Alboserpin, a Factor Xa inhibitor from the mosquito vector of Yellow Fever, binds heparin and membrane phospholipids and exhibits antithrombotic activity
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Running title: Factor Xa inhibition by alboserpin

Molecular mechanism of FXa inhibition by Alboserpin, the major salivary gland anticoagulant from the mosquito and yellow fever vector Aedes albopictus, has been characterized. cDNA of Alboserpin predicts a 45-kDa protein that belongs to the serpin family of protease inhibitors. Recombinant Alboserpin displays stoichiometric, competitive, reversible and tight binding to FXa (pM range). Binding is highly specific and is not detectable for FX, catalytic site-blocked FXa, thrombin and 12 other enzymes. Alboserpin displays high-affinity binding to heparin (Kd~20 nM), but no change in FXa inhibition was observed in the presence of the cofactor, implying that bridging mechanisms did not take place. Notably, alboserpin was also found to interact with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) but not with phosphatidylserine (PS). Further, annexin V (in the absence of Ca2+) or heparin outcompetes Alboserpin for binding to phospholipid vesicles, suggesting a common binding site. Consistent with its activity, Alboserpin blocks prothrombinase activity and increases both PT and aPTT in vitro or ex vivo. Furthermore, Alboserpin prevents thrombus formation provoked by ferric chloride injury of the carotid artery and increases bleeding in a dose-dependent manner. Alboserpin emerges as an atypical serpin that targets FXa and displays unique phospholipids specificity. It conceivably uses heparin and PC/PE as anchors to increase protein localization and effective concentration at sites of injury, cell activation or inflammation.

To acquire a blood meal, haematophagous arthropods usually break the host's skin stratum corneum (1-2). As a result, wounded tissues and injured blood vessels trigger the host hemostatic process which is a complex and redundant, interlinked biological system consisting of platelet aggregation, blood clotting, and vasoconstriction (3-5). The clotting cascade can be activated by the intrinsic or extrinsic pathways, both converging to the activation of factor X (FX) to FXa, which converts prothrombin to thrombin; the latter cleaves fibrinogen to produce fibrin (6). FXa also plays a pivotal role in inflammation through activation of protease-activated receptors (7). In order to counteract host response to injury, salivary glands (SGs) of blood-sucking arthropods express a number of inhibitors of blood coagulation, platelet aggregation, host immunity, inflammation, angiogenesis, neutrophil function, wound healing and vasodilation (1,8-16). Among anticoagulants, inhibitors targeting FVIIa/Tissue Factor, thrombin, FXa, FIXa, FXIa, and HMWK have been reported (17-
20). Of note, members of different protein families including Kunitz-, ascaris-, antistasin-like, and SALP have been identified as FXa inhibitors (17,21). Notably, the only serine protease inhibitor (serpin) described so far and found to target FXa was identified in the SG of the mosquito *Aedes aegypti* (22).

Serpins are a large and growing superfamily of structurally homologous yet functionally diverse proteins. Serpins have been identified in species of all kingdoms. These protease inhibitors act to control proteolytic activity in a myriad of contexts including coagulation, fibrinolysis, and complement activation. They can also be found in the intracellular compartment and perform biologic functions that do not necessarily require their protease inhibitory function. Approximately 500 serpins are currently known, consisting of between 350 and 400 amino acids with molecular weights ranging from 40 to 55 kDa (23-25). In this study, we describe the main anticoagulant from *Aedes albopictus* SGs. We provide experimental evidence indicating that the molecule responsible for this salivary activity belongs to the serpin superfamily (hereafter named Alboserpin). We show that recombinant Alboserpin is a highly specific, tight inhibitor of FXa. Both recombinant Alboserpin and *Ae. albopictus* saliva do not bind to FX or active site-blocked DEGR-FXa. Moreover, Alboserpin binds heparin; notably, it also interacts with phosphatidylcholine (PC), and phosphatidylethanolamine (PE) but not with phosphatidylserine (PS). In addition, it displays potent antithrombotic properties in vivo.

**Methods**

Unless otherwise indicated, the protocols followed standard procedures (26) and all experiments were performed at room temperature (25 ± 1°C). All water used was of 18 MΩ quality, produced by a MilliQ apparatus (Millipore, Bedford, MA). Heparin (low molecular weight, 4–6 kDa average) from porcine intestinal mucosa was purchase from Sigma Chemical Co. (St. Louis, MO). Egg L-α-PC and brain L-α-PS were obtained from Avanti Polar Lipids (Birmingham, AL). Phosphatidylethanolamine (PE) from bovine brain was purchased from Sigma Chemical Co.

**Phylogenetic analysis of the serpin family**

BLASTP analysis was performed with Alboserpin (GI: 56417456) against the nonredundant database, and all blood-feeding insect sequences were retrieved. Sequences were cleaned up to obtain a nonredundant set (proteins with >95% identity in the core domain were treated as identical) and aligned with ClustalX (27-28). Alignments were manually checked, adjusted, and trimmed to include the conserved serpin core. Phylogenetic analysis was performed using Neighbor-joining analysis (29). Gapped positions were treated by pairwise deletion. Poisson correction was used as a substitution model to determine pairwise distances. Confidence was determined using bootstrap analysis (10,000 replicates) with 346 informative sites.

**Modeling and electrostatic surface calculations**

The structure of Alboserpin was modeled using the automatic modeling mode in SWISS-MODEL (30). Alboserpin coordinates were loaded on Pymol and the electrostatic surface calculated using the APBS tools. The template structure was the antithrombin-S195A factor Xa-pentasaccharide complex (2GD4). Secondary structure was obtained based on alboserpin model.
**Ae. albopictus collection of saliva and SG dissection**

Samples of saliva from female *Ae. albopictus* mosquitoes were collected by oil-induced salivation. After saliva collection, the sample was spun down at 14000×*g* in a bench-top centrifuge and the lower phase, containing the saliva, was transferred to a clean Eppendorf tube. SGs were dissected as indicated (31). The protein concentration from the collected saliva and SG extracts was estimated spectrophotometrically in a ND1000 (NanoDrop Technologies, Wilmington, DE).

**Expression of Alboserpin in *Escherichia coli***

For bacterial expression of recombinant Alboserpin, a synthetic gene was designed coding for the mature protein, which also contains *NdeI* and *XhoI* restriction sites. The synthetic Alboserpin gene was subcloned into pET-17b (Biobasic Inc., Markham, Ontario, Canada) for expression in *E. coli* (BL21pLYS) cells. Recombinant protein production and inclusion body preparation were carried out as indicated (9). The inclusion bodies were solubilized in 20 mM Tris-HCl, pH 7.4, 6 M guanidinium hydrochloride, 15 mM dithiothreitol, 1 mM EDTA. The solubilized material was diluted in 4 liters of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM GSSG, 1 mM GSH, and 200 mM arginine monohydrochloride and incubated overnight. Refolded Alboserpin was concentrated and purified as indicated (9). The purified recombinant Alboserpin protein was submitted to automated Edman degradation for N-terminal sequencing. Concentration of purified Alboserpin (corrected for ε280nm = 44410) (calculated using software from DNAStar Inc., Madison, WI) was estimated by its absorbance at 280 nm using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies).

**Anticoagulant assays**

Anticlotting measurements were performed by either measuring the recalcification time as described before (32) or prolongation of the activated partial thromboplastin time (APTT) and prothrombin time (PT). APTT was carried out as supplied by the APTT Reagent kit (Helena Laboratories, Beaumont, TX). Briefly, 30 µL of recombinant Alboserpin or SG extracts at different concentrations and 30 µL normal reference plasma (American Diagnostica, Greenwich, CT) were incubated for 10 minutes at room temperature before adding 30 µL of ALEXIN LS (diluted 1:3 in 20 mM HEPES, 120 mM NaCl, pH 7.4). After 5 minutes, clotting was induced with 30 µL of 20 mM CaCl2, 20 mM HEPES, 120 mM NaCl, pH 7.4, and measured at 650 nm every 11 seconds for 30 minutes. PT was measured under the same conditions described above, replacing ALEXIN reagent for Thromboplastin reagent (Helena Laboratories) diluted 1:2 in 20 mM HEPES, 120 mM NaCl, pH 7.4. All readings were performed in a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA).

**Kinetics of FXa inhibition by Alboserpin**

All reactions were carried out at 37°C. Five SG pairs from adult female mosquitoes (2–4 days old, non-bloodfed) were dissected under stereoscopic microscope in 20 µL of PBS (0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4) and kept at −80°C until use. Factors X and Xa were obtained from Hematologic Technologies Inc. (Essex Junction, VT), and chromogenic substrate S2222 (N-Benzoyl-L-isoleucyl-L-glutamyl-L-arginine-p-nitroaniline hydrochloride) was purchased from...
DiaPharma Group Inc. (Westchester, OH). Recombinant Alboserpin at concentration ranging from 0.1 to 14.5 nM or SG extract (0.0023 to 0.3 µg/µL) were incubated with either 1, 3, or 6 nM of FXa in 50 mM Tris, 120 mM NaCl, 5 mM CaCl₂, 0.1% (w/v) BSA, pH 7.4, for 20 minutes at room temperature in a final volume of 100 µL. The reaction was initiated by adding 375 µM of S2222 and the FXa activity measured spectrophotometrically at 405 nm for 1 hour in a plate reader (Thermomax Microplate Reader; Molecular Devices).

For the inhibition constant determination of Alboserpin for FXa, the SpectroFluor FXa fluorogenic substrate (American Diagnostica) was used. Briefly, 25 pM of factor Xa was allowed to interact with increasing inhibitor concentrations (0–525 pM) in the presence of varying concentrations of substrate (100–500 µM), in 50 mM Tris, 120 mM NaCl, 5 mM CaCl₂, 0.1% BSA, pH 7.4. Assays were performed in the 96-well plate format and were initiated by the addition of the enzyme to a mixture containing substrate and Alboserpin; reactions were followed for 30 minutes. Enzymatic activity was measured from the increase in absorbance of the free chromophore (AMC) generated by substrate hydrolysis (λₑₓ = 360 nM, λₑₘ = 440 nM) using a SpectraMax Gemini XPS plate reader linked to SOFTmax Pro 3.0 software (Molecular Devices). For kinetics, the percent inhibition of the reaction at different substrate concentrations was calculated from a control reaction containing only vehicle and data fitted using Morrison equation (equation 1) as reported (19),

\[
V_s/V_o = \frac{1}{(1 + (I_0 - [I]) - K_{i*} + ([I] - K_{i*} - [I])^2 + 4K_{i*}[I]^{1/2})/2[I]}
\]

(Equation 1)

where \(K_{i*}\) is the apparent dissociation constant for the enzyme–inhibitor complex, \(V_s\) is the inhibited steady-state velocity, \(V_o\) is the control (uninhibited) velocity, \([I]\) is the total inhibitor concentration, and \([E]\) is the total FXa concentration.

**Affinity chromatography on a heparin-sepharose column**

Two µM of FXa was incubated for 2 minutes in the absence or presence of Alboserpin (5 µM) in 50 mM Tris-HCl, pH 7.4, buffer containing 5 mM CaCl₂. The sample (0.5 ml) was applied at 0.5 mL/minute on a 1 mL HiTrap heparin–sepharose (GE Healthcare, Piscataway, NJ) column pre-equilibrated with the same buffer. The column was washed with 8 mL followed by elution with a 30 mL gradient of 0–1.0 M NaCl prepared in the same buffer. Fractions (0.5 ml) were collected, and their activity toward S2222 was determined as described above.

**Prothrombinase assembly**

Activation of prothrombin by human FXa was performed in TBS-Ca²⁺ (20 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 0.3% (w/v) BSA, pH 7.5), using a discontinuous assay. FXa (10 pM; final concentrations are given) was incubated with Alboserpin (0–500 pM) for 20 minutes at room temperature. Human FVa (1 nM) and PC/PS (10 µM) were added and incubated for 5 minutes. Reactions were initiated by addition of human prothrombin (1.4 µM). Aliquots of 25 µL were removed every minute into microplate wells containing 50 µL of TBS-EDTA (20 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA, 0.1% BSA, pH 7.5) to stop reactions. After addition of 25 µL of S2238 (312.5 µM), absorbance at 405 nm was recorded at 37°C for 15 min at 11-second intervals using a Thermomax microplate reader (Molecular Devices). Initial velocities (mOD/minute) obtained were used to calculate the amount of thrombin formed, using a standard curve (33). Absence of one of the components in
the prothrombinase showed no thrombin formation.

**Surface plasmon resonance (SPR)**

Binding of recombinant Alboserpin to FXa, FX, and FXa-DEGR (active site-blocked factor Xa containing the fluorescent inhibitor Dansyl-EGRck) was analyzed at 25°C by SPR spectrometry using a BIAcore T100 instrument (BIAcore AB, Uppsala, Sweden) as described (34). Factors X, Xa, and Xa-DEGR (30 µg/mL) were covalently immobilized on the surface of a CM5 sensor chip using the amine coupling kit supplied by BIAcore at a flow rate of 10 µL/minute—aiming to reach 1500RU in 10 mM acetate buffer pH 4.8—resulting in a final immobilization of 1470.8 RU for FXa, 1764.4 RU for FX, and 1807 RU for FXa-DEGR. Blank flow cells were used to subtract the buffer effect on the sensograms. For kinetic experiments, recombinant Alboserpin in concentrations ranging from 0.04–25 nM was run for 120 seconds at 30 µL/minute in HBS-P buffer (10 mM HEPES, 150 mM NaCl, 0.005% surfactant P20, pH 7.4). The Alboserpin-coagulation factor complex dissociation was monitored for 1800 seconds, and the active cell was regenerated with 3-second pulse (50 µL/min) of 10 mM glycine-HCl, pH 1.5. Kinetic experiments were performed in triplicate on different days. The same experimental design was utilized for kinetic analysis of bacterially expressed Alboserpin. BIAcore T100 evaluation software was used for kinetic evaluation, and sensograms were fitted using the 1:1 binding model (Langmuir binding model). Alternatively, an experiment was designed to test the presence of salivary Alboserpin in the mosquito's saliva. A new sensor chip was used so as to have the FX, Xa, and FXa-DEGR on the same sensor chip. The flow cell 1 was used as a blank to subtract the buffer effect on the sensograms. The immobilization procedure was the same as described above. Four different concentrations of *Ae. albopictus* saliva (15, 25, 50, and 90 µg/mL in HBS-P) were manually injected over the four flow cells in the sensor chip for 90 seconds at a flow rate of 20 µL/minute. The complex dissociation was monitored for 500 seconds and the sensor surface regenerated by a pulse of 5 seconds of 10 mM glycine-HCl, pH 1.5, at 40 µL/minute. These experiments were carried out in duplicate.

Binding of recombinant Alboserpin to heparin was carried out by SPR using a BIAcore 3000 instrument. Heparin (4–6 kDa average MW) was biotinylated at the reducing end and injected over a SA-sensor chip (GE Healthcare) in HBS-P (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% P20 surfactant) at a flow rate of 10 µL/minute for 2 minutes. The resulting immobilization level obtained was 230.6 RU. The kinetics assay was performed at 25°C in HBS-P. Alboserpin concentrations ranging from 3.9 to 62.25 nM were allowed to interact to immobilized heparin for 180 seconds at 30 µL/minute. Alboserpin-heparin complex dissociations were monitored for 600 seconds and the sensor regenerated with 1-minute injection of 2.5 M NaCl at 30 µL/minute. Kinetic parameters (*k_1*, *k_2*, and *K_D*) were determined by global fitting of the sensograms using the 1:1 Langmuir binding model in the BIAcore Evaluation 3.1 software.

**Isothermal titration calorimetry**

Calorimetric assays for measuring FXa binding to Alboserpin were performed using a VPITC microcalorimeter (Microcal, Northampton, MA) at 30°C. Titration experiments were performed by successive injections of 10 µL each of 17 µM of FXa into the sample cell containing 2 µM of Alboserpin. Prior to the run, the proteins were dialyzed against 50 mM Tris-HCl, 0.15
M NaCl, pH 7.4, 5 mM CaCl$_2$ for binding experiments. All solutions were degassed under vacuum for 5 minutes before use.

The calorimetric enthalpy ($\Delta H^{\text{cal}}$) for each injection was calculated, and binding isotherms were fitted according to a model for a single binding site by nonlinear squares analysis using Microcal Origin software. The enthalpy change ($\Delta H$), and stoichiometry ($n$) were determined according to equation 2:

$$Q = n \theta M t \Delta H V_0$$

(Equation 2)

where $Q$ is the total heat content of the solution contained in the cell volume ($V_0$) at fractional saturation $\theta$, $\Delta H$ is the molar heat of ligand binding, $n$ is the number of sites, and $M_t$ is the bulk concentration of macromolecule in $V_0$. The binding constant, $K_a$, is described as:

$$K_a = \frac{\theta}{(1-\theta)}[X]$$

(Equation 3)

where $[X]$ is the free concentration of ligand.

**Binding of Alboserpin to FXa by size-exclusion chromatography**

Analysis of Alboserpin-FXa complex formation was carried out using a Superdex 75 PC 3.2/30 column (3.2 × 300 mm; GE Healthcare), 2.4-mL bed volume, assembled in Akta Purifier equipment (Amersham Pharmacia Biotech). The column optimal separation range is 300–70000 Da, with an exclusion limit of 100,000 Da. The column was equilibrated in HEPES-buffered saline, pH 7.4 (HBS) at 40 µL/minute. For the experiment runs, recombinant Alboserpin and FXa were loaded independently on the column. For the Alboserpin-FXa complex, equimolar concentrations of both proteins were incubated in HBS at room temperature. After 30 minutes of incubation, the mixture was loaded on the column, and the protein-protein complex was monitored at 280 nm.

**Phospholipid vesicle formation and protein binding**

Large unilamellar vesicles were formed by drying the lipids in a chloroform solution under a stream of nitrogen in a glass vial, then resuspended in 20 mM Tris HCl, pH 7.4, vortexed and extruded using a LiposoFast (Avestin Inc., Ottawa, Ontario, Canada) extrusion device. Extrusion was performed through two (stacked) polycarbonate filters (19-mm diameter) of 0.1 µm pore size (Whatman, Clifton, NJ). The final concentration of the vesicle suspensions was 1 mg/mL, and nitrogen was bubbled through the mixture during preparation. Binding was measured at various salt concentrations by diluting the vesicle suspension in TBS containing the appropriate concentration of NaCl and 3 µM Alboserpin in a final volume of 50 µL. A second set of competition experiments was carried out where Alboserpin and phospholipids were incubated in the presence of 30 µg/mL of heparin or 10 µM of annexin V. The mixtures were incubated at room temperature for 30 minutes and centrifuged at 100,000×g for 30 minutes. The supernatant was removed and the pellet resuspended in 40 µL of 1× LDS loading buffer supplemented with 1× NuPAGE reducing agent. Both the pellet and supernatant fractions were analyzed by SDS–PAGE, and the fraction of bound protein was determined by densitometry of Coomassie blue-stained gels using ImageJ software (public domain, open source; NIH).

**Animals and tail bleeding assay; ex vivo PT and aPTT assays**

Female C57BL/6 mice (6–10 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Mice were
maintained at an American Association of Laboratory Animal Care-accredited facility at the National Institute of Allergy and Infectious Diseases, National Institutes of Health. All experiments with mice were evaluated and approved by the NIAID Animal Care and Use Committee of the NIH (Rockville, MD). For the tail-vein bleeding assay, mice were injected intravenously with phosphate-buffered saline (PBS) or two doses of Alboserpin (10 and 100 µg/kg) in a 100 µL volume. After 30 minutes, mice were anesthetized and placed on a warming pad. A transverse incision was performed using a scalpel over a lateral vein at a position where the diameter of the tail is 2.5 mm. The tails were left hanging over the edge of the bench and immersed in a 12 × 75 mm tube containing 4 mL saline buffer for 30 minutes. The tubes were gently rotated from time to time to prevent the shed blood from obscuring the incision. Although only a mild bleeding was expected to occur in the assay, mice were observed for the next 6 hours for signs of delayed bleeding. The samples were properly diluted and the absorbance determined at 540 nm to estimate hemoglobin content. Results represent the mean ± SEM of three animals per group, and the assay was repeated twice.

A second group of mice identically treated with Alboserpin or PBS was used for ex vivo aPTT and PT assays. Briefly, 1 hour after Alboserpin or PBS administration, blood was collected by cardiac puncture in 3.8% trisodium citrate (9:1, v/v). Platelet-poor plasma was obtained by centrifugation at 2000×g for 15 minutes. aPTT and PT assays were carried out as described above.

**FeCl3-induced artery thrombosis**
Mice were anesthetized with intramuscular xylazin (16 mg/kg) followed by ketamine (100 mg/kg). The right common carotid artery was isolated through a midline cervical incision, and the blood flow was continuously monitored using a 0.5VB Doppler flow probe coupled to a TS420 flow meter (Transonic Systems, Ithaca, NY) as described previously (35). Alboserpin (at 50, 100, or 200 µg/kg) or vehicle was infused into the tail vein 15 minutes before injury. Thrombus formation was induced by applying a piece of filter paper (1 × 2 mm) saturated with 7.5% FeCl3 solution on the adventitial surface of the artery for 3 minutes. After exposure, the filter paper was removed, and the vessel was washed with sterile normal saline. Carotid blood flow was continuously monitored for 60 minutes or until complete occlusion (0 flow for at least 10 seconds) occurred.

**Serine protease inhibition assays**
All assays were performed at 30°C in triplicate. Protein (300 nM) was pre-incubated, with each enzyme tested for 10 minutes before the addition of the corresponding substrate as described (36).

**Statistical analysis**
Statistical differences among the groups were analyzed by analysis of variance (ANOVA) using Tukey as a multiple comparison post-test. A P value of 0.05 or less was considered statistically significant.

**Results and Discussion**

The sialotranscriptome of female *Ae. albopictus* SG (37) revealed a cluster of sequences coding for a putative serpin protein similar to the *Ae. aegypti* FXa-directed anticoagulant factor (22). The Alboserpin cDNA has 1,257 nucleotides, coding for a protein of 418 amino acids, including a signal peptide of 19 amino acids (gi:56417456). The mature protein has a calculated MW of 45,986.97 Da, with a pI of 9.21.
Alboserpin is an atypical serpin

Salivary FXa inhibitors have been classified as Kunitz-type, antistasin family, ascaris-type protease inhibitor, and SALP family (17). Sequence analysis of Alboserpin shows high sequence similarities to several other serpins, including PAI-2, antithrombin, and PCI. A CLUSTAL alignment of Alboserpin and other relevant serpins is presented in Figure 1. A comparison of the reactive site loop with Alboserpin and known serpin proteins illustrates that while there is remarkable conservation in amino acid sequences, there is also considerable divergence. For example, the reactive site loop in Alboserpin appears to be truncated in comparison with other serpins. The predicted P1 for Alboserpin aligns as an arginine that would be expected for FXa-specific serine protease inhibitor. Furthermore, P17 within the hinge region is typically a glutamic acid residue, while in Alboserpin it is a histidine residue. This P17 has been identified as glutamic acid for 39 serpins analyzed (23-25). The hinge region of serpins is proposed to be essential for protease inhibition (mousetrap mechanism) by promoting a conformational change that allows a tight interaction with the active site of serine proteases followed by cleavage of the P1–P1' peptide bond of the serpin RCL (23-25). This forms an acyl-intermediate complex in which the bond is cleaved and the P1 residue becomes covalently linked to the enzyme. This leads to a large conformational change in which the RCL inserts into the center of β-sheet A, causing the tethered proteinase to be dragged to the opposite end of the protein and inactivated by conformational deformation and irreversible binding. Other serpins have less typical mechanisms (23-25). Thus, Alboserpin shares primary amino acid sequence in common with typical serpins, including the appropriate spacing of domains, but significant divergence is evident and makes it a unique protein.

Alboserpin and phylogeny

Figure 2A shows BLASTP analysis using the FXa inhibitor from *Ae. albopictus* retrieved serpins from several blood-feeding insects that included the tsetse fly *Glossina morsitans morsitans*, the sand fly *Lutzomyia longipalpis*, the cat flea *Ctenocephalides felis*, and the mosquitoes *Anopheles gambiae*, *Ae. aegypti*, and *Ae. albopictus*. The recently sequenced genomes of *An. gambiae* and *Ae. aegypti* contributed the bulk of the serpins retrieved. Most of these grouped into well supported clades, indicating that they are orthologs and limited to the head and fat bodies, which suggest that they perform housekeeping functions conserved in the ancestor to all *Culicidae*. The proteins from the *Aedes* genus that has a confirmed localization to the SG group into a well supported clade to the exclusion of any Anopheline sequences. BLASTP analyses of the genome of *An. gambiae* using any of these sequences do not retrieve other serpins, which suggests that no orthologs exists for these proteins in *Anopheles*. The closest homologs are serpin 14 from fat bodies. This suggests that a gene duplication event in SG led to the evolution of a new class of SG-derived serpins in *Aedes* sp.

Charges and model

The structure of Alboserpin was modeled using the fully automated structure homology-modeling server. The model coordinates obtained were based on antithrombin-S195A factor Xa-pentasaccharide complex (2GD4). Modeled Alboserpin shows a typical three-
dimensional structure of native inhibitory serpin with its RCL completely exposed (Figure 2B). The electrostatic surface generated using the Adaptive Poisson Boltzmann Solver (APBS) shows a highly positively charged region similar to the one found in the antithrombin-S195A factor Xa-pentasaccharide complex (Figure 2C). This region might be the heparin binding site in Alboserpin. Further structural study is necessary to address this question.

**Anticlotting activity in saliva and SGs of *Ae. albopictus***

In an attempt to characterize the anticoagulant activity of Alboserpin, the corresponding cDNA was cloned in a Pet17b expression vector and expression induced by IPTG. Alboserpin was purified in a Mono-Q ion exchange column (not shown) followed by a last step in molecular sieving chromatography. It was eluted at 10 ml which corresponds to a protein of ~45 kDa (Figure 3A). This finding was consistent with the predicted molecular weight for Alboserpin and is indicative of high purity of the preparation as confirmed by SDS/PAGE (Figure 3A, inset). Recombinant Alboserpin produced in *E. coli* was tested in coagulation assays. Figure 3B shows that Alboserpin prolongs both PT and aPTT, while Figure 3C shows that it also blocks prothrombinase assembly in a dose-dependent manner, consistent with FXa-directed anticoagulant. *Ae. albopictus* SGs also increase the recalcification time (Figure 3D) and prolong both PT and aPTT (Figure 3E). In addition, SPR experiments demonstrated unambiguously that saliva displays FXa-binding properties (Figure 3F).

**Specificity of Alboserpin**

Table 1 shows that incubation of Alboserpin with a series of enzymes does not affect their catalytic activities, indicating that Alboserpin is a highly specific FXa inhibitor.

**Alboserpin is a tight inhibitor of FXa**

Experiments revealed that when FXa was added to the reaction buffer containing Alboserpin and chromogenic substrate, the progress curve displayed a downward concavity (Figure 4A, curve b and c). These results resemble slow-binding kinetics, as seen with many peptidic inhibitors of serine proteinases when small chromogenic or fluorogenic substrates are used to kinetically characterize the interaction. However, appropriate kinetics studies are needed to further address this question. In addition, Alboserpin significantly inhibits FXa at concentrations similar to that of the enzyme (Figure 4B), indicating that Alboserpin is also a tight-binding inhibitor. Conventional Michaelis–Menten kinetics do not apply to the study of tight-binding inhibitors, because they assume that the free inhibitor concentration is equal to the total inhibitor concentration, a reasonable approximation when the enzyme used is at a much lower concentration than the inhibitor. Therefore, Morrison’s equation for tight-binding inhibition (38) was used as described (19) to obtain apparent dissociation constants for Alboserpin. In these experiments, 25 pM of FXa was allowed to interact with increasing inhibitor concentrations (0–525 pM) for 30 minutes in the presence of varying concentrations of substrate (100–500 µM) before product rate formation for the following 30 minutes was recorded (not shown). Resulting steady-state rates were fit by nonlinear regression to Equation 1 for several substrate concentrations as described (19). When the Ki* was plotted against the substrate concentration, a linear regression line ($r^2 = 0.9923$) indicated a y intercept of
68.2 ± 5.4 pM, which is the Ki value for the binding of Alboserpin to FXa (Figure 4C).

**Kinetics and stoichiometry of Alboserpin-FXa interactions**

To investigate binding kinetics of Alboserpin-FXa interactions, SPR experiments were performed. Typical sensograms are shown in Figure 5A. Best fit was attained using a 1:1 model. Using this model, a $k_{on}$ of $1.005 \times 10^6$ M$^{-1}$ s$^{-1}$ and $k_{off}$ of $2.685 \times 10^{-5}$ s$^{-1}$ were obtained. The fast $k_{on}$ for Alboserpin-FXa interaction determined by SPR suggest that enzyme activity is blocked very shortly after binding to the serpin. Accordingly, it is plausible that interactions of FXa with Alboserpin can be better described as fast when physiological macromolecules (FXa) instead of small chromogenic substrates (S2222), are used to determine the kinetics of the interaction involving enzyme and inhibitor. Similar findings have been reported before for tick salivary FVIIa/TF inhibitor, Ixolaris (20). While the remarkably low $k_{off}$ makes it inaccurate to calculate the $K_D$, it was estimated at 26.85 pM, which is in reasonable agreement with the affinity estimated by the Morrison equation (Figure 4C). Notably, covalent binding of Alboserpin to FXa did not take place, as acidic conditions (glycine, pH 1.5) dissociates Alboserpin from FXa immobilized in the sensor chip in contrast to typical serpins. In this respect, Alboserpin more likely behaves as a Kunitz-type inhibitor, which binding to serine proteases are typically reversible (39). No binding was observed for FX or DEGR-FXa (Figure 5B), which is an indication of the catalytic site dependence for binding; however, interaction of the serpin with FXa exosite cannot be excluded (40-41).

Binding of Alboserpin to FXa was measured by isothermal titration calorimetry (ITC) with the results shown in Figure 5C. Fitting of the observed enthalpies to a single-site binding model revealed a $K_D$ of $< 1.0$ nM for Alboserpin binding to FXa. Estimation of the dissociation constant is limited by the parameter $c = K_a [P]$, where $K_a$ is the association equilibrium constant and $[P]$ is the protein concentration in the calorimeter cell. Measurement of the equilibrium constant is considered unreliable when the value for c exceeds 1000. Binding was exothermic, with a favorable enthalpy ($\Delta H = -34.77$ kcal/mol) and unfavorable entropy ($\Delta S = -73.9$ cal/mol K) for binding of the enzyme to the inhibitor. Stoichiometry of the binding ($n = 0.85 \pm 0.003$) indicates that one FXa molecule binds to one Alboserpin molecule (Figure 5C). Binding of Alboserpin to FXa was also confirmed by identification of complex formation that eluted at a MW compatible with 1:1 interaction, as estimated by gel-filtration chromatography (Figure 5D). Figure 5E demonstrates that alboserpin-FXa complex formation dissociates when the sample is warmed at 70°C for 7 min, indicating that complex is reversible in contrast to typical serpins. This result is consistent with reversible interaction also demonstrated by SPR. While Alboserpin-FXa complex formation is kinetically reversible, it is conceivable that this tight interaction results in perhaps a functionally irreversible binding in nature. Finally, saliva was found to interact with FXa but not DEGR-FXa, consistent with presence of Alboserpin in the native secretion (Figure 5F).

**Alboserpin interacts with heparin**

Heparin accelerates the rate of vertebrate plasma antithrombin (AT) inhibition of FXa by three to four orders of magnitude (40). In
fact, AT that normally regulates the proteolytic activity of FXa is a weak inhibitor of coagulation proteinases unless it binds heparin-like glycosaminoglycans. Heparin binding to both protease and serpin enhances the rate of encounter complex formation between the two proteins (bridging effects) (42). To verify whether Alboserpin binds to heparin, SPR experiments were performed as described in Methods. Figure 6A shows sensograms of Alboserpin-heparin interaction. Best fit was achieved with a 1:1 Langmuir model, yielding a $K_D$ of 20.8 nM ($k_{on}$ of $1.13 \times 10^6$ M$^{-1}$ s$^{-1}$ and $k_{off}$ of $2.36 \times 10^{-2}$ s$^{-1}$). In addition, Figure 6B demonstrates that FXa binds to heparin-sepharose column and is eluted with a NaCl gradient. Next, we studied the effects of heparin in the inhibition of FXa by Alboserpin. FXa (1 nM) was used to start reactions containing a mixture of S2222 and alboserpin, with and without heparin. Alboserpin was tested at low concentrations (<0.5 nM) producing partial inhibition of FXa catalytic activity in order to observe any possible effects of heparin. Figure 6C shows that progress curves for FXa inhibition by Alboserpin were the same in the absence or in the presence of heparin. The effects of heparin were also tested after incubation of FXa with Alboserpin followed by addition of S2222 to start reactions. Figure 6D demonstrates that the curves were superimposed indicating no change in the affinity. It is concluded that alboserpin belongs to the subfamily of serpins such as AT (40), heparin cofactor II (43), protease nexin I (44), PZI (45), PAI-1(46) and PCI (47) which reportedly binds heparin and other glycosaminoglycans. However, heparin does not operate as a bridge for Alboserpin to interact with proteinases. In other words, Alboserpin interacts with FXa irrespective of the presence of cofactors (e.g. heparin). This is not surprising, taking into account the tight binding of FXa and Alboserpin which affinity is in the range of thrombin-antithrombin complex when heparin is present. It is also important to recognize that heparan sulfate-containing proteoglycans are found membrane-associated to the cell surface and can substitute for heparin. Plausibly, heparin functions as an anchor that targets Alboserpin to endothelial cells, localizing it at the site of injury where it remains bound instead of free in solution, resulting in higher effective concentration. In this context, TFPI and VEGF binding to heparin has also been demonstrated and sought to contribute to localization in endothelial cells and other GAG-bearing cells (48-49).

**Alboserpin interacts with PC and PE, but not with PS**

Among serpin family members, PCI was initially found to bind to PE, PS and PC (50). However, PCI was later demonstrated to interact with oxidized PE or oxidized PS, but not with PC (47,51). Therefore, binding of Alboserpin to phospholipids vesicles in solution was investigated. Alboserpin was incubated with PC, PS, PE or PC/PS for 15 minutes, followed by centrifugation at 100,000×g. Phospholipid preparation was performed under N$_2$ in order to avoid oxidation. The pellet and the supernatant were collected, and bound (found in the pellet) or free (found in the supernatant) Alboserpin was detected by SDS-PAGE. Figure 7A demonstrates that Alboserpin binds to PC and PE, but surprisingly not to PS. This phospholipid specificity distinguishes Alboserpin and PCI, which is a PS-binding serpin (50). It also binds to a mixture of PC/PS (4:1), which mimics
phospholipid composition of activated platelets. When salt concentration was increased above 0.2 M NaCl, Alboserpin interaction with PC/PS was gradually lost (Figure 7B for gel; Figure 7C for quantification). In addition, as a control, salivary protein (AeDL1) also expressed in *E. coli* did not bind to phospholipids (Figure 7B). It is important to recognize that high salt concentrations may affect alboserpin binding to vesicles not only through electrostatic interference, but also by affecting vesicle structure. While we cannot exclude the later possibility, our most relevant finding is that Alboserpin binds phospholipids in solution, at physiologic ionic strength. Annexin V, a protein that binds PS in the presence of Ca$^{2+}$, but PC in its absence (52) was used to investigate whether it can compete with Alboserpin for PC/PS binding. Experiments performed in the absence of Ca$^{2+}$ demonstrate that annexin V prevents interaction of Alboserpin to PC/PS (Figure 7D) suggesting competition for the same binding site, i.e., PC. In addition, incubation of Alboserpin with heparin completely abolished its interaction with PC/PS vesicles in solution, suggesting that the heparin-binding site of Alboserpin is also required for phospholipid binding (Figure 7E). It is plausible that PC and PE, but not PS, works as an anchor that targets the inhibitor to cells at sites of injury, or which have been activated or present at sites of inflammation. For example, PE in addition to PS, is the major phospholipid component (nearly 40%) of the outer leaflet of activated platelet membrane; accordingly, PE conceivably targets Alboserpin to sites of platelet activation. Studies have also demonstrated that PE enhances the PS-mediated sensitivity of factor VIIa-tissue factor activity (53) and induces high affinity binding sites for factor VIII on surfaces containing PS (54). Moreover, PE has been reported in tumor cell membranes (55), and is known to regulate the activation of blood coagulation by enhancing APC inhibition by PCI (50-51). It is possible that occupation of these sites by Alboserpin interferes with the inhibitory function of PCI towards APC, which displays particularly important anticoagulant and anti-inflammatory activities in vivo (7,56). In addition, PC in its oxidized form is found in atherosclerotic lesions, apoptotic cells, and oxidized LDL and stimulate endothelial cells to produce inflammatory cytokines, leukocyte chemoattractants, and coagulation factors (57-58). The remarkable mechanism of action of Alboserpin—which concentrates and localizes the inhibitor at sites of injury or cell activation—suggests that it has evolved to remain bound at sites of inflammation where the concentrations of PE or PC are presumably very high. It is also clear that the phospholipid specificity of Alboserpin is unique among family members of the serpin family described so far in the sense that, in contrast to PCI, it does not recognize PS but interacts with PC. On the other hand, a lipocalin from *Rhodnius prolixus* binds to PS but not other phospholipids and affect multimolecular coagulation complex formation (59). These molecular adaptations are notable examples of how evolutionary pressure has dictated the function of salivary components from bloodsucking arthropods.

Alboserpin displays antithrombotic and anticoagulant activity in vivo

To demonstrate that the anticoagulant effects of alboserpin translate into inhibition of thrombus formation, the inhibitor was injected in mice, which were then submitted to FeCl$_3$-induced carotid artery injury. Application of FeCl$_3$ to the exterior of blood vessels causes severe endothelial damage and occlusion by platelet-rich thrombi. Times to occlusion were not significantly
different between control and mice treated with 50 µg/kg of Alboserpin (16.11 ± 1.47 vs 17.0 ± 1.63 minutes) (Figure 8A). In contrast, mice treated with 100 and 200 µg/kg of Alboserpin were resistant to arterial occlusion, and in these cases, occlusion did not take place before 60 minutes for most animals (Figure 8A). These results indicate that Alboserpin inhibition of FXa effectively blocks thrombin generation at sites of vascular injury. Next, the effects of alboserpin in bleeding were estimated using the tail transection method. Figure 8B shows that Alboserpin (100 µg/kg) produces significant bleeding, as would be expected for a potent anti-FXa inhibitor. Furthermore, injection of alboserpin (100 µg/kg) in mice prolongs PT and aPTT ex-vivo (Table 2). While the concentrations used in the experimental thrombosis model is high when compared to the amount of alboserpin injected at the site of mosquito bite, this is explained by the intensity and extension of the injury provoked by FeCl3. However, one may calculate the approximate concentration of the inhibitor injected by the mosquito, which causes a microinjury to the host. Accordingly, the volume of blood taken in each blood meal is ~ 4 µl and ~ 50% of the salivary gland content (~1 µg) is released during the bite, being Alboserpin ~ 1% of the salivary proteins (~0.01 µg). This results in a concentration ~ 40 nM, which is much above Alboserpin K_D for FXa. Therefore, it is evident that Alboserpin behaves as an anticoagulant and antithrombotic in vivo, which is consistent with its biologic activity characterized in vitro.

**Concluding remarks**

Unique binding behavior, shorter and distinct composition of the RCL, reversible interaction with the enzyme and no requirement for cofactors places Alboserpin as a useful prototype to understand structural features of FXa and serpin mechanisms of protease inhibition. Structural studies of Alboserpin will define the precise mechanism by which this molecule specifically and reversibly blocks FXa, thus preventing its role in thrombosis and inflammation (7). Alboserpin may also be regarded as a prototype to develop anticoagulants targeting FXa, an important target of antithrombotic therapy clinically illustrated by the effectiveness of oral FXa inhibitors rivaroxaban and apixaban (60).

**Acknowledgments**

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References

Table 1: Specificity of Alboserpin (150 nM)

<table>
<thead>
<tr>
<th>Enzyme (nM)</th>
<th>(%) activity</th>
</tr>
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<tbody>
<tr>
<td>FXa (0.8)</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>uPA (0.7)</td>
<td>99.8 ± 2.3</td>
</tr>
<tr>
<td>Kallikrein (0.8)</td>
<td>100.6 ± 2.2</td>
</tr>
<tr>
<td>Granzyme B (20)</td>
<td>98.9 ± 1.4</td>
</tr>
<tr>
<td>Matriptase (1.2)</td>
<td>101.2 ± 1.7</td>
</tr>
<tr>
<td>Elastase (0.01)</td>
<td>95.3 ± 1.3</td>
</tr>
<tr>
<td>α-chymotrypsin (0.05)</td>
<td>105.0 ± 0.9</td>
</tr>
<tr>
<td>Chymase (1.8)</td>
<td>106.8 ± 4.7</td>
</tr>
<tr>
<td>FXIIa (1.2)</td>
<td>105.1 ± 2.5</td>
</tr>
<tr>
<td>FXIa (0.06)</td>
<td>95.1 ± 2.4</td>
</tr>
<tr>
<td>Plasmin (0.25)</td>
<td>102.3 ± 3.7</td>
</tr>
<tr>
<td>Thrombin (0.01)</td>
<td>105.4 ± 3.8</td>
</tr>
<tr>
<td>Trypsin (0.25)</td>
<td>100.7 ± 4.1</td>
</tr>
<tr>
<td>Cathepsin G (10)</td>
<td>103.5 ± 2.0</td>
</tr>
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</table>
Table 2: Ex vivo aPTT and PT

<table>
<thead>
<tr>
<th>Alboserpin (µg/kg)</th>
<th>aPTT ± SE</th>
<th>PT ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>0.96 ± .011</td>
<td>1.66±0.12</td>
</tr>
<tr>
<td>100</td>
<td>1.41±.059</td>
<td>3.41±0.39</td>
</tr>
</tbody>
</table>
Figure Legends

Fig. 1. Clustal alignment of Alboserpin and other serpins. The secondary structure elements are indicated according to alboserpin sequence (gi_56417456): α-helices (black rectangles), β-strands (red ellipses). RCL, reactive center loop; P1 (magenta). The following serpins have been used in the alignment: alboserpin-like (gi_94468342); protein C inhibitor (PCI, gi_546321); plasminogen activator inhibitor-1 (PAI-1, gi_10835159), antithrombin (gi_28907); heparin cofactor II (HCII, gi_183908); protease nexin I (PNI, gi_225907); protein Z inhibitor (PZI, gi_154759291).

Fig. 2. Phylogeny and molecular modeling of Alboserpin. (A) Phylogenetic analysis of serpins found in bloodfeeding insects. Analysis was performed using Neighbor-joining analysis. Bootstrap support (10,000 replicates) are indicated by grey (>70%) and black (>90%) dots. Red dots indicate proteins that tested positive for FXa inhibition, while blue dots indicate proteins that tested as negative. Serpins are labeled according to the annotation found for serpins from the genome of Anopheles gambiae. Information on organ localization was obtained by tblastn analysis of the EST database. Sequences are indicated by the three letter abbreviation of the species name followed by the GI accession number. (B, C) Models were constructed using the coordinates generated with Swiss-Model (automated mode). Surface map was generated using PyMOL APBS tools. (B) Ribbon diagram of Alboserpin model with the reactive center loop side-chains highlighted. (C) Electrostatic potential surfaces of Alboserpin showing the positively charged surface (blue) corresponding to the putative heparin-binding site.

Fig. 3. Purification of recombinant Alboserpin and anticoagulant properties. (A) Alboserpin was expressed in Escherichia coli and the final purification step carried out in Sephadex G-75. Inset: PAGE of Alboserpin under denaturing, nonreducing conditions. (B) Ae. albopictus salivary gland (SG) dose-dependently increases the recalcification time. (C) Recombinant Alboserpin dose-dependently increases the PT and APTT. (D) Ae. albopictus SG dose-dependently increases the PT and APTT. (E) Alboserpin inhibits prothrombinase activity. (F) SPR shows that Ae albopictus saliva binds to FXa. Experiments were performed 3–4 times.

Fig. 4. Alboserpin is a tight inhibitor of FXa. (A) Typical progress curves for FXa-mediated S2222 hydrolysis in the absence (curve a) and presence of Alboserpin (curves b and c). Curve b: reaction was started by the addition of FXa (1 nM) to a mixture containing Alboserpin (7.25 nM and 15 nM) and S2222 (500 µM) at 37°C. Substrate hydrolysis was followed for 1 hour at 37°C, at 405 nm. (B) Dose–dependent inhibition of different concentrations of FXa by Alboserpin. FXa and Alboserpin were incubated for 30 minutes before addition of S2222. (C) Determination of the apparent dissociation constant, K*, from steady-state velocities. The solid line represents the best nonlinear fit of the data to equation 1, yielding a K* of 68.2 pM. Inset: Relationship of the apparent dissociation constant, K*, to substrate concentration when reactions were initiated by the addition of FXa. Values for K* were calculated as described in the text (FXa, 25 pM; Alboserpin, 0–525 pM; fluorogenic substrate, 100–500 µM). Linear regression of the data yields a K* of 68.2 ± 5.4 pM (r = 0.9941). The points in the figure are the mean ± SE of three independent experiments.

Fig. 5. Kinetics and stoichiometry of FXa inhibition by Alboserpin. Alboserpin binds to FXa by SPR. (A) Typical sensograms of Alboserpin interaction with FXa. Different concentrations of Alboserpin (in nM: a, 25; b, 12.5; c, 6.25; d, 3.12; and e/f, 1.56) were injected over immobilized FXa for 180 seconds. Dissociation of Alboserpin–Xa complex was monitored for 2000 seconds, and a global 1:1 binding model was used to calculate kinetic parameters. Representative sensograms are shown. RU, resonance units. (B) Alboserpin binds to FXa and des-Gla-FXa but not to FX or DEGR-FXa. (C) Molar enthalpies per injection for FX interaction with Alboserpin. Filled circles, measured enthalpies; solid line, fit of experimental data to a single site-binding model. Inset, solution binding of Alboserpin to FXa as measured by isothermal titration calorimetry. Baseline adjusted heats per injection of FXa (14.1 µM) into Alboserpin (1.0 µM). Thermodynamic parameters: ΔH = −34.77 ± 0.28 kcal/mol. (D) Binding of Alboserpin to FXa estimated by gel-filtration chromatography as described in Methods. (E) Alboserpin-FXa complex is dissociated by warming (70°C, 15 min). FXa and alboserpin (3.1 µM) were incubated for 30 min at
37°C, in TBS with 5 mM CaCl₂ and warmed at 72°C for 7 min. 1- FXa, 2-Alboserpin, 3-FXa and Alboserpin. SDS-PAGE was run under denaturing, non-reducing conditions. (F) *Aedes albopictus* saliva binds to FXa but does not interact with DEGR-FXa.

**Fig. 6.** Alboserpin interacts with heparin but does not affect the Alboserpin-FXa interaction. (A) Alboserpin (in nM: a, 62.5; b, 31.25; c, 15.6; d, 7.8; e/f, 3.9) were perfused onto a heparin-immobilized SA sensor chip for 180 seconds at 30 µL/minute and their dissociation monitored for 600 seconds. Kinetics parameters were calculated using the 1:1 Langmuir binding model. (B) Alboserpin (5µM), FXa (2µM) and Alboserpin-FXa (5:2µM) complex were independently chromatographed over a HiTrap heparin column. Samples were eluted with an increasing NaCl gradient from 0–1M over 20 minutes. (C) Different concentrations of Alboserpin (in nM, a, 0; b, 0.078; c, 0.312) were incubated at 37ºC with 250 µg/mL of S2222 in the absence (black lines) or in the presence of 30 µg/mL of heparin (red lines). After 15 minutes, the enzymatic reaction was initiated by adding 1 nM of FXa. S2222 hydrolysis was followed for 45 min, at 405 nm. The first 10 min of the reaction has been deleted to better visualize the lack of effects of heparin in the progress curves. (D) FXa (1 nM) was incubated with different concentrations of Alboserpin for 15 minutes in the absence and presence of 30 µg/mL of heparin. The reaction was started by adding 250 µM of chromogenic substrate S2222. All experiments were carried out in triplicate.

**Fig. 7.** Alboserpin binds to PC:PS, phosphatidylcholine and phosphatidylethanolamine, but does not bind to phosphatidylserine. (A) Alboserpin (3 µM) was incubated with 1 mM of PC, PS, PE, and PC:PS for 30 minutes and spun down at 100,000×g for 30 minutes. Both the pellet and supernatant were electrophoresed under reducing conditions. (B) Effect of NaCl on Alboserpin-PC:PS interaction. Alboserpin and PC:PS were diluted in TBS pH 7.4 containing the indicated concentrations of NaCl. The second panel in Fig 7B shows that a control protein (AeDL1) expressed in E. coli did not interact with phospholipids. (C) Graphic representation of Alboserpin-PC:PS interaction in the presence of NaCl. (D) Competition assay of Alboserpin (3 µM) and annexin V (10µM) for PC:PS (1 mM). The result suggests a common binding site. (E) Heparin (30 µg/ml) inhibits the interaction of Alboserpin with PC:PS under physiologic pH and salt conditions. As a control, the migration of alboserpin in the absence of PC/PS and heparin is depicted. Representative experiments are shown. P, pellet; S, supernatant.

**Fig. 8.** Alboserpin increases bleeding time and is antithrombotic in vivo. (A) Thrombosis was induced in the carotid artery of mice by local application of 7.5% FeCl₃. Blood flow was monitored with a perivascular flow probe for 60 minutes or until stable occlusion has occurred. Alboserpin was injected in the caudal vein 15 minutes before injury. Each symbol represents one individual. (B) Bleeding was estimated by the tail transaction model after intravenous infection of Alboserpin at indicated concentrations.
Figure 4

A

B

C

Ki = 68.2 ± 5.4 pM

r² = 0.9923
Figure 5
Figure 8

A

Time to occlusion (min)

control
(n=8)
50
(n=8)
100
(n=6)
200
(n=6)

Alboserpin, μg/kg

B

O.D. (540 nm)

Control
Alboserpin, 10 μg/kg
Alboserpin, 100 μg/kg

*
Alboserpin, a Factor Xa inhibitor from the mosquito vector of Yellow Fever, binds heparin and membrane phospholipids and exhibits antithrombotic activity
Eric Calvo, Daniella M. Mizurini, Anderson Sa-Nunes, Jose M.C. Ribeiro, John F. Andersen, Ben J. Mans, Robson Q. Monteiro, Michail Kotsyfakis and Ivo M.B. Francischetti

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