4) Against Human FXIa

Several approaches have been utilized to discover and/or rationally design inhibitors of FXIa. These approaches include small molecules, polypeptides, aptamers, and monoclonal antibodies.^[30] While most of the small molecules are active site inhibitors, sulfated nonsaccharide mimetics of heparin, reported as SPGG^[54,55] and SCI,^[56] are allosteric inhibitors of FXIa. They appeared to inhibit FXIa by targeting its anion-binding sites, particularly the site in the catalytic domain. Interestingly, SCI exhibited potent anticoagulant activity with no bleeding complications in rat models of thrombosis.^[57] Although their allosteric inhibition mechanism is unique for achieving a high level of functional selectivity, these molecules are highly negatively charged with at least 10 sulfate groups, a structural feature that may compromise their druggability. Thus, we have considered phosphonate and phosphate derivatives 1–4 (Figure 2) to be screened against human FXIa to identify new inhibitors of FXIa with fewer number of negative charges. Given the fact that anion-binding sites of FXI(a) recognize both sulfated heparins as well as inorganic polyphosphates, we have hypothesized that phosphonate and phosphate derivatives will likely exhibit an allosteric inhibition mechanism similar to that exhibited by the sulfated nonsaccharide mimetics of heparin. We have also hypothesized that the phosphonate and phosphate derivatives will likely enjoy a better long-term stability because of the reduced number of negative charges. Not only that, but strategies to develop phosphonate and phosphate prodrugs are also well established,^[57–59] which is necessary to be considered to enhance their overall druggability, especially as it relates to their oral bioavailability.

2.2. FXIa Inhibition Potential of Phosphonate/Phosphate Derivatives (1–4)

The four molecules were evaluated for their potential to inhibit FXIa hydrolysis of S-2366, a chromogenic tripeptide substrate, under the physiological conditions of pH 7.4 and 37 °C, as reported earlier.^[60,61] Only the tetraphosphonate derivative 1 and phosphate derivative 4 inhibited FXIa in a dose-dependent fashion. Molecules 2 and 3 did not inhibit FXIa at the highest concentration tested of 100 μM. The inhibition of FXIa by molecules 1 and 4 could be fitted using the logistic equation 1, which resulted in an IC_{50} value of 7.4 ± 0.9 μM and efficacy of $68.0 \pm 3.7\%$ for inhibitor 1 (Figure 3, Table 1) and an IC_{50} value of 59.4 ± 17.5 μM and efficacy of $111 \pm 15.6\%$ for inhibitor 4 (Table 1). The lack of inhibition potential for phosphate derivatives 2 and 3 suggests a rather selective interaction between FXIa and inhibitors 1 and 4. Of note, molecules 2–4 also have two sulfonate groups in addition to the phosphate group, yet only molecule 4 inhibited human FXIa indicating that negative charges are not the only interacting groups and that other structural features are also important.

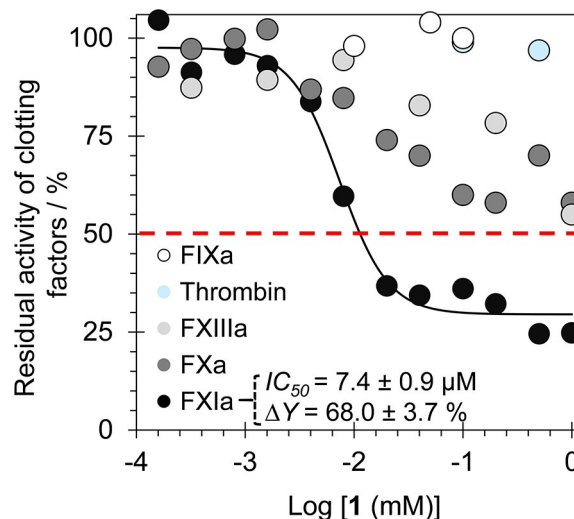


Figure 3. Direct inhibition of coagulation factors by inhibitor 1. The inhibition of FXIa (●), FXa (●), FXIIIa (●), thrombin (●), and FIXa (○) by benzyl tetraphosphonate 1 was studied using the corresponding chromogenic substrate hydrolysis assays. Solid line represents sigmoidal dose–response fits (eq 1) to the data to obtain the values of IC_{50} , ΔY , and HS.

Table 1. The inhibition of human FXIa by aryl phosphonate and phosphate derivatives (1–4)^[a]

Polyphosphate(onate)	FXIa IC_{50} [μM]	HS	ΔY [%]
1	7.4 ± 0.9 ^[b]	1.9 ± 0.5	68.0 ± 3.7
2	> 100 ^[c]	NA ^[d]	NA
3	> 100	NA	NA
4	59.4 ± 17.5	0.9 ± 0.1	111 ± 15.6

[a] The values of IC_{50} , HS, and ΔY were obtained following non-linear regression analysis of direct inhibition of human FXIa in appropriate Tris–HCl buffers of pH 7.4 at 37 °C. Inhibition was monitored by spectrophotometric measurement of residual enzyme activity. [b] Errors represent ± 1 S.E. [c] Estimated value was based on the highest concentration of the inhibitor used in the experiment. [d] Not available.

2.3. Benzyl Tetraphosphonate 1 is a Selective Inhibitor of Human FXIa Over Other Coagulation Proteins

The inhibition profiles of benzyl tetraphosphonate 1 against thrombin, FIXa, and FXa were studied using the corresponding chromogenic substrate hydrolysis assays under physiological conditions, as described earlier.^[54,55,60,61] In these assays, the inhibition potential was determined by spectrophotometric measurement of the residual protease activity in the presence of varying concentrations of inhibitor 1 (Figure 3). Furthermore, the molecule's activity against human FXIIIa was also studied using the bi-substrate, fluorescence-based trans-glutamination assay, as described earlier.^[62] Based on the highest concentration tested of inhibitor 1 against the above enzymes, the calculated IC_{50} values are estimated to be > 500 μM for thrombin, > 200 μM for FIXa, > 100 μM for FXa, and > 200 μM for FXIIIa (Table 2), suggesting selectivity indices of > 67.6 -fold, 27-fold, > 14 -fold, and > 27 -fold, respectively. Overall, the above results indicate that benzyl tetraphosphonate 1 is a selective

Table 2. The inhibition of human thrombin, FIXa, FXa, and FXIIIa by benzyl tetraphosphonate 1.

Benzyl tetraphosphonate	FXIa IC_{50} [μ M]	Thrombin IC_{50} [μ M]	FIXa IC_{50} [μ M]	FXa IC_{50} [μ M]	FXIIIa IC_{50} [μ M]
1	7.4 ^[a] ± 0.9 ^[b]	> 500 ^[c]	> 200	> 100	> 200

[a] The inhibition values were obtained following non-linear regression analysis of direct inhibition of human thrombin, factor Xa, and factor XIIIa in appropriate Tris–HCl buffers of pH 7.4 at 37 °C. Inhibition was monitored by spectrophotometric measurement (thrombin, FIXa, and FXa) or spectrofluorometric measurement (FXIIIa) of residual enzyme activity. [b] Errors represent ± 1 S.E. [c] Estimated value was based on the highest concentration of the inhibitor used in the experiment.

inhibitor for human FXIa, as determined in the corresponding *in vitro* assays.

2.4. Effect of Inhibitor 1 on Clotting Times of Normal and FXI Deficient Human Plasma

Plasma clotting assays of APTT and prothrombin time (PT) are routinely used to investigate the anticoagulation potential of new procoagulant enzyme inhibitors under *in vitro* conditions. The former time measures the effect of potential anticoagulant on the intrinsic/contact pathway-driven clotting which involves FXIIa, FXIa, and FIXa. The latter time measures the effect of potential anticoagulant on the extrinsic pathway of coagulation which involves FVIIa. The effect of different concentrations of inhibitor 1 on APTT and PT of normal human plasma was measured (Table 3 and Figure 4), as described in earlier studies.^[54,55,60,61] Results indicated that inhibitor 1 concentration-dependently prolonged APTT but not PT over the concentration range of 0–470 μ M. Figure 4A shows the effect of anti-FXIa monoclonal antibody on the clotting times. The antibody selectively recognizes human FXI, and under our testing conditions, resulted in 1.5-fold increase in APTT at a concentration of 1.4 μ g/mL. The antibody did not affect the PT at the highest concentration tested of 2.88 μ g/mL. Likewise, Figure 4B shows the variation in APTT and PT in the presence of varying concentrations of inhibitor 1. A 1.5-fold increase in APTT required 311.3 μ M of inhibitor 1. However, a 1.5-fold increase in the PT required > 750 μ M of inhibitor 1. These results indicate, as expected, that inhibitor 1 is anticoagulant in normal human

Table 3. Effects of inhibitor 1 on the clotting times of human plasma.^[a]

[1] [μ M]	APTT [sec]	PT [sec]
0	34.4 \pm 0.1 ^[b]	14.5 \pm 2.1
141	39.7 \pm 3.9	13.0 \pm 1.5
235	42.6 \pm 5.8	14.2 \pm 2.3
282	45.4 \pm 7.2	14.2 \pm 1.3
329	56.1 \pm 6.7	14.7 \pm 1.3
423	73.2 \pm 7.6	16.9 \pm 3.0
470	83.7 \pm 8.6	16.7 \pm 1.8

[a] Prolongation of clotting time as a function of concentration of inhibitor 1 in the APTT and PT assays. [b] Error represents ± 1 S.E.

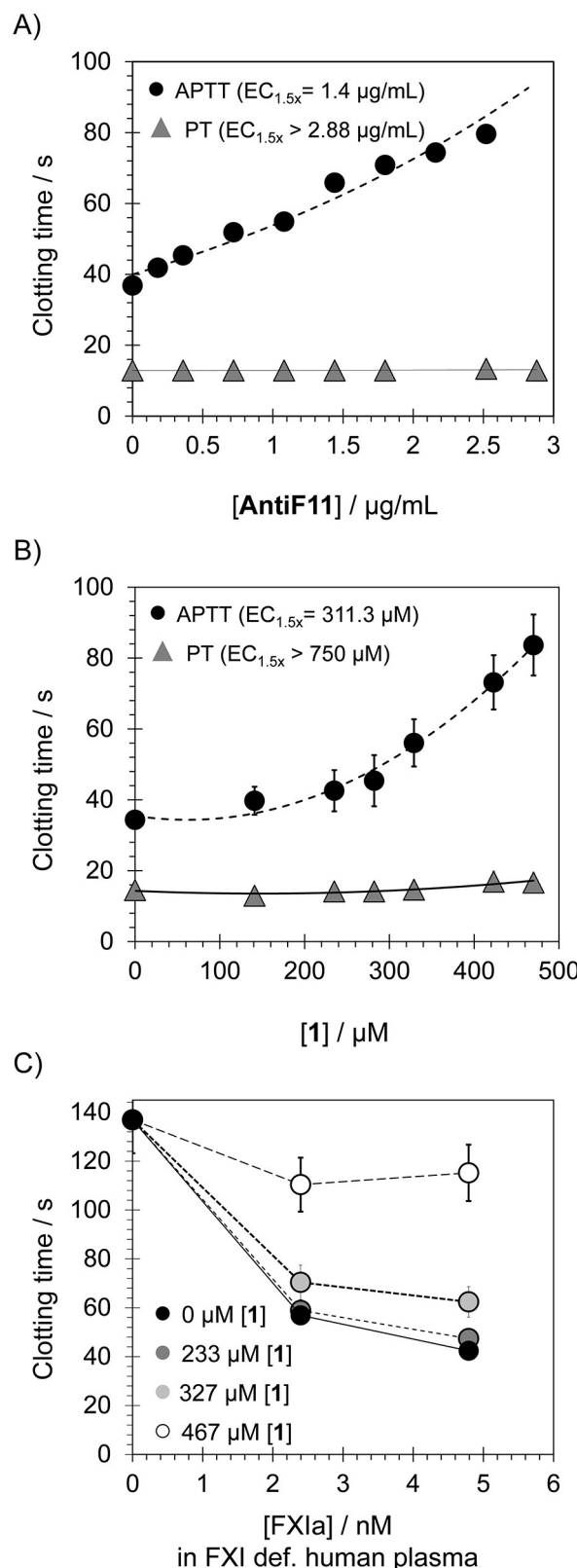


Figure 4. Effect of different concentrations of AntiF11 monoclonal antibody (A) and inhibitor 1 (B) on the clotting times of APTT (●) and PT (▲) in human plasma. Solid lines are trend lines from which the concentration necessary to increase the clotting times by 1.5-fold were deduced. Shown in (C) is the effect of different concentrations of inhibitor 1 (●) 0 μ M, (●) 233 μ M, (●) 327 μ M, and (○) 467 μ M on FXIa-induced clotting of FXI-deficient human plasma. FXIa concentrations used were 2.4 nM and 4.8 nM.

plasma and it does so by targeting proteins in the intrinsic pathway of coagulation, particularly FXIa. To confirm the involvement of FXIa in streaming the anticoagulant effect of inhibitor 1, we measured the effect of adding variable concentrations of inhibitor 1 on FXIa-induced clotting of FXI-deficient human plasma. Figure 4C shows that FXI-deficient human plasma clotted at 136.9 s, yet adding 2.4 nM or 4.8 nM of human FXIa accelerated its clotting so as to take place at 56.9 s or 42.4 s, respectively. However, the addition of 233–467 μM of inhibitor 1 significantly delayed the FXIa-induced clotting of FXI-deficient human plasma by 1.04–1.94-fold when clotting was induced by 2.4 nM FXIa or by 1.11–2.72-fold when clotting was induced by 4.8 nM FXIa. Overall, these results further establish that the anticoagulant activity of inhibitor 1 in human plasma is attributed to its effect on FXIa of the intrinsic coagulation pathway.

2.5. Allosteric Inhibition of FXIa by Benzyl Tetraphosphonate 1

To understand the mechanistic basis of molecule 1 inhibition of human FXIa, Michaelis-Menten kinetics of S-2366 hydrolysis by

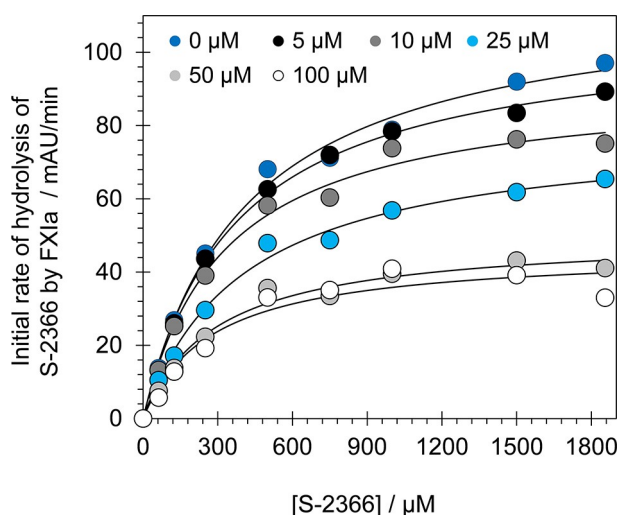


Figure 5. Michaelis–Menten kinetics of S-2366 hydrolysis by FXIa in the presence of inhibitor 1. The initial rate of hydrolysis at various substrate concentrations was measured in pH 7.4 buffer using FXIa full length wild type. The concentrations of inhibitor 1 chosen in the study were 0 (●), 5 (●), 10 (●), 25 (●), 50 (●), 100 μM (○). Solid lines represent nonlinear regressional fits to the data by the Michaelis–Menten eq 2.

[1] [μM]	S-2366 K_M [mM]	V_{MAX} [mAU/min]
0	$0.41 \pm 0.04^{[b]}$	116.2 ± 4.0
5	0.39 ± 0.02	106.5 ± 1.8
10	0.33 ± 0.04	91.8 ± 3.4
25	0.41 ± 0.04	79.3 ± 2.8
50	0.29 ± 0.05	50.0 ± 2.5
100	0.28 ± 0.09	45.7 ± 4.2

[a] K_M and V_{MAX} values of S-2366 substrate hydrolysis by FXIa were measured by Michaelis Menten kinetics. [b] Error represents ± 1 S.E.

the wild type full-length FXIa was performed in the presence of inhibitor 1 at pH 7.4 and 37 °C. Figure 5 shows the initial rate profiles in the presence of inhibitor 1 (0–100 μM). Each profile displays a characteristic rectangular hyperbolic trend, which could be fitted using equation 2 to give the apparent K_M and V_{MAX} (Table 4). The K_M for S-2366 did not significantly change (0.41 ± 0.04 mM– 0.28 ± 0.09 mM) in the presence or absence of inhibitor 1. Nevertheless, the V_{MAX} decreased steadily from 116 ± 4 mAU/min in the absence of inhibitor 1 to 45.7 ± 4.2 mAU/min at 100 μM of inhibitor 1. Thus, the inhibitor appears to bring about structural changes in the active site of FXIa which do not affect the formation of Michaelis complex but lead to a significant disruption in FXIa catalytic activity. This indicates that molecule 1 is an allosteric inhibitor of human FXIa.

2.6. Inhibition of FXIa: Activation of the Physiologically Relevant Substrate FIX by Tetra-Phosphonate 1

Although tetraphosphonate 1 inhibited the hydrolysis of chromogenic tripeptide substrate S-2366 by FXIa, yet we have aimed at establishing its physiological relevance by evaluating its effect on the physiological substrate of FXIa i.e. FIX. During coagulation, FXIa binds to and activates FIX (Figure 1) by successively cleaving two peptide bonds of Arg145-Ala146 and Arg180-Val181 so as to eventually generate FIXa β .^[50] Subsequently, FIXa β along with factor VIIIa forms the intrinsic tenase complex, in the presence of calcium ions and phospholipids, to activate factor X to FXa which eventually amplifies thrombin generation.^[63] To establish the physiological relevance of benzyl tetraphosphonate 1 inhibitory activity toward FXIa, we evaluated FXIa activation of FIX in the presence and absence of inhibitor 1 using SDS-PAGE (Figure 6A) and Western blotting (Figure 6B). The figures show that inhibitor 1 dose-dependently (0–1000 μM) inhibited the formation of the intermediate FIX i.e. FIXa as well as the fully activated FIX i.e. FIXa β (heavy chain (FXIa β -HC) and light chain (FXIa β -LC)) as indicated by the absence of the corresponding bands. These results suggest that the inhibitory activity of tetraphosphonate 1 toward FXIa is physiologically relevant and it takes place at a concentration range (≤ 250 μM) similar to that used in the chromogenic substrate hydrolysis assays.

2.7. Effect of Benzyl Tetraphosphonate 1 on FXI(a) Interactions with Macromolecules

To understand the effect of benzyl tetraphosphonate 1 beyond the inhibition of FXIa catalytic activity, we investigated three intermolecular interactions involving FXIa and its zymogen FXI. In this direction, it has been reported that FXIa can be inhibited by antithrombin, an endogenous serpin, in a reaction that is accelerated in the presence of heparin by a template- or bridging-based mechanism.^[52] In this interaction, a denaturation-resistant complex between the FXIa active site and the reactive center loop of antithrombin is formed. In this complex,

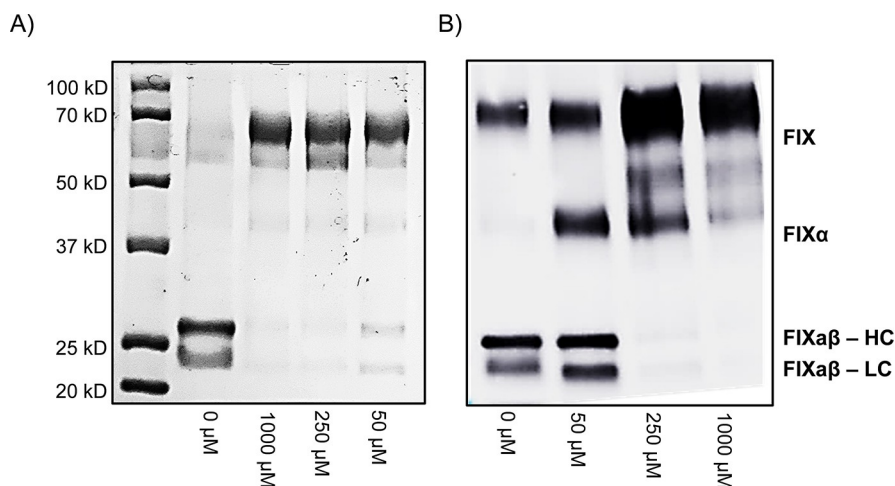


Figure 6. Effect of inhibitor 1 on the activation of FIX by FXIa as evaluated by SDS-PAGE (A) and Western blotting (B), under reducing conditions. Inhibitor 1 concentrations used were 0, 50, 250, 1000 μM . Shown is activation of FIX to FIX α by cleaving the peptide bond of Arg145-Ala146, and then, to FIX $\alpha\beta$ by cleaving the peptide bond of Arg180-Val181. Under reducing condition, the FIX $\alpha\beta$ appears as heavy chain (FIX $\alpha\beta$ – HC) and light chain (FIX $\alpha\beta$ – LC).

the light chain of FXIa (FXIa-LC) acylates antithrombin by forming a covalent bond. Figure 7A shows SDS-PAGE evidence of the 90 kDa FXIa-LC – antithrombin complex (lane 3) formation in the absence of inhibitor 1. In contrast, the presence of increasing concentrations of inhibitor 1 (50–1000 μM) inhibited the formation of such complex. In fact, the formation of the complex appears to be significantly diminished at the highest concentration tested of 1000 μM . At concentrations of 250 μM and 1000 μM , the band of 90 kDa complex on SDS-PAGE significantly diminished and that of FXIa-LC appeared again at 30 kDa (lanes 5 and 6). These results suggest that inhibitor 1 disrupts the formation of FXIa-antithrombin complex, potentially by competing with heparin for its anion-binding sites on the catalytic domain and/or apple 3 domain.

Furthermore, it is well documented that the zymogen FXI is activated by the action of thrombin or FXIIa in processes that are accelerated in the presence of inorganic polyphosphates or dextran sulfate.^[51,64,65] Specifically, dextran sulfate is a sulfated polysaccharide that appears to accelerate the activation reactions via template-dependent mechanism in the which the polysaccharide engages with the anion binding sites on both the substrate i.e. FXI as well as the activators i.e. thrombin or FXIIa.^[64–69] Figure 7B shows that inhibitor 1 inhibited the activation of FXI by thrombin, as shown by the decreased intensity of FXIa-HC and FXIa-LC bands (lanes 6–10), over the concentration range of 2–1000 μM . Likewise, Figure 7C shows that inhibitor 1 inhibited the activation of FXI by FXIIa, as shown by the decreased intensity of FXIa-HC and FXIa-LC bands (lanes 7–9), over the concentration range of 50–1000 μM . Taken together, benzyl tetraphosphonate 1 has been found to inhibit the dextran sulfate-mediated activation of FXI by thrombin as well as by FXIIa at comparable concentrations to those determined in the above experiments. This suggests that inhibitor 1 potentially competes with dextran sulfate for anion-binding sites on the catalytic domain and/or apple 3 domain. Overall, the above results suggest that benzyl tetraphosphonate

1 is likely to bind to an allosteric site on FXI(a) rather than the active site. Similar to sulfated non-saccharide mimetics of heparins, the binding site is likely to be the anion-binding site in the catalytic domain.

2.8. Molecular Modeling of Potential Binding of Benzyl Tetraphosphonate 1 to the Anion – Binding Site in the Catalytic Domain of FXIa

To identify a plausible binding mode for benzyl tetraphosphonate 1, we performed molecular docking studies by considering the anion-binding site on the catalytic domain of FXIa. The rationale for considering this site is that its lysine and arginine residues have been implicated in FXI(a) interactions with the negatively charged functional groups of several macromolecules including the sulfate groups of heparins^[52] and the phosphate groups of inorganic polyphosphates.^[53] The anion binding site on the catalytic domain of FXIa has also been implicated in the action of SPGG and that of SCI, two small molecule heparin mimetics that act as allosteric inhibitors of human FXIa.^[54–56] The docking studies of benzyl tetraphosphonate 1 onto the anion-binding site were carried out using Glide,^[70] as described in the experimental part. Initial coordinates for FXIa catalytic domain were taken from the crystal structure of PDB ID: 2FDA.^[71] The studies revealed that one phosphonate group on one end of the inhibitor structure interacts via electrostatic/hydrogen bond with K529 and T523 residues (Figure 8). The studies also revealed that the central urea carbonyl oxygen interacts via hydrogen bond with N524 residue and that another phosphonate group on the other end of inhibitor 1 interacts with K535 residue (Figure 8). In particular, the K529 residue (chymotrypsin number is K170) and the K535 (chymotrypsin number is K175) have been implicated in heparin-mediated inhibition of FXIa by antithrombin^[52] as well as in polyphosphate-mediated activation of FXI.^[51] Impor-

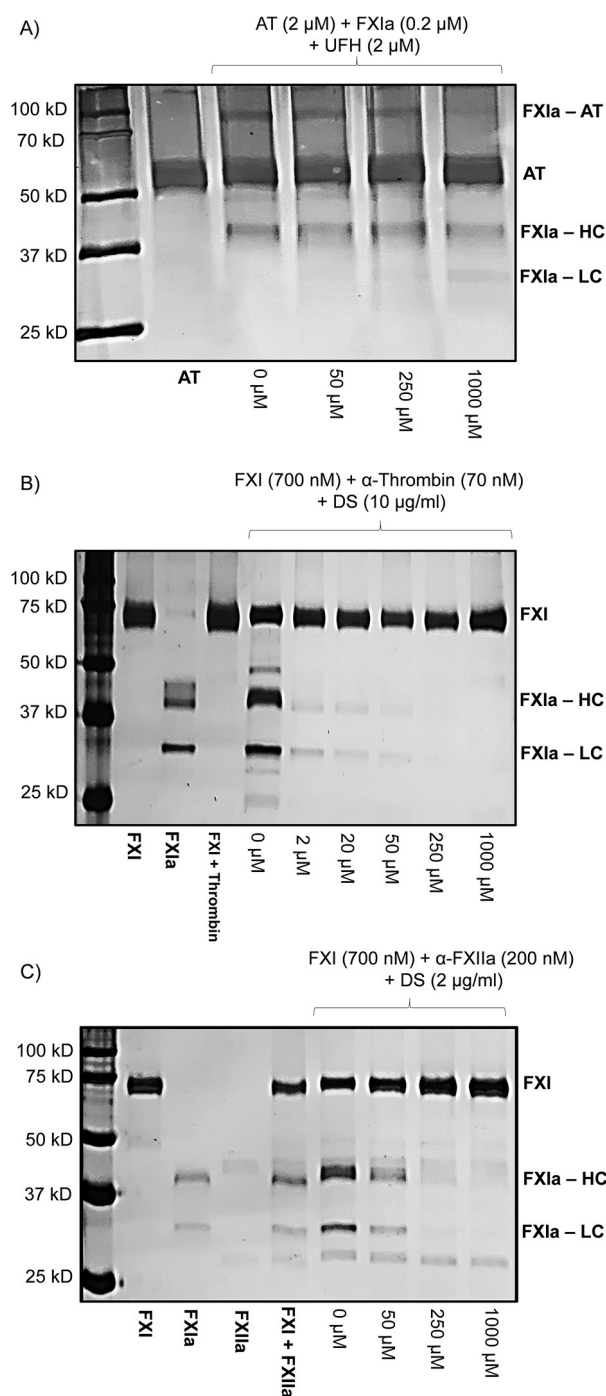


Figure 7. Effect of different concentrations of inhibitor 1 on FXI(a) interactions with macromolecules including FXIa-antithrombin complex formation in the presence of heparin (A), dextran sulfate-mediated activation of FXI by thrombin (B), and dextran sulfate-mediated activation of FXI by FXIIa. Frequent concentrations of benzyl tetraphosphonate 1 used are 50, 250, and 1000 μM. The inhibitor disrupted all of the above interactions indicating that the inhibitor recognizes both the enzyme FXIa and the zymogen FXI, potentially by binding to the anion-binding site of the catalytic domain of FXIa and the corresponding site on FXI.

tantly, these results highlight the essential requirement of at least two phosphonate groups for the inhibitor action toward FXIa. Although these results are to be experimentally confirmed via crystallography studies and/or mutagenesis studies, how-

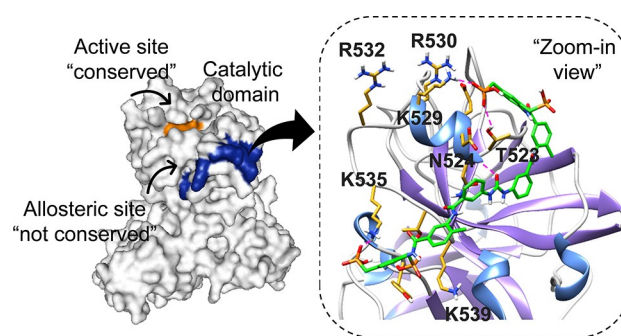


Figure 8. The anion-binding site in the catalytic domain of FXIa is presented as a potential binding site for inhibitor 1. In contrast to the active site, this anion-binding site of FXIa is relatively not conserved. In this molecular modeling study, inhibitor 1 has been found to engage with K529 and K535 residues. The two residues have been implicated in heparin-mediated inhibition of FXIa by antithrombin and in polyphosphate-mediated activation of FXI. The chymotrypsin-based numbering for the presented amino acids is as follows: T523 = T164, N524 = N165, K529 = K170, R530 = R171, R532 = R173, K535 = K175, and K539 = K179.

ever, the above computational exercise is important as the identified putative binding site can be used to guide subsequent efforts to optimize the potency and selectivity of inhibitor 1.

3. Discussion

Oral as well as parenteral anticoagulants are widely used to prevent and/or treat thromboembolic diseases.^[13–15] In addition to having potent and selective pharmacodynamic profile, ideal anticoagulants should have predictable pharmacokinetics and do not require continuous monitoring and/or dose adjustment. They should also be rapidly reversed via the use of affordable and effective antidotes. In contrast to the existing ones, ideal anticoagulants should be devoid of hepatotoxicity, osteoporosis, or thrombocytopenia. They should also be safe for use in compromised patients with a high risk of thrombosis as in pregnant patients and cancer patients. Importantly, they should induce no bleeding complications.^[72–74]

While a significant progress has been made towards satisfying the above criteria, bleeding risk continues to be a significant side effect of heparins and warfarin. Newer direct anticoagulants inhibiting thrombin or FXa are increasingly replacing heparins and warfarin, yet they appear to be still associated with significant risks of major bleeding.^[17–22] Accordingly, patients who may benefit from anticoagulation therapy do not receive it or receive a lower dose which is often not effective. This is the case of patients of atrial fibrillation and chronic kidney diseases.^[23–27] Thus, the search for safer anticoagulant drugs with little-to-none bleeding risk continues. Towards this goal, several FXI(a)-targeting agents are in development including small molecules (BMS-986177 and EP-7041),^[35–41] monoclonal antibodies (AB023, MAA868, and Osocimab),^[42–45] aptamers (FELIAP),^[48,49] and sulfated glycosaminoglycan mimetics (SPGG and SCI).^[54–56] Osocimab and BMS-986177, in particular, are currently in advanced clinical trials.

Among the above molecules, SPGG and SCI were developed as small molecule allosteric inhibitors of FXIa. They have been projected to target the anion-binding site in the catalytic domain.^[54–56] In fact, their structural features were designed in relevance to heparin, a heterogenous mixture of sulfated glycosaminoglycans that is known to allosterically inhibit FXIa by targeting the anion-binding sites in the catalytic domain and the apple 3 domain. Inorganic polyphosphates are also known to engage with FXI(a) anion-binding sites. However, the inorganic polyphosphates facilitate the autoactivation of the zymogen FXI as well as its activation in the presence of thrombin or FXIIa, and thus, leads to a procoagulant outcome. Inspired by the anionic nature of heparin and inorganic polyphosphates, several aptamers have also been designed to potentially disrupt the inorganic polyphosphates' procoagulant role as well as the procoagulant properties of FXI(a). These include aptamers 12.7 and 11.16 (RNA aptamers which bind to FXI(a) anion binding site on the catalytic domain resulting in allosteric inhibition)^[46] and FELIAP (DNA aptamer which binds to or near active site of FXIa).^[49]

In this study, we report on a small molecule (~1180.74 Da), benzyl tetraphosphonate **1**, that directly inhibits the catalytic activity of FXIa with an IC_{50} value of ~7.4 μ M (K_i value is estimated to be ~7.4 μ M,^[75] given the testing conditions), as determined in the corresponding chromogenic substrate hydrolysis assay. Importantly, the *in vitro* inhibition of FXIa-mediated hydrolysis of the chromogenic tripeptide substrate i.e. S-2366 has been found to translate into an *in vitro* inhibition of FXIa-mediated hydrolysis of its physiological substrate i.e. FIX. Thus, the activity of inhibitor **1** appears to be physiologically relevant. Furthermore, the inhibitor has demonstrated significant selectivity over other procoagulant serine proteases including thrombin, FIXa, and FXa as well as over the transglutaminase FXIIIa, as determined by the corresponding *in vitro* enzyme assays. Inhibitor **1** has also demonstrated a substantial anticoagulant effect by targeting FXIa in human plasma as demonstrated by its relatively selective effect on APTT as well as by its ability to abolish the FXIa-induced clotting of FXI-deficient human plasma.

Despite the direct effect of the inhibitor, its biological effects beyond the direct inhibition of FXIa activity are very interesting. In this arena, the molecule has been found to inhibit the dextran sulfate-mediated activation of the zymogen FXI by thrombin and FXIIa at concentrations comparable to those that inhibit FXIa hydrolysis of S-2366 as well as FIX. This has suggested that the benzyl tetra-phosphonate **1** may bind to a cluster of basic amino acids presented by both the enzyme FXIa and its zymogen FXI. This cluster is likely to be the anion binding sites, particularly the one in the catalytic domain. In fact, the ability of the inhibitor to disrupt FXIa-antithrombin complex formation in the presence of heparin lends support to the projection of the inhibitor targeting the anion-binding site on the catalytic domain. In line with these observations is also the fact that the molecule only affected the V_{MAX} parameter of FXIa hydrolysis of the chromogenic substrate but not the K_M parameter, as determined by Michaelis-Menten kinetics experiment. As a result, the above studies indicate that benzyl

tetraphosphonate **1** is allosteric inhibitor of FXIa and it binds to similar anion-binding sites on both FXIa and FXI. In fact, molecular modeling studies show that the inhibitor can favorably bind to two key basic residues in this binding site. The two residues are K529 and K535 which have previously been implicated in heparin-mediated inhibition of FXIa by antithrombin^[52] as well as in polyphosphate-mediated activation of FXI.^[51]

In contrast to the previous small molecule allosteric inhibitors of FXIa i.e. SPGG and SMI, which were associated with ~100% inhibition of the enzyme activity, inhibitor **1** induces sub-maximal inhibition of FXIa at saturation, with an efficacy value of ~68%. Such phenomenon is not possible for orthosteric inhibitors and can only be exhibited by allosteric inhibitors. This further supports the allosteric behavior of inhibitor **1** which, in turn, is important because it permits partial enzyme inhibition (modulation) rather than complete inhibition. Partial allosteric inhibition may translate into a lesser bleeding risk as previously proposed for partial allosteric inhibitors of thrombin.^[76,77] Importantly, targeting allosteric sites on FXIa also better permits the realization of selective enzyme modulation, considering the fact that other coagulation proteases share significant active site similarity. Thus, the allosteric nature of inhibitor **1** will likely result in a better safety profile. Overall, the discovery of this inhibitor is extremely promising for the prospect of discovering more clinically relevant partial modulators of FXIa.

4. Conclusions and Outlook

In this study, we have identified benzyl tetraphosphonate **1** as the first of its kind inhibitor of human FXIa. The inhibitor demonstrates substantial potency and selectivity toward FXIa and over other coagulation factors of thrombin, FIXa, FXa, and FXIIIa. The molecule not only inhibits the catalytic activity of FXIa, but it also inhibits the activation of its zymogen by thrombin and FXIIa. Importantly, the inhibitor represents an important step forward towards designing allosteric inhibitors of FXIa with submaximal efficacy. This is certainly important to modulate the catalytic activity of an enzyme belonging to a superfamily of largely conserved enzyme members so as to achieve a higher level of enzyme inhibition selectivity, and subsequently, a higher margin of safety. Furthermore, inhibitor **1** also exhibits a significant anticoagulant activity as demonstrated by extending the APTT of human plasma. Accordingly, we put forward this inhibitor as a lead molecule to develop a new generation of effective anticoagulants that are devoid of bleeding complications so as to be safely used in a wide range of patients populations, particularly those who are at a high risk of bleeding.

Future studies will focus on establishing the structure-activity relationship of inhibitor **1** to further enhance its potency and selectivity. The structural manipulation will focus on optimizing the number and the position of phosphonate groups as well as on the length and the substituents of the urea-based linker. The druggability of phosphonate derivatives

can also be further advanced by preparing the corresponding prodrugs to achieve meaningful oral bioavailability. It is also worth to mention here that the toxicity profile of inhibitor **1** has been evaluated in three cell lines of breast (MCF-7), intestine (CaCo-2), and kidney (HEK-293) (unpublished data). Initial results suggest that 10 μM of the inhibitor does not significantly affect the proliferation of the above cell lines. Testing at higher concentrations will be performed and reported in due time.

Lastly, a recent report has suggested that drugs that target the contact activation enzymes including FXIa may serve as potential therapeutics for patients with COVID-19.^[78] Earlier, targeting FXI(a)/FXIIa interface by pharmacological means has been shown to prevent coagulopathy, systemic inflammation, and mortality in experimental sepsis.^[79,80] In nonhuman primates, inhibition of contact activation also prevented death from *Staphylococcus aureus*-induced systemic inflammatory response syndrome.^[81,82] Overall, these studies suggest that the new class of allosteric FXIa inhibitors can be further developed as adjunct therapy for COVID-19 and similar microbial outbreaks that are typically associated with excessive coagulopathy and inflammation.

Experimental Section

Materials

Phosphonate derivative (**1**) and phosphate derivatives (**2–4**) were purchased from Santa Cruz Biotechnology (Dallas, TX). Reagents for clotting assays including thromboplastin D, APTT reagent, and CaCl_2 solution were all from Fisher Scientific (Pittsburgh, PA). Chemicals used to prepare enzyme assay buffers were from Milipore-Sigma (Burlington, MA), Fisher Scientific, or Bio-Rad laboratories (Hercules, CA). *N,N*-dimethyl-casein, dansylcadaverine, and dithiothreitol for FXIIIa assay were also from Milipore-Sigma. All types of plasmas were purchased from George King Bio-Medical, Inc. (Overland Park, KS). Antithrombin, coagulation zymogens, and coagulation enzymes including thrombin, FIXa, FXa, FXIa, and FXIIIa were from Haematologic Technologies, Inc. (Essex Junction, VT). Chromogenic substrates: Spectrozyme TH, Spectrozyme FIXa, and Spectrozyme FXa were obtained from Biomedica-Diagnostics (Windsor, NS Canada). Factor XIa chromogenic substrate (S-2366; L-pyroglutamyl-L-prolyl-L-arginine *p*-nitroaniline hydrochloride) was obtained from Diapharma (West Chester, OH). These substrates have a nitro-anilino chromophore and they were designed based on the physiological substrate of the corresponding clotting factor to ensure its specificity. Heparin was from Milipore-Sigma. F11 (mouse monoclonal antibody from AbnovaTM) for plasma studies, dextran sulfate (MW ca > 500,000), and Coomassie Brilliant Blue for gel electrophoresis were also from Fisher Scientific. Human FXIIa (α -form) and antibodies for western blot were from Enzyme Research Laboratories (South Bend, IN). The buffers used for enzyme assays were: a) 50 mM Tris-HCl buffer, pH 7.4, containing 100–150 mM NaCl, 0.1% PEG8000, and 0.02% Tween 80 for human thrombin, FXa, and FXIa; b) 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl_2 , 0.1% PEG8000, 0.02% Tween 80, and 33% v/v ethylene glycol for human FIXa; and c) 50 mM TrisHCl buffer, pH 8.0, containing 10 mM CaCl_2 and 100 mM NaCl for human FXIIIa.

Inhibition of FXIa in Chromogenic Substrate Hydrolysis Assay by Phosphonate/Phosphate Derivatives (1–4)

Direct inhibition of human FXIa was measured by the corresponding chromogenic substrate hydrolysis assay, as reported earlier^[54,55,60,61] at pH 7.4 and 37 °C. Each well of the 96-well microplate contained 85 μL of the buffer to which 5 μL of molecules **1–4** (or high pure water) and 5 μL of FXIa (0.765 nM) were sequentially added. Following 10-min incubation, 5 μL of FXIa substrate (345 μM) was rapidly added and the residual FXIa activity was measured from the initial rate of increase in absorbance at the wavelength of 405 nm. Stocks of the potential inhibitors were serially diluted. Relative residual FXIa activity at each concentration of the inhibitor was calculated from the ratio of FXIa activity in the presence and absence of the inhibitor. Logistic eq. 1 was used to fit the concentration dependence of residual FXIa activity so as to obtain the potency (IC_{50}) and efficacy ($\Delta Y\%$) of inhibition.

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

In this equation, Y is the ratio of residual FXIa activity in the presence of inhibitor to that in its absence, Y_M and Y_0 are the maximum and minimum possible values of the fractional residual FXIa activity, IC_{50} is the concentration of the inhibitor that leads to 50% inhibition of enzyme activity, and HS is the Hill slope. Y_M , Y_0 , IC_{50} , and HS values are determined by nonlinear curve fitting of the data.

Effect of Benzyl Tetraphosphonate **1** on Other Coagulation Factors

The inhibition potential of benzyl tetraphosphonate **1** against thrombin, FIXa, and FXa was also evaluated using the corresponding chromogenic substrate hydrolysis assays reported in our previous studies.^[54,55,60,61] Briefly, to each well of a 96-well microplate containing 185 μL of 20–50 mM Tris-HCl buffer, pH 7.4, containing 100–150 mM NaCl, 0.1% PEG8000, and 0.02% Tween80 at either 25 °C (thrombin) or 37 °C (FIXa and FXa) was added 5 μL of 0–1 mM benzyl tetraphosphonate **1** (or high pure water) and 5 μL of the enzyme. The final concentrations of the enzymes were 6 nM (thrombin), 89 nM (FIXa), and 1.09 nM (FXa). Following 10-min incubation, 5 μL of Spectrozyme TH (final conc. 50 μM), Spectrozyme FIXa (850 μM), or Spectrozyme FXa (125 μM), was rapidly added and the residual enzyme activity was measured from the initial rate of increase in absorbance at the wavelength of 405 nm. Relative residual enzyme activity as a function of the concentration of the inhibitor was calculated. Likewise, to measure the effect of benzyl tetraphosphonate **1** on human FXIIIa, a bi-substrate, fluorescence-based trans-glutamination assay was performed as we reported previously.^[62] Generally, 1 μL of molecule **1** was diluted with 87 μL of pH 7.4 buffer (50 mM Tris-HCl, 1 mM CaCl_2 , 100 mM NaCl, and 2 mg/mL *N,N*-dimethylcasein) and 5 μL dithiothreitol (20 mM) at 37 °C followed by the addition of 2 μL of human FXIIIa (0.3 μM) and incubation for 10 min. The activity of FXIIIa was monitored following the addition of 5 μL of dansylcadaverine (2 mM) by measuring the initial rate of increase in fluorescence emission ($\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 490$ nm). As with the above enzymes, relative residual FXIIIa activity as a function of the concentration of the inhibitor was calculated. In all of the above enzyme experiments, data were plotted using equation 1 above to obtain the corresponding IC_{50} values, only if 50% or more of enzyme inhibition was obtained.

Effect of Benzyl Tetraphosphonate 1 on Clotting Times in Human Plasmas

Clotting times (APTT and PT) were measured using the BBL Fibrosystem fibrometer (Becton-Dickinson, Sparks, MD), as reported in our previous studies.^[54,55,60,61] For the APTT assay, 10 μL of benzyl tetraphosphonate 1 was mixed with 90 μL of citrated human plasma and 100 μL of prewarmed APTT reagent (0.2% ellagic acid). After incubation for 4 min at 37 $^{\circ}\text{C}$, clotting was initiated by adding 100 μL of prewarmed 25 mM CaCl_2 , and the time to clotting was recorded. For the PT assay, thromboplastin-D was prepared according to the manufacturer's directions by adding 4 mL of distilled water, and then, the resulting mixture was warmed to 37 $^{\circ}\text{C}$. A 10 μL of benzyl tetraphosphonate 1 was then mixed with 90 μL of citrated human plasma and was subsequently incubated for 30 sec at 37 $^{\circ}\text{C}$. Following the addition of 200 μL of prewarmed thromboplastin-D preparation, the time to clotting was recorded. In the two assays, seven concentrations of the inhibitor were used over the concentration range of 0–500 μM to establish a concentration vs effect curve. The concentrations vs clotting times data were fitted to a quadratic trend line, which was eventually used to determine the concentration of the inhibitor necessary to increase the clotting time by 1.5-fold. Clotting times in the absence of an anticoagulant was determined in a similar fashion using 10 μL of deionized water and was found to be 34.4 ± 0.1 sec for APTT and 14.5 ± 2.1 sec for PT. To establish the FXIa-dependent effect of the inhibitor in human plasma, the APTT assay was repeated using FXI-deficient human plasma to which human FXIa (0, 2.4, and 4.8 nM) was added in the absence and the presence of benzyl tetraphosphonate 1 (0, 233, 327, and 467 μM).

Michaelis-Menten Kinetics for Chromogenic Substrate (S-2366) Hydrolysis by Human FXIa in the Presence of Benzyl Tetraphosphonate 1

The initial rate of S-2366, a chromogenic tripeptide substrate, hydrolysis by purified human FXIa was obtained from the linear increase in absorbance at the wavelength of 405 nm corresponding to the consumption of < 10% of the chromogenic substrate, as reported in our previous studies.^[54–56] The initial rate was measured as a function of various concentrations of the substrate (0–2000 μM) in the presence of a fixed concentration of benzyl tetraphosphonate 1 in 20 mM Tris–HCl buffer, pH 7.4, containing 100–150 mM NaCl, 0.1% PEG8000, and 0.02% Tween80 at 37 $^{\circ}\text{C}$. The experiment was conducted at five concentrations of the inhibitor: 0, 5, 10, 25, 50, and 100 μM . The data was fitted using the standard Michaelis–Menten equation 2 to determine the K_M (the affinity of the substrate to the active site of FXIa) and V_{MAX} (the maximum hydrolysis reaction velocity).

$$V = \frac{V_{\text{MAX}} [S]}{K_M + [S]} \quad (2)$$

Effect of Benzyl Tetraphosphonate 1 on FXIa-Mediated Activation of FIX

The experiments were done as reported in our earlier studies.^[54–56] FIX (6.2 μM) was incubated with FXIa (10 nM) in the presence of inhibitor 1 (0, 50, 250, and 1000 μM) in 50 mM HEPES supplemented with 5 mM CaCl_2 , at room temperature. Samples were incubated for 30 min. Following the 30-min incubation, the reactions were quenched using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing dithiothreitol and electrophoresed on a 10% SDS-

polyacrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue.

For Western blot experiment, plasma FIX (200 nM) was incubated at room temperature with human FXIa (40 nM) in 50 mM HEPES buffer supplemented 5 mM CaCl_2 . After 30-min incubation, SDS-PAGE loading buffer containing dithiothreitol was added, fractionated on 10% polyacrylamide-SDS gels, and then transferred to nitrocellulose membrane. The primary antibody was goat anti-human FIX polyclonal IgG, and the secondary antibody was horseradish peroxidase-conjugated anti-goat IgG. Detection was by chemiluminescence. The relative positions of FIX and FIXa bands were confirmed using Western blots of known standards for each protein.

Effect of Benzyl Tetraphosphonate 1 on FXIa – Antithrombin Complex Formation

The effect of inhibitor 1 on the complex formation between FXIa and antithrombin was performed in HEPES buffer supplemented with 5 mM of CaCl_2 , as reported earlier.^[49] Briefly, FXIa (300 nM) was pre-incubated with inhibitor 1 (0, 50, 250, and 1000 μM) at 37 $^{\circ}\text{C}$ for 5 minutes, and then combined with 2 μM purified human antithrombin in the presence of 2 μM sodium heparin for a further 30 minutes. At the incubation time, samples were quenched using SDS-PAGE loading gel buffer containing dithiothreitol and subjected to electrophoresis on 10% SDS-PAGE. Protein bands were visualized by staining with silver stain.

Effect of Benzyl Tetraphosphonate 1 on FXI Activation by Thrombin

Thrombin-mediated FXI activation was analyzed by SDS-PAGE, as reported earlier.^[49,64–68] FXI (700 nM) was incubated with α -thrombin (70 nM) with or without dextran sulfate (10 $\mu\text{g}/\text{ml}$) in the presence of different concentrations of inhibitor 1 (2, 20, 50, 250, and 1000 μM) in HEPES buffer supplemented with 5 mM of CaCl_2 . Samples were incubated for 60 mins. After the incubation period, reactions were quenched using argatroban (2 μM) and polybrene (6 $\mu\text{g}/\text{ml}$). samples were placed into reducing sample buffer, size fractionated on 10% polyacrylamide-SDS gel and stained with silver staining.

Effect of Benzyl Tetraphosphonate 1 on FXI Activation by FXIIa

FXIIa-mediated activation of FXI activation was analyzed by SDS-PAGE, as reported earlier.^[49,64–68] FXI (700 nM) was incubated with α -FXIIa (200 nM) with or without dextran sulfate (2 $\mu\text{g}/\text{ml}$) in the presence of different concentrations of inhibitor 1 (50, 250, and 1000 μM) in HEPES buffer supplemented with 5 mM of CaCl_2 . Samples were incubated for 60 mins. After the incubation period, reactions were quenched using corn trypsin inhibitor (1 μM) and polybrene (6 $\mu\text{g}/\text{ml}$). samples were placed into reducing sample buffer, size fractionated on 10% polyacrylamide-SDS gel and stained with silver staining.

Molecular Modeling Studies of FXIa and Benzyl Tetraphosphonate 1

The docking studies were carried out using Glide of Schrodinger Suite 2017–1.^[70] Initial coordinates for FXIa were taken from the crystal structure of FXIa in complex with α -ketothiazole arginine-based ligand (PDB: 2FDA).^[71] The protein structure was prepared by removing the crystallographic water molecules and the crystal

ligand, and by adding hydrogen atoms consistent with the physiologic pH of 7.0 using Maestro 11.1 of Schrodinger Suite. The protein molecule was then energy minimized with an RMSD cutoff value of 0.3 Å for all heavy atoms. Initial coordinates for benzyl tetraphosphonate **1** were built and energy minimized using the Schrodinger Suite. The basic residue of K529, R530, R532, K535, and K539 in the catalytic domain and the surrounding area of these amino acids were specified as the ligand binding site. The grids for the target protein were generated using the OPLS3 forcefield.^[83] The grid center was set to be the centroid of the above basic residues, with a cubic grid box of 10 Å on each side. No constraints were used in the grid generations. The docking calculations were done using the default parameters under the stand precision mode. All the poses were subjected to post-docking minimization. The best-docked structure based on the docking score was selected for subsequent analysis. The chymotrypsin-based numbering for the presented amino acids is as follows: T523=T164, N524=N165, K529=K170, R530=R171, R532=R173, K535=K175, and K539=K179.

Abbreviations

APTT, activated partial thromboplastin time; COVID-19, coronavirus disease of 2019; FVIIa, factor VIIa; FIXa, factor IXa; FXI, factor XI; FXIa, factor XIa; FXIIa, factor XIIa; PT, prothrombin time; SDS, sodium dodecyl sulfate.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: factor XIa · allosteric inhibitors · anticoagulants · molecular modeling · phosphonate derivatives

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