Lufaxin, a Novel Factor Xa Inhibitor From the Salivary Gland of the Sand Fly Lutzomyia longipalpis Blocks Protease-Activated Receptor 2 Activation and Inhibits Inflammation and Thrombosis In Vivo

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Objective—Blood-sucking arthropods’ salivary glands contain a remarkable diversity of antithrombotics. The aim of the present study was to identify the unique salivary anticoagulant of the sand fly Lutzomyia longipalpis, which remained elusive for decades.

Methods and Results—Several L. longipalpis salivary proteins were expressed in human embryonic kidney 293 cells and screened for inhibition of blood coagulation. A novel 32.4-kDa molecule, named Lufaxin, was identified as a slow, tight, noncompetitive, and reversible inhibitor of factor Xa (FXa). Notably, Lufaxin’s primary sequence does not share similarity to any physiological or salivary inhibitors of coagulation reported to date. Lufaxin is specific for FXa and does not interact with FX. Dansyl-Glu-Gly-Arg-FXa, or 15 other enzymes. In addition, Lufaxin blocks prothrombinase and increases both prothrombin time and activated partial thromboplastin time. Surface plasmon resonance experiments revealed that FXa binds Lufaxin with an equilibrium constant $K_{d} = 3 \text{nM}$, and isothermal titration calorimetry determined a stoichiometry of 1:1. Lufaxin also prevents protease-activated receptor 2 activation by FXa in the MDA-MB-231 cell line and abrogates edema formation triggered by injection of FXa in the paw of mice. Moreover, Lufaxin prevents FeCl$_3$-induced carotid artery thrombus formation and prolongs activated partial thromboplastin time ex vivo, implying that it works as an anticoagulant in vivo. Finally, salivary gland of sand flies was found to inhibit FXa and to interact with the enzyme.

Conclusion—Lufaxin belongs to a novel family of slow-tight FXa inhibitors, which display antithrombotic and anti-inflammatory activities. It is a useful tool to understand FXa structural features and its role in prohemostatic and proinflammatory events. (Arterioscler Thromb Vasc Biol. 2012;32:2185-2196.)

Key Words: hemarthrophy ■ leishmaniasis ■ microcirculation ■ thrombosis ■ vector biology

Salivary glands (SGs) of blood-sucking arthropods display a notable variety of negative modulators of vascular biology,1 Several molecules have been studied in detail, including anticoagulants,2 platelet aggregation inhibitors,3 and vasodilators.4 These molecules are aimed to block the host response to an injury (ie, bite), therefore contributing to successful blood feeding.1 In some cases, saliva also contributes to the transmission of infection agents to the vertebrate host, a phenomenon most notably demonstrated for leishmaniasis,5 a vector-borne disease transmitted by the bite of sand flies, Lutzomyia longipalpis sp. and Phlebotomus papatasii sp, among others. Saliva of these phlebotomines expresses many pharmacologically active components which modulate hemostasis and the immune response. For instance, saliva of sand flies contain an apyrase that inhibits platelet aggregation,6 a potent vasodilator (maxadilina) that promotes an increase in blood flow,7 and at least 1 immunomodulator (eg, salivary protein 15) that skews the host immune response toward Th1 polarization.8 However, several proteins coded by their corresponding SG transcripts remain without a defined
function. Likewise, some biological functions described in the SG have not been associated with a specific protein. For example, the anticoagulant of L. longipalpis or P. papatasi remained elusive for decades.

A remarkable diversity of anticoagulants' targeting factor VIIa (FVIIa)/tissue factor (TF), FIXa, FXa, thrombin, and of the contact pathway have been reported in other blood-sucking animals, including mosquitoes, ticks, bugs, leeches, and bats, but not sand flies. Among several coagulation factors, FXa is a particularly attractive target because it plays a central role in the coagulation cascade, where both extrinsic and intrinsic pathways converge, leading to prothrombinase assembly with subsequent thrombin generation and fibrin formation. FXa pathways converge, leading to prothrombinase assembly with subsequent thrombin generation and fibrin formation. FXa also activates protease-activated receptor (PAR) 1 or PAR2 in different cell types, which enables this enzyme to promote inflammation and immune modulation in the absence of fibrin formation.

The importance of FXa in the coagulation cascade is illustrated by the tight regulation of its activity by 3 physiological inhibitors: TF pathway inhibitor, antithrombin, and protein Z. TF pathway inhibitor is a multidomain Kunitz-type inhibitor that binds to the active site of FXa by the second Kunitz domain, and this complex blocks FVIIa/TF. Antithrombin is a serpin that binds to the active site of FXa by the second Kunitz domain, and this complex blocks FXa. Antithrombin is a serpin that binds heparin and regulates proteolytic activity of FXa by binding to the active site and trapping the enzymes in an inactive complex. Protein Z serves as a cofactor for the inhibition of FXa by protein Z-dependent protease inhibitor. Notably, only 5 distinct salivary inhibitors targeting FXa have been molecularly cloned and expressed from blood-sucking animals, including mosquitoes, ticks, bugs, leeches, and bats, but not sand flies. Among several coagulation factors, FXa is a particularly attractive target because it plays a central role in the coagulation cascade, where both extrinsic and intrinsic pathways converge, leading to prothrombinase assembly with subsequent thrombin generation and fibrin formation. FXa also activates protease-activated receptor (PAR) 1 or PAR2 in different cell types, which enables this enzyme to promote inflammation and immune modulation in the absence of fibrin formation.

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Deglycosylation of Lufaxin
This was performed using the Enzymatic DeGlycoMx Kit from QA-Bio (Palm Desert, CA), which contains a mixture of N-glycosidase F, sialidase, β-galactosidase, glucosaminidase, and O-glycosidase. The assay was performed following the manufacturer’s instructions, and described in the online-only Data Supplement.

Platelet Aggregation Assays
Platelet-rich plasma was obtained by plateletpheresis from medication-free platelet donors at the Department of Transfusion Medicine/ National Institutes of Health blood bank. Aggregation was performed as described previously.28

Contraction of Rat Aorta
Contraction of rat aortic ring preparations by U46619 was measured isometrically and recorded with transducers from Harvard Apparatus Inc (Holliston, MA) as reported, and described in detail in the online-only Data Supplement. Briefly, aortic rings were suspended in a 0.5-mL bath kept at 36°C and were preconstricted by 100 mmol/L U46619 before addition of Lufaxin to give final concentrations of 1 µmol/L, or SGHs of Rhodnius prolixus (0.04 of 1 pair of glands/mL, with an approximate final concentration of nitrophorins of 2 µmol/L positive control).

Recalcification Time
Clotting activity was measured by the recalcification time of human plasma using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA) with a kinetic module, as described in the online-only Data Supplement.

aPTT and PT Assays
The effect of Lufaxin on coagulation tests aPTT and PT was evaluated on an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany) as reported in the online-only Data Supplement.

Prothrombinase Assembly
Activation of prothrombin by human FXa was performed in Tris-buffered saline-CaCl2 (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L CaCl2, 0.3% BSA, pH 7.5), using a discontinuous assay as reported before, and described in the online-only Data Supplement.

Kinetics Studies
This was performed as described using chromogenic substrate (S2222) hydrolysis specific for FXa and described in detail in the online-only Data Supplement.

Determination of the Active Site of Lufaxin
In a polymerase chain reaction tube, Lufaxin (1.9 µmol/L) was incubated with human FXa (2.7 µmol/L) for 3 or 24 hours at 37°C, with and without 5 mmol/L CaCl2. Lithium dodecyl sulfate loading buffer and dithiothreitol were added to the tubes, warmed for 10 minutes at 70°C, and the mixture was loaded in a 4% to 12% NuPAGE gel (2-[N-morpholino]ethanesulfonic acid buffer) and Coomassie Blue stained. See Blue standard was used as molecular mass marker (Invitrogen).

Surface Plasmon Resonance
All surface plasmon resonance (SPR) experiments were carried out in a T100 instrument (Biacore Inc, Uppsala, Sweden) following the manufacturer’s instructions. For immobilization using an amine coupling kit (Biacore), carboxymethylated dextran chips were activated with 1-ethyl-3-(dimethylaminopropyl) carbodiimide, and N-hydroxysuccinimide before injection of Lufaxin (6.5 µg/mL) or FXa (30 µg/mL) in aceyte buffer, pH 5.5. Remaining activated groups were blocked with 1 mol/L ethanolamine, pH 8.5, resulting in a final immobilization of 604.5 RU (for Lufaxin) or 661.7 RU (for FXa). Kinetic experiments were carried out by injecting FXa for a contact time of 180 seconds at a flow rate of 30 µL/minute at 25°C and described in the online-only Data Supplement.

Isothermal Titration Calorimetry
Lufaxin binding to FXa was performed using a isothermal titration calorimetry (ITC) microcalorimeter (Microcal, Northampton, MA) at 30°C. Before the run, the proteins were dialyzed against 20 mmol/L Tris-HCl, 0.15 M NaCl, pH 7.4 for binding experiments. Titrations were performed by making successive injections of 5 µL each of 20 µmol/L FXa into the 2-µL sample cell containing 2 µmol/L Lufaxin until near-saturation was achieved as described in the online-only Data Supplement.

Cell Culture
MDA-MB-231 breast cancer cells were maintained in Iscoves medium (Invitrogen) supplemented with 10% fetal bovine serum in culture flasks in a 5% CO2-air mixture at 37°C. Subconfluent cultures were washed twice with PBS, and cells were detached with Hank’s solution containing 10 mmol/L HEPES and 0.2 mmol/L EDTA. Cells were seeded at 5×10⁴ cells per well in 6-well tissue culture plates for signaling assays.

PAR2 Signaling Assay
MDA-MB-231 cells were serum starved for 90 minutes and stimulated with 10 mmol/L FXa (in Iscoves medium, no fetal bovine serum) for 10, 15, 30, and 60 minutes. Lufaxin (50 µmol/L) was added 1 hour before stimulation with FXa. To avoid PAR1 activation by thrombin, assays were performed in the presence of 10 mol/L hirudin as described. Detection of phosphorylation of ERK (pERK) was carried out as described in the online-only Data Supplement.

Paw Edema in Mice
Female C57BL/6 mice, 6 to 8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the National Institute of Allergy and Infectious Diseases Animal Care Facility and experiments were performed as reported. Briefly, the posterior footpad thickness of each mouse was recorded using a caliper, before each injection (Mitutoyo America Corp, Aurora, IL). Subsequently, PBS, FXa alone, or FXa incubated with Lufaxin were injected intra-dermally in the paw using a 0.3-mL syringe. As an index of edema formation, paw thickness was then measured at 15, 30, 45, and 60 minutes as described in the online-only Data Supplement.

FeCl3-Induced Artery Thrombosis
Thrombus formation was induced by applying a piece of filter paper (1x2 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 as described in detail in the online-only Data Supplement.

Tail Bleeding Assay
Bleeding was estimated by the tail transection method as described in the online-only Data Supplement.

Protease Inhibition Assays
This was performed as described in the online-only Data Supplement.

Statistical Analysis
Results are expressed as mean±SE. Statistical differences among the groups were analyzed by t test, or ANOVA using Bonferroni as a
Figure 1. Lufaxin is a novel, specific factor Xa (FXa) inhibitor from Phlebotominae sp. A, Clustal alignment of Lufaxin from L. longipalpis (Lon, gi41397464) and orthologues from P. arabiensis (Para, gi242564823), P. aniasi (Pari, gi61807168), P. tobbi (Ptoob, gi299829388), P. permicous (Pper, gi76446599), P. argentipes (Parg, gi74466551), P. sergenti (Pser, gi299829440), and P. dubosqi (Pdub, gi112496879). B, Phylogeny for the sequences described in A. Analysis was performed with neighbor-joining algorithm using Bootstrap support (10,000 replicates). The numbers indicate the 10% amino acid divergence in the sequences. C, Lufaxin was expressed in human embryonic kidney 293 cells and purified with a Ni²⁺ column followed by a gel-filtration chromatography. Samples were loaded in a NuPAGE gel under nonreducing (NR) or reducing (R) conditions. Gels were stained with Coomassie Blue. On the left, molecular mass markers are indicated. D, Lufaxin is glycosylated. Lufaxin incubated with a combination of glycosidases migrates as a lower molecular mass protein in a NuPAGE gel. E, Lufaxin does not affect platelet aggregation. Platelet-rich plasma was stimulated with collagen (0.33 µg/mL) with or without Lufaxin (100 nmol/L). Aggregation response was monitored by turbidimetry using a Lumi-Aggregometer. F, Lufaxin does not produce vasodilation. Lufaxin (100 nmol/L) was added to rat aorta strips at the time indicated (30 minutes) by the arrow. No vasodilation was detectable.
multiple comparison posttest. Significance was set at P≤0.05 (GraphPad Prisma software, La Jolla, CA).

Results

Identification of Lufaxin as a Novel Specific FXa Inhibitor

Lufaxin (gi 41397464) from L. longipalpis is a 32.4-kDa protein of 278 amino acids, 8 cysteines, and pI 8.27. It belongs to a family of highly conserved salivary proteins present in other Phlebotominae sp. including P. arabiensis, P. ariasi, P. perniciosus, P. tobbi, P. sergenti, P. argentipes, and P. dubosi (Figure 1A). The function of this family of protein has remained elusive.3–11 Phylogenetic analysis revealed that these molecules are closely related suggesting that Lufaxin family of anticoagulant evolved from a common ancestor (Figure 1B). It is also evident that Lufaxin members found in the Phlebotominae clade are separated from the L. longipalpis homolog (Figure 1B), consistent with the separation of Old World and New World sand flies >60 million years ago after the split of Gondwanaland.

In an attempt to identify the function of Lufaxin, its corresponding cDNA was cloned in a VR2001-TOPO27 expression vector and described in Methods, and used for transfection of human embryonic kidney 293-F cells. Supernatants containing recombinant Lufaxin (containing a 6×His-tag) were loaded in a Ni²⁺-column followed by gel-filtration chromatography. Purified Lufaxin was analyzed by NuPAGE and found to migrate as a single band of ~38 kDa under denaturing or reducing conditions (Figure 1C). The N-terminal for Lufaxin obtained by Edman degradation identified the sequence DGDEYFIGKYKED, which is in agreement with the N terminus predicted for the mature protein, according to the cDNA. To verify whether the higher molecular mass of Lufaxin was attributable to glycosylation, the inhibitor was incubated with a combination of glycosidases, or D.W. (control) followed by NuPAGE. Figure 1D shows that the migration pattern of Lufaxin changes to lower molecular mass after incubation with the mixture of enzymes. This indicates that recombinant Lufaxin is glycosylated, explaining the difference between the actual and predicted molecular masses. This experimental finding is also in agreement with the prediction of 1 N-linked glycosylation (calculated value 0.6638; threshold at 0.5) in the sequence NKTC (amino acids 239) of mature Lufaxin. However, Lufaxin sequence does not display potential O-linked glycosylation sites.

Lufaxin was tested in screening assays in an attempt to identify its molecular target. Lufaxin (100 nmol/L) did not inhibit platelet aggregation by collagen (Figure 1E) or promote vasodilation (Figure 1F). When tested in the recalcification time assay, Lufaxin dose-dependently prolonged clotting time, suggesting that it is an anticoagulant (Figure 1G). To determine whether Lufaxin blocks coagulation factors involved in the intrinsic, extrinsic, or common pathway, PT and aPTT were performed. Figure 1H shows that Lufaxin dose-dependently prolongs PT in both human and mouse plasma. Likewise, Lufaxin prolonged aPTT in a dose-dependent manner in either species and at a similar concentration (Figure 1I). These results indicated that Lufaxin interferes with a common pathway of the coagulation cascade. Concentrations of Lufaxin to block PT and aPTT were higher than observed for inhibition of the recalcification time likely because of the stronger activation of the coagulation (ie, FXa production) by the PT or aPTT reagents when compared with CaCl₂ only. In fact, control clotting time for PT and aPTT were 16.67±0.20 seconds and 42.92±2.08 seconds, respectively, whereas it took 200 to 250 seconds for the plasma to clot in the recalcification time assay. Next, specificity of Lufaxin was tested by incubating a molar excess of the inhibitor with FXa or other enzymes involved in coagulation/inflammation (eg, thrombin, FXa, FXIIa, kallikrein, chymase, tryptase, matrypsin, elastase, cathepsin G, proteinase 3), fibrinolysis (eg, plasmin, urokinase-type plasminogen activator, tissue plasminogen activator), or digestive processes (eg, trypsin and chymotrypsin). Incubation was followed by addition of small fluorogenic substrates specific for each enzyme. Figure 1J demonstrates that only FXa was inhibitable by Lufaxin, which thus characterizes it as a specific inhibitor for FXa.

FXa plays a critical role in prothrombinase assembly, through conversion of prothrombin to thrombin in the presence of membrane phospholipids, FVa, and calcium.12 To ascertain whether Lufaxin blocks prothrombinase, inhibitor and enzyme were incubated followed by addition of other components of the complex. Lufaxin incubated with FXa dose-dependently blocks thrombin formation, indicating that the inhibitor not only prevents small chromogenic substrate hydrolysis by FXa but also interferes with the productive assembly of the prothrombinase (Figure 1K).

Lufaxin Is Present in the SG of L. longipalpis

To verify whether an FXa inhibitor was expressed in the SG of L. longipalpis and other Phlebotominae, fresh SGHs were obtained and tested for inhibition of FXa catalytic activity by addition of recombinant inhibitor protein (NP2) to the chamber (arrows) at different time points, which was followed by vasodilation. G, Indicated concentrations of Lufaxin were tested by the recalcification time assay. Citrated human plasma was mixed with recombinant proteins and reactions were initiated by the addition of CaCl₂. Clotting formation was monitored at 650 nm. H, Lufaxin prolongs prothrombin time (PT). Lufaxin was added to plasma, followed by addition of PT reagent and Ca²⁺: Clotting was estimated using a coagulometer. PT time; human plasma, 16.67±0.20 seconds; mice plasma, 15.79±0.40 seconds. I, Lufaxin prolongs aPTT. Lufaxin was added to plasma, followed by addition of activated partial thromboplastin time (aPTT) reagent and Ca²⁺: Clotting was estimated using a coagulometer. aPTT time; human plasma, 42.92±2.08 seconds; mice plasma, 37.61±3.70 seconds. J, Lufaxin does not inhibit activity of several enzymes, except FXa. Catalytic activity was estimated by fluorescent substrate hydrolysis. K, Lufaxin inhibits thrombin formation by prothrombinase. FXa was incubated with Lufaxin, followed by addition of FVas, phosphatidylcholine/ phosphatidylserine, and prothrombin in the presence of Ca²⁺: After 6 minutes, aliquots were taken to estimate thrombin generation and expressed as percentage of control (no inhibitor). Data are the average±SE of triplicate determinations (n=3). u-PA indicates urokinase-type plasminogen activator; t-PA, tissue plasminogen activator.
using S2222. Figure 2A shows that SGH from *L. longipalpis*, *P. papatasi*, or *P. duboscqi* cause dose-dependent inhibition of FXa catalytic activity, with a half maximal inhibitory concentration (IC$_{50}$) $\approx 0.5$ pairs per assay. Experiments performed side by side with Lufaxin (0–60 nmol/L) or SGH (0–2 pairs per assay), which were incubated with FXa (0.5 nmol/L) followed by addition of S2222 (250 µmol/L), indicate that the concentration of Lufaxin in 1 pair of gland is $\approx 15$ to $30$ nmol/L (Figure I in the online-only Data Supplement). In addition, Figure 2B demonstrates that both recombinant Lufaxin and a protein with similar migration properties from the SGH were recognized by anti-Lufaxin antibody. Next, SGH was tested for FXa-binding activity using SPR. FXa was immobilized in a carboxymethylated dextran chip, and *L. longipalpis* SGH was used as an analyte. Figure 2C shows that SGH produces an increase in resonance units in a dose-dependent manner, which is consistent with FXa-binding protein present in the SGH. This was congruent with the finding that *L. longipalpis* SGH also prolongs recalcification time (Figure 2D). Finally, many recombinant proteins from the SG of *L. longipalpis* which were expressed in the same system as Lufaxin were tested side by side. Figure 2E demonstrates that Lufaxin is the only inhibitor that prolongs recalcification time, whereas 7 other recombinant proteins were without effect.

**Lufaxin Is a Slow, Tight, and Noncompetitive Inhibitor of FXa**

We examined whether inhibition by Lufaxin could be characterized as a tight inhibitor of FXa using S2222. For these experiments, Lufaxin (0–60 nM) and FXa (2, 4, and 8 nmol/L) were incubated for 60 minutes followed by addition of S2222 (250 µmol/L) to start reactions. Figure 3A shows that inhibition was immediate, resulting in linear progress curves. The data were transformed as $V_{s}/V_{o}$ and fitted with the Morrison equation for tight inhibitors, which allows calculation of the IC$_{50}$ for each FXa concentration tested in the assay (Figure 3B). It has been demonstrated that an IC$_{50}$ value obtained from this treatment of the data is similar to the concentration of total enzyme in the sample (ie, within a factor of 10) when an inhibitor is of the tight-binding type. This is because a tight-binding inhibitor interacts with the enzyme in nearly stoichiometric fashion. In other words, the higher the concentration of enzyme present, the higher the concentration of inhibitor required to reach half-maximal saturation of the inhibitor-binding sites. Thus, a plot of IC$_{50}$ as a function of [E] (at a single, fixed substrate concentration) would show linearity.

**Figure 2.** Salivary glands (SGs) from *Lutzomyia* and *Phlebotominae sp.*, exhibit antifactor Xa (FXa) activity. A, SG homogenate (SGH) from *L. longipalpis*, *P. papatasi*, and *P. duboscqi* were incubated with FXa (0.5 nmol/L) in Tris-buffered saline-BSA buffer for 1 hour, followed by addition of S2222 (250 µmol/L). Reactions were followed for 1 hour and inhibition of FXa activity estimated as percent of control (PBS only). B, Detection of Lufaxin in the SGH of *L. longipalpis*. Lufaxin or SGH were loaded in a NuPAGE gel, followed by Western blotting using anti-Lufaxin polyclonal antibody. The arrowhead indicates the bands corresponding to recombinant Lufaxin and native Lufaxin (from the SGH). C, Surface plasmon resonance experiments demonstrate that SGH of *L. longipalpis* binds to immobilized FXa. SGH (a, 2 pairs; b, 1 pair; c, 0.5 pair; d, 0.25 pair; e, 0.125 pair; and f, 0.06 pair in 100 µL of HEPES-buffered saline-P) was used as an analyte to bind immobilized FXa. Association phase of 60 seconds was followed by 600-second dissociation. D, *L. longipalpis* SGH prolongs recalcification time. SGH (0–2 pairs per assay) was added to plasma and CaCl$_2$ was added to start reactions (7.5 mmol/L, final concentration). Clot formation was estimated by turbidimetry at 650 nm. Assay volume was 100 µL. E, Lufaxin and several recombinant proteins (50 nmol/L each) from the SG of *L. longipalpis* were incubated with plasma and recalcification time estimated as in D. Only Lufaxin prolonged the clotting time. Experiments were performed at least 3× and confirmed with different preparations of SGH or batches of recombinant Lufaxin.
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Figure 3. Lufaxin is a tight and slow inhibitor of factor Xa (FXa). A, Typical progress curves for FXa-mediated S2222 hydrolysis in the absence (curve a) and presence of Lufaxin (curves b, 0.93 nmol/L; c, 1.8 nmol/L; d, 3.75 nmol/L; e, 7.5 nmol/L; f, 15 nmol/L; g, 30 nmol/L; and h, 60 nmol/L). Reactions started with addition of S2222 (250 μmol/L) to a mixture containing Lufaxin incubated for 1 hour with FXa (2 nmol/L). Substrate hydrolysis was followed for 2 hours at 37°C, at 405 nm. B, The experiment was performed as in A but FXa concentrations were 2, 4, and 8 nmol/L. The ratio of Vs/Vo was plotted against Lufaxin concentration and data fitted with the Morrison equation to calculate the half maximal inhibitory concentration (IC50) at each enzyme concentration. C, Plot of IC50 and FXa produces a straight line typical of tight inhibitors. D, Slow-type inhibition of FXa by Lufaxin. Typical progress curves for FXa-mediated S2222 (1 mmol/L) hydrolysis in the absence (curve a) and presence of Lufaxin (curves b, 0.93 nmol/L; c, 1.8 nmol/L; d, 3.75 nmol/L; e, 7.5 nmol/L; f, 15 nmol/L; g, 30 nmol/L; h, 60 nmol/L). Reactions started with addition of FXa (0.5 nmol/L) to a mixture containing Lufaxin and S2222 (1 mmol/L). Substrate hydrolysis was followed for 2 hours at 37°C and 405 nm. E, Experiments were performed as in D, but S2222 concentrations were 150, 300, 450, 600, 750, 900 μmol/L. The ratio Vs/Vo obtained between 45 and 60 minutes was plotted against S2222 concentration and data fitted with the Morrison equation to calculate the IC50 at each substrate concentration. F, Plot IC50 and S2222 concentrations. Values were fitted by linear regression. The pattern of curves is typical for noncompetitive inhibitors. Six experiments were performed, and each data point is the average of duplicate determinations.

concentration) is expected to yield a straight line with a slope of 0.5, and y intercept equals apparent Ki (Ki*). The value Ki* is related to true Ki by factors involving substrate concentration and Km, depending on the mode of interaction between inhibitor and enzyme.34,35 Figure 3C reveals that inhibition of FXa by Lufaxin obeys typical graphic properties of a tight inhibitor, with a slope of 0.4504 and calculated Ki* of 0.95±0.07 nmol/L.

To verify whether Lufaxin is a slow or fast inhibitor of FXa, the enzyme was added to reaction mixture containing inhibitor and S2222. Reactions were initiated with FXa. This protocol also allows the characterization of the inhibitor as competitive, uncompetitive, or noncompetitive. Figure 3D shows that the progress curves displayed a downward concavity, which is followed by a linear component after 40 minutes which indicates that the reaction reached a steady state. This kinetic pattern is typical of slow-binding inhibitor. The Vs/Vo values obtained between 45 to 60 minutes (Vmax mode) were fitted by the Morrison equation for each substrate concentration.34,35 Figure 3E shows that the curves obtained using this approach were almost superimposable and the calculated IC50 was determined from 6 repeated duplicate experiments with a <10% error.

For tight inhibitors, a plot of IC50 versus substrate concentration and data fitted with the Morrison equation to calculate the half maximal inhibitory concentration (IC50) at each enzyme concentration. C, Plot of IC50 and FXa produces a straight line typical of tight inhibitors. D, Slow-type inhibition of FXa by Lufaxin. Typical progress curves for FXa-mediated S2222 (1 mmol/L) hydrolysis in the absence (curve a) and presence of Lufaxin (curves b, 0.93 nmol/L; c, 1.8 nmol/L; d, 3.75 nmol/L; e, 7.5 nmol/L; f, 15 nmol/L; g, 30 nmol/L; h, 60 nmol/L). Reactions started with addition of FXa (0.5 nmol/L) to a mixture containing Lufaxin and S2222 (1 mmol/L). Substrate hydrolysis was followed for 2 hours at 37°C and 405 nm. E, Experiments were performed as in D, but S2222 concentrations were 150, 300, 450, 600, 750, 900 μmol/L. The ratio Vs/Vo obtained between 45 and 60 minutes was plotted against S2222 concentration and data fitted with the Morrison equation to calculate the IC50 at each substrate concentration. F, Plot IC50 and S2222 concentrations. Values were fitted by linear regression. The pattern of curves is typical for noncompetitive inhibitors. Six experiments were performed, and each data point is the average of duplicate determinations.

Scheme 1

In our experiments with Lufaxin and FXa, the IC50 values were higher at or below the Km of the enzyme (0.3 mmol/L), but essentially the same at higher substrate concentration,
indicating a noncompetitive pattern and \( \alpha <1 \) (Figure 3F). Linear regression analysis indicates a negative slope \((-0.00222)\) with \( P=0.0104\), leading us to accept the hypothesis of Lufaxin being a noncompetitive inhibitor with an \( \alpha <1 \). Next, the IC\(_{50}\) values obtained for each substrate concentration of the 6 experiments were used to calculate the best \( \alpha \) and \( K_i \) (as determined by the least sum of squares to the observed points) using the equation for noncompetitive tight-binding inhibitors.

**Scheme 2**

\[
\text{IC}_{50} = \frac{[S] + K_m}{K_i} + \frac{[E]}{\alpha K_i}
\]

This transformation yield \( \alpha \) value of 0.28±0.049 and Ki of 7.9±0.70 nmol/L, which is the Ki in the limiting condition of substrate absence. The product \( \alpha K_i \) is thus equal to 2.21 nmol/L, being the Ki observed when all the enzymes are in the enzyme-substrate complex form (see Discussion).

**Ca\(^{2+}\) Is Not Required for FXa–Lufaxin Complex Formation**

Progress curves initiated by addition of FXa to a mixture containing Lufaxin and S2222 were superimposable when 0, 0.5 mmol/L, or 5 mmol/L Ca\(^{2+}\) was added to the reaction mixture (not shown, \( n=3 \)), indicating that Ca\(^{2+}\) is not required for FXa interaction with Lufaxin. Our results also did not detect interaction of Lufaxin with vesicles of phosphatidylcholine/phosphatidylserine (10 \( \mu \)mol/L), in the presence of Ca\(^{2+}\) (not shown).

Inhibition of FXa by Lufaxin was also unaffected by phosphatidylcholine/phosphatidylserine (20 \( \mu \)mol/L; not shown).

**Lufaxin Displays High-Affinity and Stoichiometric Interaction With FXa**

Kinetics of FXa interaction with Lufaxin were studied by SPR. Lufaxin was immobilized in a carboxymethylated dextran sensor chip, and FXa was used as analyte. Typical sensograms are shown in Figure 4A. Best fit was attained using a 1:1 Langmuir equation, with a calculated \( K_a \) of 2.29±1.325×10\(^{5}\) \( \text{M}^{-1}\)\( \text{s}^{-1}\) and \( k_{off} \) of 7.203±1.709×10\(^{-5}\) \( \text{s}^{-1}\) and equilibrium constant (KD) of 3.86±2.7 nmol/L (\( \chi^2=0.44\pm0.1 \)). Fitting was not significantly improved using other models (eg, 2-state reaction). When data points were fitted using steady-state kinetics, binding was saturable and yielded a KD of 9.69±0.38 nmol/L (\( \chi^2=0.083\pm0.03 \) (Figure 4B). Our results also show that Lufaxin interacts with bovine FXa, but does not bind to catalytic site-blocked FXa (DEGR-FXa), thrombin, FXaI, FIXa, or zymogen FX (Figure 4C). Lufaxin binding to FXa is noncovalent, as it was readily dissociated by the acidic pH needed to regenerate the sensor chip.

To determine the stoichiometry and enthalpy of the interaction between FXa and Lufaxin, ITC was performed. Fitting of the observed enthalpies to a single-site-binding model revealed a KD of 2.1±0.62 nmol/L for Lufaxin binding to FXa with a stoichiometry of binding \( n=1.38\pm0.006 \), compatible with 1:1 enzyme/inhibitor complex formation (Figure 4D). Binding was exothermic, with a favorable enthalpy \( \Delta H \) of −23.56 kcal/mol and unfavorable entropy \( \Delta S \) of −11.5 cal/mol K.

**Figure 4.** Kinetics and stoichiometry of Lufaxin interaction with factor Xa (FXa). A, SPR experiments demonstrate that FXa interacts with immobilized Lufaxin. FXa at 62.5 nmol/L (a), 31.25 nmol/L (b), 15.6 nmol/L (c), 7.8 nmol/L (d), and 3.9 nmol/L (e) were injected over immobilized Lufaxin for 180 seconds. Dissociation of the Lufaxin-FXa complex was monitored for 600 seconds, and a global 1:1 binding model was used to calculate kinetic parameters. Representative sensograms are shown in black, and fitting of the data points using the Langmuir equation is depicted in red. B, Lufaxin-FXa interaction calculated by steady-state kinetics. C, Lufaxin binds to human and bovine FXa, but does not interact with FX. Dansyl-Glu-Gly-Arg (DEGR)-FXa, FXaI, FIXa, or thrombin. All analytes were tested at 100 nmol/L and injected in a sensor chip containing immobilized Lufaxin. D, Solution binding of Lufaxin to FXa as measured by isothermal titration calorimetry. Top, base line-adjusted heats per injection of FXa (20 nmol/L) into Lufaxin (2.0 µmol/L). Bottom, molar enthalpies per injection for FXa interaction with Lufaxin. Filled squares, measured enthalpies; solid line, fit of experimental data to a single site-binding model. Thermodynamic parameters: \( \Delta H \) in kcal/mol, \( \Delta S \) in kcal/mol, and equilibrium constant (KD) are indicated in the inset. E, Lufaxin (1.9 µmol/L) was incubated with FXa (2.7 µmol/L) for 3 or 24 hours, with and without 5 mmol/L Ca\(^{2+}\). The mixture was loaded in a NuPAGE gel. The bands corresponding to FXa or Lufaxin are indicated. 1, FXa; 2, Lufaxin; 3, mixture of FXa and Lufaxin. No cleavage of Lufaxin is observed.
Lufaxin Is Not Cleaved by FXa

Our results demonstrate that no change in the pattern of Lufaxin migration was observed after overnight incubation at 37°C with molar excess of FXa, with or without 5 mmol/L Ca²⁺ (Figure 4E).

Lufaxin Displays Anti-Inflammatory Activity

Although FXa is a critical component of the coagulation cascade, it also induces cell activation through binding to PARs. This response induces cell signaling, which is accompanied by expression of adhesion molecules, procoagulant TF, and cytokine production. To verify whether Lufaxin blocks FXa-induced PAR2 activation in vitro, MDA-MB-231 cells, which express high levels of PAR2, were incubated for 1 hour with Lufaxin and stimulated with FXa for 10, 15, 30, or 60 minutes. Read-out for PAR2 activation was pERK1/2, which migrates as a p42–44 protein in the gel. All experiments were performed in the presence of hirudin to block (ectopic) thrombin activation of PAR1. As a control for loading, non-phosphorylated ERK was also used in Western blottings and probed with anti-ERK antibodies. Figure 5A demonstrates that FXa induces a robust response of the cells in a time-dependent manner that reaches a maximum at 60 minutes. In contrast, Lufaxin incubated with the cells for 1 hour, followed by addition of FXa, completely blocked pERK. Figure 5B depicts the quantification of the signal for pERK:ERK ratio by band densitometry for the experiments presented in Figure 5A.

In vivo, subplantar injection of FXa produces a time- and dose-dependent edema in the paws of mice that resembles the effects observed after administration of carrageenan. This effect has been demonstrated to be mediated by PAR2 activation. In an attempt to determine whether Lufaxin also inhibits the inflammatory activity of FXa in vivo, the enzyme was injected either alone in the mouse paw or in the presence of 2 concentrations of Lufaxin. Figure 5C shows that FXa induces a time-dependent increase in paw edema that reached a maximum at 15 minutes and returned to basal level at 60 minutes, as previously reported. When a mixture of FXa (7.3 µmol/L) and subsaturating concentrations of Lufaxin (2.5 µmol/L) were injected in the paw, edema was partially reduced; however, when FXa (7.3 µmol/L) was injected in the presence of molar excess of Lufaxin (9.3 µmol/L), complete blockade of edema formation was attained.

Lufaxin Is Antithrombotic In Vivo

To test whether Lufaxin displays antithrombotic activity, a mouse model of thrombosis was used in which FeCl₃ was used to induce carotid artery injury. Thrombus formation was estimated using a Doppler flow probe that allows monitoring of carotid blood flow for 60 minutes or until complete occlusion takes place. Times to occlusion were not significantly different between control and mice treated with 200 µg/kg Lufaxin (16.1 ± 1.47 versus 17.0 ± 1.63 minutes); however, mice treated with 500 µg/kg Lufaxin were resistant to arterial occlusion. In these cases, occlusion did not take place before 60 minutes for most animals (Figure 6A). Injection of Lufaxin (500 µg/kg) in mice also prolongs aPTT ex vivo (Figure 6B). Next, the effects of Lufaxin in bleeding were estimated using the tail transection method. Figure 6C shows that Lufaxin (500 µg/kg) produces significant bleeding, as would be expected for an inhibitor targeting FXa.

Discussion

Sand flies are blood-sucking arthropods that rely on the composition of their saliva to obtain a blood meal. Many pharmacologically active components have been identified in these secretions, including inhibitors of platelet aggregation, vasodilators, and immunomodulators. Notably identification of the major anticoagulant in sand fly saliva remained elusive for decades. Furthermore, analysis of the SG transcriptomes from several Phlebotominae has not revealed a candidate (eg, Kunitz-type proteins) with the potential to block...
FXa. In an attempt to identify the major anticoagulant of *L. longipalpis*, many molecules found in the SG were selected for transient expression in mammalian cells (human embryonic kidney 293 cells). Recombinant proteins obtained using this approach were initially tested for inhibition of platelet aggregation or clotting, or for vasodilation activity. As a result of this screening, we discovered that 1 candidate, herein named Lufaxin, prolonged recalcification time and both PT and aPTT, suggesting inhibition of the common pathway. A screening for inhibition of a panel of enzymes identified only FXa, of 16 enzymes, as the target for Lufaxin. Inhibition of FXa was also observed with the SGs of *P. papatasi* and *P. dubosci*. In addition, components from the SGH of *L. longipalpis* dose-dependently interact with FXa immobilized in a carboxymethylated dextran sensor chip (Figure 2). Moreover, a band corresponding to Lufaxin was identified by Western blot using homogenates implying that Lufaxin is expressed in the SGs of sand flies.

Notably, the primary sequence of Lufaxin is novel and it does not display similarity to the sequences or molecular domains of other proteins deposited in the databases. Therefore, it does not belong to any known family of physiological or salivary coagulation inhibitors such as Kunitz-type proteins (eg, TF pathway inhibitor, tick anticoagulant peptide, simulikinin, ixolaris), *Ascari*s-type inhibitors (eg, nematode anticoagulant peptide 5, ancylostoma ceylanicum anticoagulant peptide-1), antistasin-like inhibitors from leeches (eg, antistasin, ghilanten, therostasin), serpins (eg, antithrombin, mosquitos serpin), or members of the Salp family (eg, Salp14, Salp9). Remarkably, each of these inhibitors displays a particular mode of FXa inhibition, either mechanistically or through unique structural features, or both. For example, in tick anticoagulant peptide, the 3 N-terminal residues of tick anticoagulant peptide make multiple contacts with the catalytic site of FXa noncanonically, whereas in nematode anticoagulant peptide 5 or antistasin, both inhibitors canonically interact with FXa active site through the reactive-site loop that possesses a P1 Arg. In this respect, FXa has specificity for Ile-Glu-Gly-Arg-X, and many protein inhibitors of serine proteases inhibit their target enzyme by presenting a substrate- or bait-like region that resembles the natural substrate of enzyme which display Arg or Lys as P1 position. In Lufaxin, a Lys residue is present adjacent to the fifth cysteine; several other positively charged residues that form a consensus C(K/L)LVFKK(R/K)(R/K) sequence between the fifth and sixth cysteines have also been identified (Figure 1). However, our attempt to identify the P1-P1′ reactive site of Lufaxin was unsuccessful, as no cleavage of the inhibitor was observed after 3- and 24-hour incubation with a molar excess of FXa, with or without Ca²⁺ (Figure 4). This result is not entirely surprising, because cleavage at the active-site cleft (P1-P1′) of other FXa inhibitors (eg, TF pathway inhibitor) occurs only slowly or not at all. Therefore, it remains to be identified how Lufaxin, a molecule whose sequence has no precedents, interacts with FXa at the molecular level.

Kinetic studies using chromogenic substrate S2222 revealed that Lufaxin is a slow, tight, reversible, Ca²⁺-independent, and noncompetitive inhibitor of FXa, which blocks the enzyme with a KD = 8.0 nmol/L (Figure 3F). Consistent with these results, SPR experiments revealed the KD of Lufaxin interaction to FXa as = 3.5 nmol/L. This value is also in good agreement with the KD (= 2 nmol/L) calculated by ITC experiments which is based on thermodynamic parameters. ITC experiments also determined that 1 molecule of Lufaxin binds to 1 molecule of FXa, validating Lufaxin as a tight inhibitor. Of note, Lufaxin–FXa complex formation is considered fast (Kon = 2.2 × 10⁶ mol/L⁻¹.s⁻¹) according to SPR results. Therefore, the slow-inhibition of FXa by Lufaxin observed with small substrate may in part be attributable to the use of the low molecular mass of S2222 instead of the natural macromolecular substrate prothrombin. Conceivably, this complex slowly converts to a stable one involving the catalytic site, which explains
why slow-binding kinetics was observed in the experiments using S2222. It also suggests that exosites may be involved in the interaction of Lufaxin with FXa. Also, inhibition of catalytic activity of FXa assessed with S2222 was not complete at equimolar concentration of Lufaxin/FXa (Figure 3C), despite a 1:1 interaction confirmed by ITC. It is concluded that S2222 retains some degree of accessibility to the active site of FXa even when Lufaxin is bound to the enzyme. This is congruent with the finding that Lufaxin is a noncompetitive type inhibitor of FXa with respect to S2222. This means that the inhibitor is not likely to have an overlapping binding site with that of the chromogenic substrate. Furthermore, the smaller αK, compared with K, indicated that the conformational changes in FXa on S2222 binding have positive influence on Lufaxin interaction with the enzyme. It is also important to recognize that SPR experiments did not detect binding of Lufaxin to DEGR-FXa, which in many respects mimics the enzyme–substrate transition state complex. This result suggests that occupancy of the catalytic site of FXa by DEGR may not entirely reproduce the interactions observed with S2222; of note, DEGR is an irreversible glu-gly-arg-chloromethyl ketone containing a dansyl group which is not present in the cleavable chromogenic substrate. Conceivably, the kinetics of FXa inhibition by Lufaxin in the presence of S2222 and described by scheme 1 may not fully apply when DEGR-FXa is used as [S]. Additional kinetics and structural studies with catalytic site-mutated FXa (eg, FXaα195A) among other residues will be required to identify which specific amino acids contribute to FXa/Lufaxin complex formation and how DEGR interferes with this interaction.

The fact that Lufaxin inhibits FXa has relevant implications to the feeding behavior of the sand fly. FXa is central enzyme in coagulation and plays a critical role in prothrombinase complex assembly leading to amplification of the coagulation cascade.12 FXa is also a direct inflammatory molecule that acts in PARs, producing many effects such as mitogenic endothelial cells, including cytokine induction and NO-mediated hypotension, and hyperalgesia through activation of afferent sensory neurons.13–16 In other words, PAR expressed around hypotenus at the interface of vector–host interactions. In fact, sand flies mouth parts work as scissors, which produce tissue damage, creating microhemorrhages from which blood is taken.13–16,50 Conceivably, intense exposure of TF leads to strong activation of the coagulation cascade at sites of bites. Accordingly, blocking FXa is relevant to prevent clot formation under the prohemostatic environment generated by the feeding behavior of sand flies. Identification of Lufaxin as a novel FXa inhibitor is also relevant experimentally to study the participation of FXa in ischemic events, tumor growth, or metastasis.13–16,50 Because of its unique sequence, Lufaxin may also be regarded as a useful tool to understand FXa structure and function.

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Disclosures
None.

References


Lufaxin, a Novel Factor Xa Inhibitor From the Salivary Gland of the Sand Fly Lutzomyia longipalpis Blocks Protease-Activated Receptor 2 Activation and Inhibits Inflammation and Thrombosis In Vivo

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Supplement Material: Material and Methods

Reagents

FXa, FX, and FIXa were from Enzyme Research Laboratories (South Bend, IN). DEGR-FXa, FXIa, FVa, prothrombin and thrombin were from Hematologic Technologies (Essex Junction, VT). Fibrillar collagen (from equine tendons) was from Chrono-log (Haverton, PA), and U46619 was purchased from Cayman Chemicals (Ann Arbor, MI). APTT (STA-PTT Automate) and PT (Neoplastine CI Plus) reagents were from Diagnostica Stago (Asnieres, France). S2222 (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroaniline hydrochloride) and S2238 (H-d-phenylalanyl-l-pipecolyl-l-arginine-p-nitroaniline dihydrochloride) were obtained from Diapharma (West Chester, OH). Phosphatidylcholine and phosphatidylserine were from Sigma Co. (Saint Louis, MO). Anti-phospho-ERK 1/2 and polyclonal anti-ERK 1/2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with biotin and peroxidase-conjugated streptavidin were obtained from Zymed (Invitrogen, San Diego, CA).

Sand Flies and Preparation of SG Homogenate (SGH)

*L. longipalpis* (Jacobina strain), *P. papatasi*, and *P. duboscqi* were reared at the Laboratory of Malaria and Vector Research (NIAD/NIH) using as larval food a mixture of fermented rabbit feces and rabbit food. Adult sand flies were offered a cotton swab containing 20% sucrose, and females were used for dissection of SGs at 4–7 days following emergence. SGs were stored in groups of 10 pairs in 10 µL NaCl (150 mmol l⁻¹), Hepes buffer (10 mmol l⁻¹; pH 7.4) at −70°C until needed. SGs were disrupted by ultrasonication within 1.5-mL conical tubes. Tubes were
centrifuged at 16,000×g for 5 minutes, and the resultant supernatant was diluted in PBS and used for the assays.

**Lufaxin Properties**

cDNA for mature Lufaxin (gi41397464; clone LJL143) codes for a protein of predicted molecular weight of 32495.78 da (278 amino acids [aa]) with an estimated pI 8.27. Extinction coefficient at 280 nm is 36180 (all disulfide bonds); A280 nm/cm0.1% (1 mg/ml), 1.0975.

**Cloning of *L. longipalpis* cDNAs in His-Tagged TOPO Vector**

VR2001-TOPO is a topoisomerase adaptation of VR1020 plasmid (Vical, Inc., San Diego, CA) described in a previous report.1 cDNA of Lufaxin (and other candidates) were amplified by PCR using a specific forward primer deduced from the amino-terminus region and a specific reverse primer containing an ATGATGATGATGATGATG motif between the stop codon and the carboxy-terminus region to introduce a 6xHis tag. The expected amplified sequences were predicted to code for proteins starting after the natural cleavage site and containing a 6xHis tag at the C-terminus region. PCR amplification conditions were: 1 hold of 94ºC for 5 minutes, 2 cycles of 94ºC for 30 seconds, 48ºC for 1 minute, 72ºC for 1 minute, 23 cycles of 94ºC for 30 seconds, 58ºC for 1 minute, 72ºC for 1 minute, and 1 hold of 72ºC for 7 minutes. Amplified products were extracted from a 1.0% agarose gel using Ultrafree-MC extraction kit (Millipore, Billerica, MA). Three µL of each PCR product was immediately incubated with 0.5 µL of VR2001-TOPO, 1 µL of salt solution (1.2 M NaCl, 0.06 M MgCl₂), and 1.5 µL H₂O for 5 minutes at room temperature. Transformation and selection of positive clones by sequencing were performed following standard procedures.1
Production and purification of recombinant proteins

VR2001-TOPO plasmids coding for Lufaxin (clone LJL143; gi 41397464, AY445936) and other salivary proteins (LJM17, AF132518; LJM04, AF132517; LJL15, DQ190946; LJM11, AY445935; LJL13, AF420274; LJM111, DQ192488; LJM26, AY455913) were used for protein expression in HEK-293 F cells at the Protein Expression Laboratory at NCI-Frederick (Frederick, Maryland), and reported elsewhere. All proteins were expressed containing a 6xHis tag. The supernatant was collected after 72 hours and concentrated from 500 ml to 300 ml using a Stirred Ultrafiltration Cell unit (Millipore) with a 30 kDa ultrafiltration membrane (Millipore). The volume was returned to 1 L by the addition of 500 mM NaCL and 10mM Tris, pH 8.0. The protein was purified by an HPLC system (DIONEX) using two 5ml HiTrap Chelating HP columns (GE Healthcare) in tandem and charged with 0.1 M NiSO₄. The protein was detected at 280nm and eluted by an imidazole gradient as follows: 0-5 min, 100% Buffer A (20 mM NaH₂PO₄, 20 mM Na₂HPO₄, pH 7.4, 500 mM NaCl); 5-15 min, a gradient of 0% to 100% Buffer B (Buffer A+50 mM imidazole); 15-17 min, a gradient of 0% C (Buffer A + 500 mM imidazole) to 10% C (90% B); 17-22 min, 90% B and 10% C; min 22-27, a gradient of 10%C to 20% C (80% B); 27-35 min, 80% B and 20% C; 35-40 min, a gradient of 20% C to 100%C; and 40-50 min, 100% C. Eluted proteins were collected every minute in a 96-well microtiter plate using a Foxy 200 fraction collector (Teledyne ISCO). Fractions corresponding to peak(s) were selected and run on a NuPage Bis-Tris 4-12% Gel (Novex) with MES running buffer under reducing conditions as per manufacturer’s instructions. Briefly, NuPage LDS sample buffer (Invitrogen), NuPage reducing agent (Invitrogen), and sample were combined and heated to 70°C for 10 min. Samples were loaded in gel with SeeBlue Plus2 Pre-Stained Standard (Invitrogen) and run at 200V for 35 min with an expected current of 100-125mA (start), 60-
80mA (end). After run, gel was stained with Comassie Blue (0.025%) to visualize proteins. Appropriate fractions as determined by molecular weight compared to standard in gel were pooled and concentrated to 1ml using a 30kDa Amicon Ultra Centrifugal Filter (Millipore). The protein sample was then injected into a g2000sw molecular sieving column (Tosoh Biosciences) with a 1 ml loop connected to HPLC (DIONEX) with PBS pH 7.2 as the buffer for further purification. The protein was detected at 280 nm and the fractions were collected as described above. Appropriate fractions were determined as described above and pooled. Concentration was measured by using a NanoDrop ND-1000 spectrophotometer at 280 nm and calculated using the extinction coefficient of the protein.

**Polyclonal Antibodies against Lufaxin**

VR2001-TOPO plasmids containing coding sequence of Lufaxin without His-tag extension were used to inject mice (20 μg, intradermally in the ear of mice) and generate polyclonal antibodies. Pre-immune samples were drawn before the first injection, and immune serum samples were taken by retroorbital bleeding after 3 injections given at 2-week intervals. Each mouse serum sample was pooled for experimentation.

**PAGE and Western Blotting**

The samples were treated with 4× NuPAGE LDS sample buffer and analyzed in NuPAGE 4–12% gels with MES running buffer. SeeBlue® Plus2 molecular weight marker was used. The proteins in the gel were transferred to nitrocellulose membrane using an iBlot™ device. After blocking with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), pH 8.0, the membrane was incubated with sera of mice immunized with Lufaxin (1:100 in TBS-T 5% milk).
After two washes with TBS-T, the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (ZyMax™) at 1:10000 in TBS-T 5 % milk for 40 minutes at room temperature. After 3 washes with TBS-T, the blots were developed by addition of Western Blue® stabilized substrate for alkaline phosphatase (Promega). For N-terminal sequencing, Lufaxin was loaded in a 4-12% NU-PAGE and transferred to PVDF membranes (0.45 μM) in 10 mM CAPS, 10% methanol, pH 11. All non-specified reagents were from Invitrogen.

**Deglycosylation of Lufaxin**

This was performed using the Enzymatic DeGlycoMx Kit from QA-Bio (Palm Desert, CA) which contains a mixture of PNGase F, Sialidase, β-Galactosidase, Glucosaminidase and O-Glycosidase. The assay was performed following the manufacturer instructions. Briefly, in a PCR tube, 5 μl of Lufaxin (5 μg) was added to 33 μl of water, followed by addition of 2.5 μl of denaturing solution. The samples were mixed and heated at 99°C for 10 min. Then, 2.5 μl of Triton-X was added, followed by addition of 2 μl of DeGlycoMx for 3 hr at 37°C. Samples were analyzed by NU-PAGE, and Coomassie Blue stained.

**Platelet Aggregation Assays**

Platelet-rich plasma was obtained by plateletpheresis from medication-free platelet donors at the DTM/NIH blood bank. Aggregation was performed as described previously.²

**Contraction of Rat Aorta**

Contraction of rat aortic ring preparations by U 46619 was measured isometrically and recorded with transducers from Harvard Apparatus Inc. (Holliston, MA) as reported.³ A modified Tyrode
solution was prepared with the addition of 5 mM Hepes; the pH was adjusted to 7.4, and the solution was oxygenated by continuous bubbling of air throughout the assays. In the first assay, aortic rings were suspended in a 0.5-mL bath kept at 36°C and were pre-constricted by 100 nM U-46619 before addition of Lufaxin to give final concentrations of 1 μM, or salivary gland homogenates of *Rhodnius prolixus* (0.04 of one pair of glands/ml, with an approximate final concentration of nitrophorins of 2 μM - positive control). Additions to the bath were never greater than 5% of the volume of the bath.

**Recalcification Time**

Clotting activity was measured by the recalcification time of human plasma using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA) with a kinetic module. Briefly, 30 μL of citrated human platelet-poor plasma and 30 μL of sample (SGH or recombinant Lufaxin diluted in TBS) were mixed in 96-well flat-bottom plates. After incubation of 10 minutes at 37°C, 30 μL of pre-warmed 25 mM CaCl₂ (final concentration 7.5 mM, diluted in TBS) were added using an 8-channel multipipette for delivering calcium aliquots. The plate was immediately mixed and maintained at 37°C during the kinetic experiment. Absorbance readings at 650 nm were taken at 10-second intervals for 20 minutes. Onset time was defined as the time to a linear increase in the OD, which reflects the maximal rate of formation of insoluble fibrin set at a OD which is approximately half of the maximum obtained in the presence of calcium only (no inhibitor).

**aPTT and PT Assays**

The effect of Lufaxin on coagulation tests aPTT and PT was evaluated on an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany). Human blood samples were collected from
healthy donors in 3.8% trisodium citrate (9:1, v/v), and platelet-poor plasma was obtained by centrifugation at 2,000×g for 10 minutes. Mouse plasma samples were obtained following the same procedure after blood collection by cardiac puncture. Plasma (50 μL) was incubated with Lufaxin for 2 minutes at 37ºC, followed by addition of the aPTT reagent (50 μL, 1 minute) or the PT reagent (100 μL) and then 25 mM CaCl₂ (100 μL). Time for clot formation was then recorded. For ex vivo assays in mice, phosphate-buffered saline (PBS) or Lufaxin were given i.v. 15 minutes before cardiac puncture. Blood collection, plasma preparation, and aPTT procedure were performed as 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% BSA, pH 7.5), using a discontinuous assay. FXa (20 pM, final concentration), was incubated with Lufaxin (0-10 nM) for 20 minutes at room temperature. Human FVa (1 nM, final concentration) and PC/PS vesicles (10 μM, final concentration) were added and incubated for 5 minutes. Reactions were initiated by addition of human prothrombin (1.4 μM, final concentration). 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 S-2238 (312.5 μM), absorbance at 405 nm was recorded at 37°C for 15 minutes at 11-second intervals using a Thermomax microplate reader (Molecular Devices). Initial velocities (Vmax mode, mOD/minute) obtained were used to calculate the amount of thrombin formed, using a standard curve. Absence of one of the components in the prothrombinase showed no thrombin formation.
Interaction of Lufaxin with phospholipid vesicles

Lufaxin (1.25 μM) was incubated with PC/PS (10 μM) in 10 mM TBS (pH 7.4) supplemented with CaCl₂. After 30 min, the mixture was centrifuged and NUPAGE sample buffer was added to the pellet and to the supernatant. The samples were loaded in a 4-12% NUPAGE gel, and Coomassie blue stained. Lufaxin which has not been incubated with PC/PS was used as control.

Kinetics Studies

This was performed as described ⁵ using chromogenic substrate (S2222) hydrolysis specific for FXa. Substrate hydrolysis was estimated by color development at 405 nm at room temperature, using an ELISA reader (Molecular Devices). All reagents were diluted in the reaction buffer, TBS-BSA (10 mM Tris, 0.15 M NaCl, 0.3% BSA, pH 7.4). To characterize the interaction of Lufaxin and FXa as tight, reactions were started by addition of S2222 (250 μM) to a mixture containing enzyme (2, 4 and 8 nM) and inhibitor (0-60 nM) pre-incubated for 1 hour at room temperature. The total volume of the reaction was 100 μL, and reactions were followed for one hour. Initial velocities (Vmax mode, mOD/minute) obtained were used to determine the ratio of substrate hydrolysis by $V_s$(inhibited)/$V_o$(uninhibited) by the enzyme in the presence of the inhibitor, which allows determination of the IC₅₀. ⁵, ⁶ For assays designed to determine whether Lufaxin is a slow or fast inhibitor of FXa, and the type of inhibition (competitive or non-competitive), the inhibitor (0-60 nM) was incubated with S2222 (150, 300, 450, 600, 750 and 900 μM) at room temperature for 5 minutes followed by addition of FXa (0.5 nM). Reactions were followed for 2 hours, in a 200 μL volume. In all kinetic measurements, care was taken to ensure that substrate was less than 20% hydrolyzed. The linear part of the progress curves between 45-60 min was chosen to determine the steady state kinetics of Lufaxin-FXa complex.
formation. These values were used to plot $V_s/V_o$ versus Lufaxin for each S2222 concentration, at a constant FXa concentration. Data points were fitted with the Morrison equation using GraphPad Prism software, which allows the determination of IC$_{50}$ for each S2222 concentration:\textsuperscript{5,6}

$$
\frac{V_s}{V_o} = \frac{[E_i] - [I_i] - K_i^* + ([I_i] + K_i^* - [E_i])^2 + 4K_i^*[E_i]^{1/2}}{2[E_i]}
$$

where $E_i$ is the total enzyme concentration, $I_i$ is the total inhibitor concentration and $K_i^*$ is the IC$_{50}$ or apparent Ki. The plot IC$_{50}$ vs S2222 was then used to calculate the type of inhibition and true Ki. Data points are the mean of 6 determinations, each performed in duplicates. In some experiments to determine the role of Ca$^{2+}$ or phospholipids in Lufaxin interaction with FXa, Ca$^{2+}$ (0.5 or 5 mM), or PC/PS vesicles (20 µM) was added to the reaction mixture.

**Determination of the active site of Lufaxin**

In a PCR tube, Lufaxin (1.9 µM) was incubated with human FXa (2.7 µM) for 3 or 24 hours at 37°C, with and without 5 mM Ca$^{2+}$. LDS loading buffer and DTT was added to the tubes, warmed for 10 min at 70°C, and the mixture was loaded in a 4-12% NU-PAGE gel (MES buffer) and Coomassie Blue stained. See Blue standard was used as molecular weight marker (Invitrogen).

**Surface Plasmon Resonance (SPR)**

All SPR experiments were carried out in a T100 instrument (Biacore Inc., Uppsala, Sweden) following the manufacturer's instructions. For immobilization using an amine coupling kit (Biacore), CM5 chips were activated with 1-ethyl-3-(dimethylaminopropyl) carbodiimide, and N-hydroxysuccinimide before injection of Lufaxin (6.5 µg/mL) in acetate buffer, pH 5.5.
Remaining activated groups were blocked with 1 M ethanolamine, pH 8.5, resulting in a final immobilization of 604.5 RU. Kinetic experiments were carried out by injecting FXa for a contact time of 180 seconds at a flow rate of 30 µL/minute at 25°C. For all runs, HBS-P buffer was used (10 mM HEPES, 150 mM NaCl, 0.005% surfactant P20, pH 7.4). FXa-Lufaxin complex dissociation was monitored for 600 seconds, and the sensor surface was regenerated by a pulse of 30 seconds of 10 mM HCl at 30 µL/minute. In some experiments, other coagulation factors (thrombin, FXIa, FIXa, DEGR-FXa, or FX) were tested (100 nM) as analytes. For some experiments, FXa (30 µg/mL in acetate buffer pH 5.0) was immobilized in CM5 sensor chips at 661.7 RU, and SGH from *L. longipalpis* was used as analyte. Blank flow cells were used to subtract the buffer effect on sensorgrams. After subtraction of the contribution of bulk refractive index and nonspecific interactions with the CM5 chip surface, the individual association (*ka*) and dissociation (*kd*) rate constants were obtained by global fitting of data using the 1:1 model (Langmuir) interaction model using BIAevaluation™ (Biacore, Inc.). Values were then used to calculate the equilibrium constant (*KD*). The values of average squared residual obtained were not significantly improved by fitting data to models that assumed other interactions. Conditions were chosen so that the contribution of mass transport to the observed values of *KD* was negligible. Also, models in the T100 evaluation software fit for mass transfer coefficient to mathematically extrapolate the true *ka* and *kd*.

**Isothermal Titration Calorimetry (ITC)**

Lufaxin binding to FXa was performed using a VP-ITC microcalorimeter (Microcal, Northampton, MA) at 30°C. Prior to the run, the proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4 for binding experiments. Titration experiments were performed by
making successive injections of 5 µL each of 20 µM FXa into the 2-mL sample cell containing 2 µM Lufaxin until near-saturation was achieved. The calorimetric enthalpy (ΔHcal) for each injection was calculated after correction for the heat of Lufaxin dilution obtained in control experiments performed by titrating Lufaxin into buffer. The binding isotherms were fitted according to a model for a single set of identical binding sites by nonlinear square analysis using Microcal Origin software (Microcal, Northampton, MA). The enthalpy change (ΔH) and stoichiometry (n) were determined according to equation 1:

\[ Q = n \theta M_t \Delta H V_0 \]  

(1)

where Q is the total heat content of the solution contained in the cell volume (V₀), at fractional saturation θ, ΔH is the molar heat of ligand binding, n is the number of sites, and Mt is the bulk concentration of macromolecule in V₀. The binding constant, Kₐ, is described as in (2):

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

(2)

where [X] is the free concentration of ligand.

The free-energy (ΔG) and entropy term (−TΔS) of association were calculated according to (3) and (4):

\[ \Delta G = -RT \ln(K_a) \]  

(3)

\[ \Delta G = \Delta H - T \Delta S \]  

(4)

**Cell Culture**

MDA-MB-231 breast cancer cells were maintained in ISCOVES medium (Invitrogen, CA) supplemented with 10% FBS in culture flasks in a 5% CO₂-air mixture at 37°C. Subconfluent cultures were washed twice with PBS, and cells were detached with Hank’s solution containing
10 mM HEPES and 0.2 mM EDTA. Cells were seeded at $5 \times 10^5$ cells/well in 6-well tissue culture plates for signaling assays.

**PAR2 Signaling Assay**

MDA-MB-231 cells were serum starved for 90 minutes and stimulated with 10 nM FXa (in ISCOVES medium, no FBS) for 10, 15, 30, and 60 minutes. Lufaxin (50 nM) was added 1 hour prior to stimulation with FXa. To avoid PAR1 activation by thrombin, assays were performed in the presence of 10 nM hirudin as described. After the incubation period, cells were washed with PBS and lysed in cold buffer containing a phosphatase inhibitor cocktail (Sigma). Cell lysates (15 µL) were separated by SDS-PAGE (10%). Proteins were transferred onto polyvinylidene fluoride membranes (Millipore) and blocked with TBS supplemented with 5% BSA and 0.1% Tween 20 for 1 hour at room temperature. Membranes were then probed with primary antibody anti-pERK (1:000 in TBS, 2% BSA, 0.1% Tween 20, TBS-BSA) overnight at 4ºC. The membranes were washed 3 times with TBS-BSA before the addition of goat anti-mouse biotin-labeled secondary antibody (1:20,000 for p-ERK; 1:30,000 for ERK) for 1 hour at room temperature. The membranes were washed in TBS-BSA and probed with peroxidase-conjugated streptavidin (1:30,000; Zymed-Invitrogen, CA) for 1 hour at room temperature. After washing the membranes, immunodetection was carried out side-by-side by a chemiluminescent method using the Western Lightning ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and Amersham Hyperfilm ECL (General Electric, UK). The blots were quantified by Scion Image software (Scion Corporation, USA). The relative levels of pERK were estimated as a ratio to ERK 1/2.
Paw Edema in Mice

Female C57BL/6 mice, 6–8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the NIAID Animal Care Facility. Mice were maintained at an American Association of Laboratory Animal Care-accredited facility at NIAID, National Institutes of Health. All experiments with mice were evaluated and approved by the NIAID Animal Care and Use Committee of the National Institutes of Health (Rockville, MD). Prior to each injection, the posterior footpad thickness of each mouse was recorded using a caliper (Mitutoyo America Corp., Aurora, IL). Subsequently, 30 μL of PBS, or 30 μL of FXa (7.3 μM, 10 μg), or 30 μL of a mixture containing 30 μL FXa (7.3 μM) plus Lufaxin (2.4 μM), or 30 μL FXa (7.3 μM) plus Lufaxin (9.8 μM) were injected intradermally in the paw using a 0.3-mL syringe U-100 29g1/2 (ref 309301) from Becton Dickinson (Franklin Lakes, NJ). As an index of edema formation, paw thickness was then measured at 15, 30, 45, and 60 minutes. Control groups of mice received the same volume of PBS (vehicle). For each data point, 4 posterior paws were injected. Statistical analysis of variance using Tukey as a multiple comparison post-test was used. A p value of 0.05 or less was considered statistically significant.

FeCl$_3$-Induced Artery Thrombosis

BALB/c mice were anesthetized with intramuscular xylazine (16 mg/kg) followed by ketamine (100 mg/kg). The right common carotid artery was isolated through a midline cervical incision, and blood flow was continuously monitored using a 0.5 VB Doppler flow probe coupled to a TS420 flow meter (Transonic Systems, Ithaca, NY). Fifteen minutes before induction of thrombosis, animals were injected in the tail vein with 50 μl Lufaxin (0.2 or 0.5 mg/kg) or vehicle. 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. Statistical analysis of variance using Tukey as a multiple comparison post-test was used. A p value of 0.05 or less was considered statistically significant.

**Tail Bleeding Assay**

Mice were anesthetized as described above and injected intravenously with PBS or Lufaxin in a 100-μL volume. After 15 minutes, the distal 2-mm segment of the tail was removed and immediately immersed in a 50-mL Falcon tube filled with saline buffer warmed to 37°C. The samples were properly homogenized and the absorbance determined at 540 nm to estimate hemoglobin content. No animal was allowed to bleed for more than 30 minutes.

**Protease inhibition assays**

All assays were performed at 30°C in triplicates. One hundred nM of Lufaxin was pre-incubated with each enzyme for 20 min before the addition of the corresponding substrate. Hydrolysis rate of the fluorescent substrate was estimated from the slope that results from the linear fit (arbitrary fluorescence units per sec; χ² > 0.95) of the data (each experiment was performed in triplicate, and the mean of the three experiments and the standard error of the mean were calculated). The linear fit of the fluorescence increase as a function of time was verified with the Magellan™ - Data Analysis Software (Tecan group Ltd). The observed substrate hydrolysis rate in the absence of protein was considered as 100% and compared with the remaining enzymatic activity in the presence of the protein.
All enzymes used were of human origin, purified or recombinant. The source and assay concentration of the different enzymes follow: thrombin (0.01 nM), α-chymotrypsin (0.04 nM), plasmin (0.8 nM), and chymase (0.45 nM) were purchased from Sigma; trypase (0.01 nM) was purchased from Promega; FXa (0.5 nM) was purchased from EMD Biosciences (Madison, WI); FXIIa (0.15 nM) was purchased from Haematologic Technologies Inc.; kallikrein (0.06 nM) was purchased from Fitzgerald Industries International; elastase (0.18 nM) was purchased from Elastin Products; cathepsin G (4.4 nM), FXIa (0.06 nM), uPA (0.125 nM), and tPA (0.025 nM) were from Molecular Innovations; matriptase (0.04 nM) was from R&D Systems; proteinase 3 (5.5 nM) was from Merck; and sequencing-grade trypsin (0.2 nM) was purchased from Roche. Assay buffers were: for elastase, proteinase 3 and chymase, 50 mM Hepes buffer, pH 7.4, 100 mM NaCl, 0.01% Triton X-100; for trypsin and chymotrypsin, FXIa and FXIIa, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM CaCl₂, 0.01% Triton X-100; for thrombin, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Triton X-100; for trypase, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.02% Triton X-100; for FXa, 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM CaCl₂, 0.1% BSA; for uPA and tPA, 20 mM Tris-HCl, pH 8.5, 0.05% Triton X-100; and for cathepsin G, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Triton X-100. The substrates used were Suc-Ala-Ala-Pro-Val-AMC for elastase and proteinase 3, Boc-Asp-Pro-Arg-AMC for thrombin and plasmin, Boc-Gln-Ala-Arg-AMC for trypsin, factor XIa and uPA (Sigma), Boc-Phe-Ser-Arg-AMC for trypase, Suc-Leu-Leu-Val-Tyr-AMC for chymase (Bachem Bioscience, Inc.), Suc-Ala-Ala-Pro-Val-AMC for chymotrypsin (EMD Biosciences) and methylsulfonyl-D-cyclohexylalanyl-Gly-Arg-AMC acetate for FXa, FXIIa, t-PA, matriptase, and kallikrein (American Diagnostica Inc.). All substrates were used in 250 μM final concentration in all
assays. Substrate hydrolysis rate was followed in a Tecan Infinite M200 96-well plate fluorescence reader (Tecan group Ltd, Switzerland) using 365 nm excitation and 450 nm emission wavelengths with a cutoff at 435 nm. \( t\)-test was used for statistical analysis and a \( p \) value of 0.05 or less was considered statistically significant.

References