



## Allosteric activation of human $\alpha$ -thrombin through exosite 2 by suramin analogs

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### ABSTRACT

Thrombin is a serine protease that plays fundamental roles in hemostasis. We have recently elucidated the crystal structure of thrombin in complex with suramin, evidencing the interaction through the anion binding exosite 2. Here, we show that the activity of thrombin toward natural and synthetic substrates is enhanced by suramin as well as analogs of suramin at a low micromolar range prior to an inhibitory component at higher concentrations. Suramin analogs substituted by phenyl and chlorine instead of methyl were the most efficient in promoting allosteric activation, with an enhancement of enzymatic activity of 250% and 630% respectively. We discuss the importance of exosite 2 as a regulatory site for ligands in both the procoagulant and inhibitory scenarios.

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### Introduction

Alpha-thrombin is a serine protease that plays fundamental roles in the coagulation and fibrinolytic pathways and is therefore an important target in drug design. Thrombin plays a central role in the coagulation cascade in injuries and other conditions related to hemostasis and system homeostasis. It mediates the conversion of fibrinogen to fibrin and the activation of platelets and coagulation factors V, VIII, XI, and XIII [1–5]. The precise control of coagulation is inherently dependent on a fine-tuned regulation of both the procoagulant and the anticoagulant events. Thrombin has few substrate requirements. In contrast, it can be modulated by a large number of cofactors at distinguished physiopathological condi-

tions, a reflex of its correspondence between conformational diversity and activation-inhibition profile [6–9].

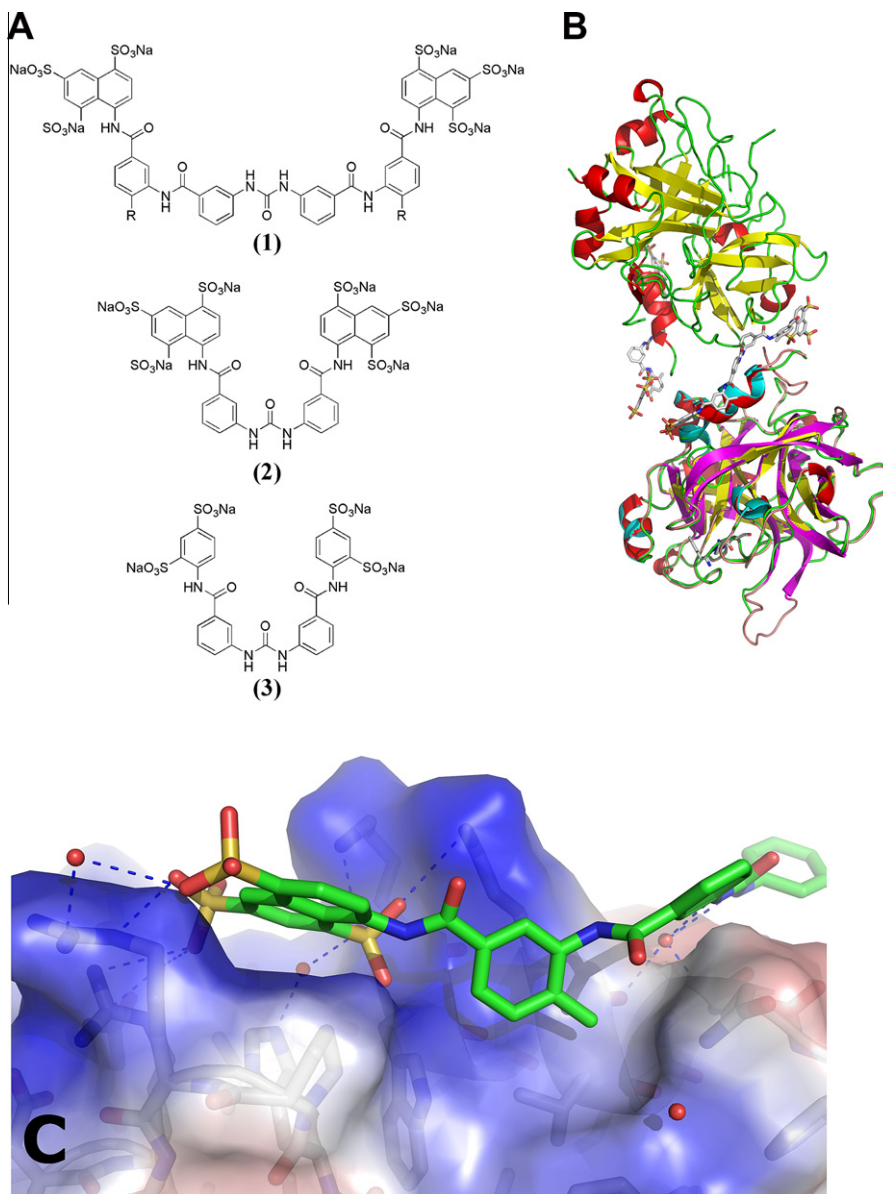
Thrombin is composed of two disulfide-linked chains, L (“light”) and H (“heavy”), with the catalytic site located in the H chain. The understanding of the ligand recognition at the active site on a molecular basis is key in the development of new compounds as drug candidates targeting the coagulation process. The recognition and binding of thrombin is mainly performed by two patches in the thrombin surface, known as anion binding exosites 1 and 2. Exosite 1 is responsible for the recognition of fibrin, fibrinogen, protease-activated receptor 1 (PAR-1) [10], and thrombomodulin, among other ligands [11,12]. Exosite 2 is well known for its role as the heparin-binding exosite.

**Abbreviations used:** FPRCK, D-Phe-Pro-Arg-chloromethylketone; Suramin, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-methylphenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF037, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-phenylenecarbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF127, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-ethylphenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF151, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-isopropylphenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF198, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-phenylphenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF157, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-fluorophenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF258, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-chlorophenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF260, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-methoxyphenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF222, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-(methoxymethyl)phenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF023, 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; MK012, 4,4'-[carbonylbis[imino-3,1-phenylenecarbonylimino]]bis[benzene-1,3-disulfonic acid] tetrasodium salt; S-2238, H-D-Phe-Pip-Arg-p-nitroanilide; TBS, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5.

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**Fig. 1.** Suramin, suramin analogs and interaction with thrombin. (A) Chemical structure of suramin and suramin analogs. Compound nomenclatures are as stated in Table 1. (B) Ribbon representation of the thrombin:suramin complex (PDB entry 2H9T; red for alpha-helices, yellow for beta-sheet) in superposition with free thrombin (PDB entry 1PPB; cyan for alpha-helices, magenta for beta-sheet), rotated 45° in the X-axis from the standard orientation. (C) Details of the suramin interaction with the thrombin exosite 2. Dotted lines represent water (red spheres) mediated contacts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Suramin (Fig. 1A; compound (1b)) is a hexasulfonate compound that has been frequently used in the treatment of African trypanosomiasis and filariasis and has served as a lead compound in the development of derivatives designed for the treatment of tropical diseases, cancer, and hemostatic effects of snake venom, among others [13–17].

Suramin was found to interact with thrombin through the anion binding exosite 2, thus inhibiting the activity of thrombin toward fibrinogen and the synthetic substrate S-2238 [18,19]. Calorimetric measurements suggested that the interaction occurred through at least two separate events in a concentration-dependent mode [18]. In a previous study, we have shown that a thrombin:suramin dimer can exist in solution [19]. Molecular dynamic simulation showed that while a dimer of the thrombin:suramin complex (Fig. 1B) is conformationally stable compared to the crystal structure, the monomeric form of the thrombin:suramin complex

undergoes deviations from the initial conformation in the crystal matrix, as well as suramin, whose coordinates are affected except for the naphthalenesulfonate moiety, which is responsible for anchoring to the anion binding exosite 2 (Fig. 1C) [19].

Another important insight obtained from the thrombin:suramin crystal structure is the presence of a shallow cavity close to one of the suramin methyl groups [19]. This cavity is occupied by a water molecule trapped within the interactions with the Gln244 side chain, the phenolic hydroxyl from Tyr89, and the amidic carbonyl from Val241 (Fig. 1C). These features create a polar environment in this cavity, providing a repulsive interaction with the suramin methyl group. Functionalization of suramin at this position would allow modulation of the interaction potential with thrombin.

In the present work, we report the investigation of a series of suramin analogs (Fig. 1A) and their effects on thrombin activity against the natural substrate fibrinogen. A dual effect over the

catalytic properties of thrombin was observed, with an inhibitory component preceded by an activation phase. We discuss the allosteric nature of these phenomena in the light of a possible procoagulant regulatory function of the anion binding exosite 2.

## Experimental procedures

### Materials

Distilled water was deionized to less than 1.0  $\mu\text{S}$  and filtered through a 0.22  $\mu\text{m}$  pore-sized membrane with a water purification system prior to use. S-2238 was obtained from Chromogenix (Möln dal, Sweden). Suramin was purchased from Sigma Chemical (St. Louis, MO). Suramin analogs were synthesized as described elsewhere [16]. Human fibrinogen was purchased from Calbiochem (La Jolla, CA). Fluorescein-D-Phe-Pro-Arg-chloromethyl ketone (PPACK) was from Hematologic Technologies Inc (Essex Junction, VT). Human thrombin was purified as previously described [20]. Protein concentrations were determined by UV absorbance at 280 nm with an extinction coefficient of 1.82  $\text{mg mL}^{-1} \text{cm}^{-1}$  [21,22]. All other reagents were of analytical grade. All buffers and solutions were prepared immediately prior to use.

### Assay for fibrinogen clotting

Fibrinogen clotting was measured on a microplate reader (Molecular Devices, Menlo Park, CA), as described elsewhere [23]. After a 5 min incubation of thrombin with suramin analogs at 37 °C, the reaction was initiated by the addition of human fibrinogen (4 mg/mL, final concentration). The initial rate of fibrinogen clotting was determined by the increase in the absorbance at 405 nm. All experiments were performed in triplicate, with three protein batches.

### Assay for S-2238 hydrolysis

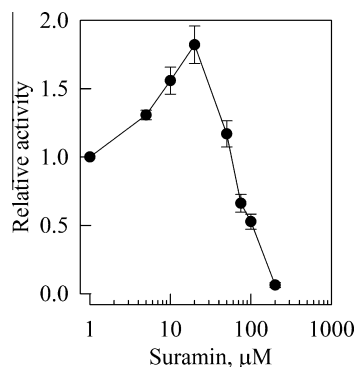
The hydrolysis of S-2238 (1 mM) by thrombin (1 nM) was measured in 20 mM Tris-HCl, 100 mM NaCl, pH 7.5, on a microplate reader (Molecular Devices, Menlo Park, CA), after 5 min incubation at 37 °C, with varying suramin analog concentrations serially diluted. The reaction was initiated by the addition of S-2238, and the initial rate of p-nitroaniline release was determined by following the increase in absorbance at 405 nm. The original activity of thrombin was calculated as the rate of S-2238 hydrolysis in the absence of ligands. All experiments were performed in triplicate, with three protein batches.

### Fluorescence polarization measurements

Fluorescence polarization measurements were performed in a Spectramax M5 microplate reader (Molecular Devices), using fluorescein-D-Phe-Pro-Arg-chloromethyl ketone (F-PPACK) – labeled thrombin (70 nM) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 25 °C as previously described [6], by setting excitation to 490 nm and measuring the fluorescence emission at 520 nm. All measurements were performed in triplicate.

## Results

It has been previously shown that suramin (1b) exerts an inhibitory effect on the hydrolytic activity of thrombin toward both the natural substrate fibrinogen and the synthetic substrate S-2238 [18]. This effect is observed at moderate to high micromolar concentrations (Fig 2). Measurements performed at lower suramin



**Fig. 2.** Suramin exerts dual effects over fibrinogen clotting by thrombin. Effects of increasing concentrations of suramin in the activity of thrombin (1 nM) against fibrinogen (2 mg/mL) clotting. The ordinate is the relative enzymatic activity, corresponding to the ratio between the initial rate of fibrinogen clotting in the presence/initial rate (in the absence of suramin). Data represent the mean  $\pm$  S.D. of three independent determinations performed in triplicate.

concentrations revealed another catalytic component resulting from their interaction. Interestingly, at low micromolar concentration ranges and below, suramin led to a pronounced increase in thrombin activity, followed by the inhibitory component (Fig 2). Maximum activation occurred at 16  $\mu\text{M}$  suramin, reaching approximately 80% of activity enhancement (Table 1). The concentration of suramin that produced half-maximal inhibition ( $\text{IC}_{50}$ ) was approximately 65  $\mu\text{M}$ , in accordance with the previous evaluation [18].

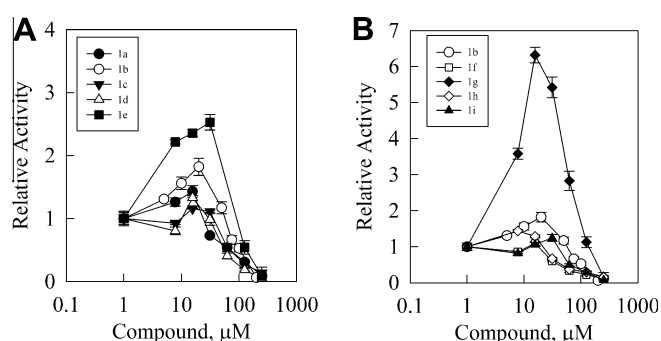
To characterize the molecular origins of the activation profile, we have evaluated a number of suramin analogs bearing groups of different polarity (e.g., alkyl, phenyl, halogen and methoxy substituents) in place of the methyl groups (1a–i; Table 1)<sup>a</sup>. This homologous series of large ureas (1a–i) showed similar behavior as suramin (1b) itself, with an activation profile at low concentrations (7–30  $\mu\text{M}$ ), which lies within the same potency range (Fig. 3A and B). They displayed maximum relative activities in the range of 20–50% enhancement, as shown in Table 1, except for the phenyl (1e) and chlorine (1g) substituents, which exerted enhanced abilities of thrombin activation against fibrinogen, with relative maximum activities of 2.6 and 6.3  $\mu\text{M}$ , respectively. The inhibitory phase occurred at higher compound concentrations, with  $\text{IC}_{50}$  values in the range of 40–70  $\mu\text{M}$ , except for the phenyl (1e) and chlorine (1g) substituents, which have  $\text{IC}_{50}$  values of 129 and 197  $\mu\text{M}$ , respectively.

Fibrinogen interacts with thrombin through its active site and exosite 1[24–26]. In contrast, suramin interacts with thrombin through exosite 2, as shown by enzymatic [18] and crystallographic data[19]. Therefore, it is likely that the catalytic modulation by suramin is an allosteric event mediated by the ligand binding to exosite 2. To address this hypothesis, we evaluated the effects of suramin and its analogs on the hydrolysis of the small synthetic substrate S-2238 (Fig. 4), an active site-specific ligand, by thrombin. As seen with fibrinogen, the suramin analogs induced a dual catalytic effect on thrombin activity over S-2238, supporting the evidence for the allosteric nature of thrombin modulation by suramin for both the activation and inhibition components (Fig. 4).

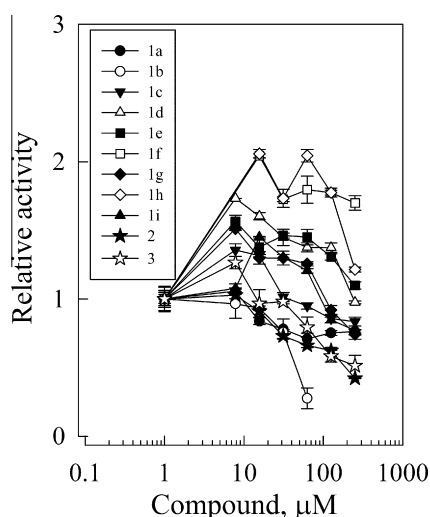
It is interesting to note that inhibitory effect of suramin towards S-2238 cleavage is much less pronounced than the inhibition of the fibrinogen cleavage by thrombin. Suramin is a large symmetric urea linking two polysulfonated naphthyl rings, comprising two strongly anionic clusters. In our previous work, we have shown by small-angle X-ray scattering and protein crystallography that thrombin forms a dimer upon binding to suramin [19]. This dimerization of thrombin occurs by the anchoring of two suramin molecules, each one binding to both thrombin molecules. Therefore, we

**Table 1**  
Activity of suramin analogs. Data were calculated from Fig 3.

Compound	-R	Concentration at maximum activity ( $\mu\text{M}$ )	Maximum relative activity	EC <sub>50</sub> ( $\mu\text{M}$ )	IC <sub>50</sub> ( $\mu\text{M}$ )
1a (NF037)	-H	16	1.43 $\pm$ 0.09	6	63
1b (Suramin)	-CH <sub>3</sub>	20	1.82 $\pm$ 0.14	7	65
1c (NF127)	-CH <sub>2</sub> CH <sub>3</sub>	16	1.16 $\pm$ 0.01	13	61
1d (NF151)	-CH(CH <sub>3</sub> ) <sub>2</sub>	16	1.33 $\pm$ 0.04	14	56
1e (NF198)	-C <sub>6</sub> H <sub>5</sub>	32	2.53 $\pm$ 0.06	5	129
1f (NF157)	-F	16	1.09 $\pm$ 0.06	13	36
1g (NF258)	-Cl	16	6.32 $\pm$ 0.21	7	197
1h (NF260)	-OCH <sub>3</sub>	8	1.44 $\pm$ 0.04	4	41
1i (NF222)	-CH <sub>2</sub> OCH <sub>3</sub>	32	1.24 $\pm$ 0.09	21	60
2 (NF023)	-H	32	1.40 $\pm$ 0.05	21	180
3 (MK012)	-H	32	2.11 $\pm$ 0.02	13	182



**Fig. 3.** Effects of suramin analogs on thrombin catalytic activity over fibrinogen. Effects of increasing concentrations of suramin analogs over the activity of thrombin (1 nM) against fibrinogen (2 mg/mL) clotting. Ordinates are the relative enzymatic activity, corresponding to the ratio between the initial rate of fibrinogen clotting in the presence/initial rate in its absence of the suramin analogs. Data represent the mean  $\pm$  S.D. of three independent determinations performed in triplicate. (A) Compounds (1a) to (1e); (B) Compounds (1f) to (1i) and suramin (1b). The enzymatic parameters obtained from these datasets, such as maximum activity and IC<sub>50</sub>, are presented in Table 1.

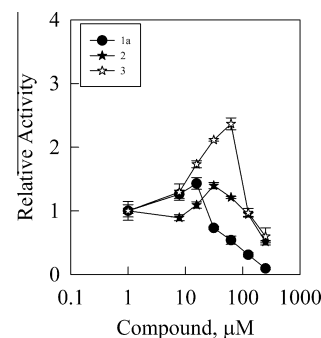


**Fig. 4.** Effects of suramin analogs on the thrombin hydrolysis of the chromogenic substrate S-2238. Effects of increasing concentrations of suramin analogs over the activity of thrombin (1 nM) against S-2238 (1 mM). The ordinate is the relative enzymatic activity, corresponding to the ratio between the initial rate of S-2238 hydrolysis in the presence/initial rate in its absence of suramin analogs (1a) to (1i), (2) and (3), as stated in the figure. Data represent the mean  $\pm$  S.D. of three independent determinations performed in triplicate.

hypothesize that a biphasic event would be independently mediated by separated binding events: at first, a 1:1 thrombin:suramin complex resulting in the activation pattern, and thus a dimerization event in the [thrombin:suramin]<sub>2</sub> complex leading to the inhibitory process.

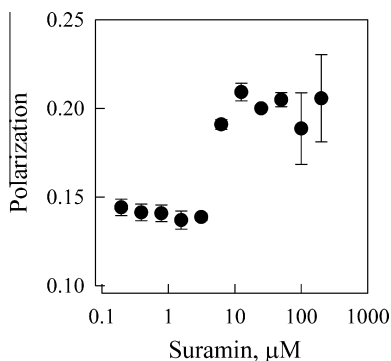
To address this issue, we further tested smaller suramin analogs with varying size and sulfonation degree, all of them with the methyl group replaced by a hydrogen atom. Compound (3) is a urea similar to compound (2), but with a phenyl-disulfonate instead of a naphthalene trisulfonate. Reduction of the size and thus the distance between the two naphthalene trisulfonate groups progressively led to an enhancement of activation potency at the cost of the reduction of inhibition efficacy (Fig. 5). This finding might be due to the reduced ability of this shorter urea in promoting thrombin dimerization, while the activation element would rely mostly on suramin binding to the thrombin exosite 2.

In order to gain further insights on the mechanisms of thrombin activation and inhibition induced by suramin, we have assayed for their interaction by fluorescence polarization, using thrombin labeled with fluorescein through the irreversible active-site inhibitor D-Phe-Pro-Arg-chloromethyl ketone (PPACK). Fluorescence polarization has long been used for the quantitative analysis of macromolecular association [27–30]. Free fluorescein-labeled thrombin showed a polarization value of about 0.14, which is compatible with a monomeric form of thrombin (Fig. 6). Incubation of fluorescein-thrombin with varying amounts of suramin up to 10  $\mu\text{M}$  did not result in changes in fluorescence polarization. Further increase of suramin concentration resulted in the progressive augment in the fluorescence polarization of fluorescein-labeled thrombin from 0.14 to about 0.20, which can be attributed to an increase in its hydrodynamic radii. The binding of a single suramin molecule to



**Fig. 5.** Minimal domains for thrombin activation. Suramin analogs were screened for their activities over thrombin (1 nM) against fibrinogen (2 mg/mL) clotting. The ordinate is the relative enzymatic activity, corresponding to initial rate of fibrinogen clotting in the presence/initial rate in its absence of suramin analogs, as stated in the figure. Data represent the mean  $\pm$  S.D. of three independent determinations performed in triplicate.





**Fig. 6.** Thrombin association induced by suramin probed by fluorescence polarization. Fluorescein-PPACK-thrombin (70 nM) was incubated with varying concentration of suramin and the fluorescence polarization was monitored. Data represent the mean  $\pm$  standard error of three independent determinations.

thrombin is unlikely to be the underlying cause of the rise in fluorescence polarization due to its low molecular weight. Instead, we attribute such phenomena to the association between thrombin molecules, such as the formation of dimers of the complex [thrombin:suramin], in accordance with previous evidences obtained by SAXS data [19].

## Discussion

The anion binding exosite 2 has long been seen as a target for the design of sulfated allosteric inhibitors of thrombin [31–34]. Exosite 2 may interact with a number of ligands, including sulfated compounds, such as the prothrombin activation fragment 2 (F2) [35], fibrin  $\gamma'$  peptide (sulfated at tyrosine; fibrin itself interacts with exosite 1) and platelet glycoprotein Ib [36,37], which is one of the four proteins in the GPIb-IX-V platelet receptor complex.

GPIb is sulfated at tyrosine residues 276, 278 and 279, which are known for their interaction with thrombin at exosite 2 [38] at the same interaction site as the sulfonated naphthyl group of suramin [19]. The crystal structure of thrombin in complex with the fibrin  $\gamma'$  peptide (PDB ID 2HWL) reveals a dimer in the asymmetric unit (as modeled from crystal symmetry) that is maintained by the cross interaction of one fibrin  $\gamma'$  peptide with the exosite 2 from two distinct thrombin monomers [39]. The sulfated Tyr422 of the fibrin  $\gamma'$  peptide interacts at the same epitope of thrombin that is responsible for heparin recognition [39], which is also the site of interaction for the sulfonated moiety of suramin.

These cofactors that recognize the allosteric procoagulant activities that exosite 2 exerts over thrombin have a common feature, which is that they have at least one sulfated amino acid at the protein domain which is responsible for the recognition and binding to thrombin. For suramin, we have found two opposing behaviors in its interaction with thrombin: the activation and inhibition of catalytic activity. Previous calorimetric evidences have also suggested two molecular events in the interaction of thrombin with suramin [18], which has been corroborated by crystal and solution structural studies [19]. However, the molecular binding mechanism between thrombin and suramin and its consequence on the behavior of single-phased enzyme activity was not exactly clear.

In this study, we have shown that submicromolar concentrations of suramin and suramin analogues, below the concentration previously investigated, were sufficient to increase thrombin enzymatic activities toward both natural and synthetic substrates. In fact, only part of the symmetric suramin molecule seems to exert the activation effect over thrombin, as it was observed that decreasing the urea size progressively increases its activation

efficacy over thrombin activity (Fig. 5). Moreover, the large urea series varies only at the methyl group position. However, the chlorine (1e) and phenyl (1g) substituted suramin analogs displayed higher activations compared to the other compounds in this series. These data suggest that the molecular volume of the substituents may play an important role on the allosteric regulation of thrombin. Such substituents are likely to occupy a cavity in the thrombin delimited by side chains from Gln256 Trp237, Tyr89, and Val241 (Fig. 1C), creating a peculiar, mixed environment.

At present, our data indicate that part of the suramin molecule delimited by the region comprised between the methyl-substituted ring and the disulfonated aromatic ring moiety comprise a minimal ligand motif for thrombin activation through exosite 2. Moreover, in conjunction with the previous crystal structure of thrombin in complex with heparin, which reveals an oligomer in the asymmetric unit [40] through exosite 2, these data indicate that thrombin dimerization can be mediated by exosite 2 cofactors, leading to potential inhibitory effects over thrombin. These data allow us to raise the hypothesis for a cofactor-mediated, oligomerization-driven mechanism for the reduction of clotting rate.

In the present study we have evaluated the effect of suramin over the enzymatic activity of thrombin against two dissimilar substrates, the small chromogenic peptide S-2238 and the natural ligand fibrinogen. We have observed that suramin exerts a more pronounced inhibitory effect over fibrinogen cleavage by thrombin compared to the cleavage of the short S-2238 molecule. While S-2238 interaction with thrombin relies in contacts established between the ligand and the active cleft of the enzyme, fibrinogen cleavage by thrombin requires contacts at both the active site and the anion binding exosite 1. In fact, the ability of ligands to exosites 1 and 2 in promoting allosteric changes on the catalytic site of thrombin has been well documented, and several evidences suggest a long range allosteric linkage between exosites 1 and 2 [41–43]. Thus, exosite 2 ligands would induce allosteric change in thrombin resulting in altered affinity of ligands to exosite 1, which could be related to the ability of suramin in altering the efficacy of thrombin in promoting fibrinogen clotting.

The two anion binding exosites in thrombin are important for substrate and/or cofactor binding and regulation. It has been suggested that the binding features of exosite 1 contain an extensive contribution from the hydrophobic component, while the cofactor binding to exosite 2 is largely driven by electrostatic forces [44]. In fact, it is likely that the interaction of suramin and suramin analogs with exosite 2 is highly favorable by the electrostatic contributions of their sulfonated groups and basic residues from thrombin [19]. It is even more likely that most natural cofactors of exosite 2 are sulfated compounds.

Collectively, these data shed light on a distinguished feature of exosite 2 that exerts a key role in thrombin regulation as a procoagulant allosteric site in the immediate beginning of the clotting event in the blood coagulation cascade through the binding of sulfated cofactors. Additionally, it is highly desirable to develop procoagulants (small-molecule compounds targeting coagulation enzymes such as thrombin) as a therapeutic strategy to control bleeding and manage blood loss in clinical situations, such as during surgical procedures. This strategy can be implemented to avoid antibody-mediated coagulopathy against bovine thrombin or immunogenic contaminants in recombinant human thrombin, which are the current therapeutic strategies.

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