FXa-α2-Macroglobulin Complex Neutralizes Direct Oral Anticoagulants Targeting FXa In Vitro and In Vivo

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Abstract
Increasing number of patients are treated with direct oral anticoagulants (DOAC). An antidote for dabigatran inhibiting thrombin (idarucizumab) is available but no antidote is yet approved for the factor Xa (FXa) inhibitors (xabans). We hypothesized that a complex between Gla-domainless FXa and α2-macroglobulin (GDFXa-α2M) may neutralize the xabans without interfering with normal blood coagulation.

Purified α2M was incubated with GDFXa to form GDFXa-α2M. Affinity of apixaban and rivaroxaban for GDFXa-α2M was only slightly decreased compared to FXa. Efficacy and harmlessness of GDFXa-α2M were tested in vitro and in vivo. Stoichiometric excess of GDFXa-α2M neutralized rivaroxaban and apixaban as attested by clot waveform assay and rotational thromboelastometry, whereas GDFXa-α2M alone had no effect on these assays. Efficacy and pro-thrombotic potential of GDFXa-α2M were also assessed in vivo. Half-life of GDFXa-α2M in C57BL6 mice was 4.9 ± 1.1 minutes, but a 0.5 mg/mouse dose resulted in uptake saturation such that 50% persistence was still observed after 170 minutes. Single administration of GDFXa-α2M significantly decreased the rivaroxaban-induced bleeding time (p < 0.001) and blood loss (p < 0.01). GDFXa-α2M did not increase D-dimer or thrombin–antithrombin complex formation, suggesting a lack of pro-thrombotic potential.

GDFXa-α2M is therefore an attractive candidate for xaban neutralization neither pro- nor anticoagulant in vitro as well as in vivo.

Keywords
► antidote
► apixaban
► rivaroxaban
► oral anticoagulant

Introduction

Following decades of vitamin K antagonist prescription as unique oral anticoagulant bridging therapy with heparin or its low molecular weight (MW) derivatives, direct oral anticoagulants (DOACs) have been developed targeting thrombin (dabigatran) or coagulation factor Xa (FXa; xabans). As prescriptions expand, experience underlines that bleeding complications also occur with DOAC.2–5 This is specially threatening in the absence of available antidote or reversal agent in clinical scenarios such as massive haemorrhage, trauma, stroke requiring thrombolysis or urgent surgery.

Effective specific antidotes are available for vitamin K antagonists, heparin and partially for its low MW derivatives.5–9 An antidote for dabigatran had been approved which consists in a humanized monoclonal Fab (idarucizumab) that can be used in case of severe active bleeding and emergency surgery or invasive procedure.10–12 Xabans still lack approved antidote.
Pro-haemostatic agents (prothrombin complex concentrate activated or not) increase thrombosis risk, thus their use is currently restricted to life-threatening situations. Andexanet-α is the most advanced candidate as antidote to xabans. It is a modified FXa lacking its Gla domain that had been inactivated by a S195A mutation. Andexanet-α efficiently binds FXa inhibitors and does not activate prothrombin nor consume antithrombin albeit still forms a low affinity Michaelis complex with the serpin. ANNEXA-4 clinical trial is currently in progress to confirm its efficacy and safety. Ciraparantag is a small cationic compound antagonizing DOAC as well as heparin and derivatives. Little data are yet available on this molecule which is currently in phase II trial. Besides anticoagulants, potent-by-passing agents are also in the pipeline, such as the 116L FX variant, acting as a zymogen-like acquiring prothrombin activator potential through factor Va binding, or the DOAC resisting 99-loop FX variant. Their key advantage resides in the limited amount needed to correct DOAC-induced bleeding, in contrast to stoichiometric lute or bait anticoagulants.

We hypothesized that Gla-domainless FXa (GDFXa) sequestered by α2-macroglobulin (GDFXα-α2M) would be prevented from interacting with pro- as well as anticoagulant macromolecules (factor Va, prothrombin, antithrombin and tissue factor pathway inhibitor [TFPI]), whereas its active site remaining functional still binds xabans.

α2M is a broad-spectrum molecular trap inhibitor mainly targeting thrombin, FXa and plasmin in blood. Native α2M is a homotetrameric glycoprotein (MW 720 kDa; plasma concentration, 3.5 µM in adults). Each sub-unit includes a bait region targeted by numerous pro- and anti-thrombin serpins, and a cysteinyl-glutamyl thiol ester bond. Cleavage induces a major conformational change trapping the protease within a cage-like quaternary structure. Native α2M and α2M that had reacted with a protease have markedly different shapes, the latter having a paradoxical enhanced mobility (fast form) in native gel electrophoresis. Cleavage also unmasks the γ-glutamyl groups which react with NH2-ε-lysyl group of the protease covalently linking the entrapped protease. Steric hindrance prevents macromolecules to interact with the entrapped protease, which nevertheless still cleaves small peptidyl substrates and is neutralized by peptidyl chloromethyl ketone. Thus, it was reasonable to expect that entrapped GDFXa would still bind DOAC inhibiting FXa.

We prepared GDFXα-α2M complex and assessed its potential regarding xabans neutralization in vitro in platelet-poor plasma (PPP) and whole blood as well as in vivo in a preclinical bleeding model.

Materials and Methods

Whole Blood and Platelet-Poor Plasma

Blood was collected by venipuncture (0.105 M buffered trisodium citrate 9/1 v/v) from healthy volunteers who gave their written informed consent (Etablissement Français du Sang, Paris, France; convention C CPSL UNT n°13/EF5/064). Pooled normal PPP was purchased from Cryocep (Montpellier, France).

Drugs, Proteins and Reagents

Apixaban and rivaroxaban were kindly provided by Bristol-Myers Squibb/Pfizer (Princeton, New Jersey, United States) and Bayer Healthcare AG (Leverkusen, Germany), respectively. About 4 mg apixaban or rivaroxaban were dissolved in dimethyl sulfoxide (DMSO). Just prior to use, rivaroxaban was rapidly diluted at 1/100 in H2O and further diluted in 50 mM Tris-HCl pH 7.5 containing 0.15 M NaCl (tris-buffered saline [TBS]) and 1% DMSO. Dilutions were performed directly in TBS containing 1% DMSO for apixaban. Effective (final) concentrations of xabans were measured in PPP by anti-Xa activity on a STA-R and a set of specific calibrators (Stago, Asnières, France). For in vivo studies, pills of rivaroxaban (Xarelto, Bayer) were dissolved in 10 mM HCl (vehicle) and used to force-feed mice at the indicated dose. Exonaparin (Lovenox) was purchased from Sanofi Aventis (Gentilly, France) and fondaparinux (Arixtra) from Aspen (Marly-le-Roi, France). Dilutions were performed in TBS. Human GDFXa expressed in bacteria was purchased from Cambridge ProteinWorks (Cambridge, UK). Antithrombin was purchased from LFB (Aclotain, Courtбавouef, France), TFPI from American Diagnostica (Greenwich, Connecticut, United States), recombinant human tissue factor (TF) from Dade Behring (Innovin, Marburg, Germany) and aprotinin from Nordic Group Pharmaceuticals (Paris, France). Phospholipid vesicles were prepared by sonication (2 minutes in pulse mode 0.15/ s, 80 W, 4°C) of a 1 mg/mL mixture of L-α-phosphatidylcholine (66%, w/w) with L-α-phosphatidylserine (33%, w/w), both from Avanti Polar Lipids (Alabaster, Alabama, United States) as previously described. Chromogenic substrate N-α-benzoxycarbonyl-d-Arg-Gly-Arg-pNA (S2765) and the inhibitors D-Phe-Pro-Arg-chloromethyl ketone (PPACK) were purchased from Cryocep, phenylmethylsulfonyl fluoride (PMSF) and methylamine (40% solution) from Sigma Aldrich (Steinheim, Germany). 1,5 Dansyl-Glu-Gly-Arg chloromethyl ketone (DEGCRk) was purchased from Merck KGaA (Darmstadt, Germany).

GDFXα-α2M Preparation and Characterization

Human α2M was purified according to published protocols. Briefly, a cocktail of inhibitors (1 µM PPACK, 2 mM PMSF, 2 mM ethylenediaminetetraacetic acid (EDTA) and 100 KIU/mL aprotinin) was added to freshly thawed frozen plasma. Plasminogen was removed by chromatography on Lysine-Sepharose 4B and vitamin K-dependent factors by phospholipid-Sepharose column (25 × 160 mm, 3 ml/min) saturated by 50 mM zinc acetate then eluted with 0.1 M EDTA pH 8.0. Following concentration by ultrafiltration (Amicon Ultra-15 100K, Merck KGaA), α2M was further purified by gel filtration on Superose 6B column (16 × 500 mm, 1 ml/min). Purified α2M was concentrated by ultrafiltration up to 15 mg/mL estimated by immunonephelometry (BN II, Siemens). GDFXα-α2M was formed by incubating GDFXa (2 µM) with...
α2M (8 μM) for 20 minutes at 37°C in TBS containing 5 mM
MnCl2. Preparations were kept at 4°C until use. Complete
sequestration of GDFXa by α2M was controlled by comparing
velocity of S2765 hydrolysis in the presence or absence of
2.6 μM antithrombin and 1 IU anti-Xa/mL enoxaparin. Active
site concentration of GDFXa–α2M was verified by titration
with DEGRck. GDFXa (0.5 μM) free or fully sequestered by α2M
were incubated with various DEGRck dilutions prepared
immediately before use. Following 30 minutes of incubation
at 37°C, residual activities were measured after a 1/20 dilution
using S2765. The reported amount of GDFXa–α2M refers to the
concentration of active GDFXa sequestered. Inhibition con-
stants (Ki) of apixaban and rivaroxaban for GDFXa–α2M were
determined as previously described. Apparent values were
corrected for the S2765 competition by taking into account its
Michaelis constant for GDFXa–α2M (60.3 ± 4.4 μM) estimated
as previously reported for FXa. For reference purpose, a small
amount of purified α2M was activated into the fast form by
incubation with 200 mM methylamine (2 hours at room
temperature) followed by extensive dialysis. Native α2M, GDFXa–α2M, and methyleamine-treated α2M were analysed
by native polyacrylamide gel electrophoresis (Invitrogen
NuPAGE 3–8% Tris-Acetate Gel, Thermo FisherScientific,
France).

Clot Waveform and Rotational Thromboelastometry
Assays
The kinetic of fibrin polymerization (clot waveform) was
recorded by measuring A405 every 8 seconds at 37°C on a
Tecan Infinite M200 Pro reader. Coagulation was initiated in
80 μL PPP containing 5 pM TF and 4 μM phospholipid vesicles
by adding 20 μL 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid 50 mM pH 7.35 containing 60 mg/mL
bovine serum albumin and 100 mM CaCl2. Data were
normalized and the lag time of clot formation was defined as
the time needed to reach 15% of the maximum turbidity
measured through A405. Rotational thromboelastometry
was performed on a ROTEM delta (Werfen, Baden-Dättwil, Swit-
zerland). Coagulation was initiated through addition of
300 μL pre-warmed whole blood containing or not contain-
ing xaban and/or GDFXa–α2M to 40 μL triggering solution
ensuring final concentrations of 2.5 μM TF, 10 μM phospho-
lipid vesicles and 20 mM CaCl2. Reported clotting times
are as defined by the manufacturer. Whole blood and PPP were
spiked (1/20; v/v) with TBS containing 1% DMSO and the
xaban as required.

Mice
C57Bl/6J/Rj male mice (25–30 g) were purchased from Janvier
Laboratory (Le Genest-Saint-Isle, France) and all assays performed at
the Animal Platform, CRP-UMS 3612 CNRS – US25 Inserm-
IRD (Université Paris Descartes). Mice were anaesthetized by
intra-peritoneal injection of a ketamine (80 mg/kg) and xyla-
zeine (16 mg/kg) mixture and were euthanized by cervical
dislocation. All animal experiments were approved by the
Ethic Committee on Animal Resources of Université Paris
Descartes (registration number 201506151109793–V5 APAFIS
#2677).

GDFXa–α2M Half-Life in Mice
Half-life of GDFXa–α2M was evaluated after a single 100 μL
retro-orbital plexus injection of 150 nM GDFXa–α2M
(0.34 mg/kg). Following tail vein transsection (see below),
25 μL of blood were collected through challenges at timed
intervals and immediately diluted at 1/5 in TBS containing
5 mM EDTA. Residual activity was evaluated in PPP after
centrifugation (1,500 × g, 10 minutes, 20°C), by measuring
rate of S2765 hydrolysis. Data were normalized with respect
to hydrolysis measured in the sample collected 1 minute
post-injection. The dependence of the normalized rate of
hydrolysis on time was analysed by non-linear regression
analysis using a single exponential decay equation to esti-
mate the in vivo half-life of GDFXa–α2M. Persistence of
GDFXa–α2M in blood was evaluated through 100 μL injection
in each retro-orbital plexus of 3.6 μM GDFXa–α2M (17 mg/kg).
Blood was collected in 0.11 M buffered trisodium citrate
(9/1; v/v) by cardiac puncture at different
time points (1–6 hours post-injection). Residual GDFXa–
α2M activity was evaluated by measuring the rate of S2765
hydrolysis, as above.

Mouse Tail Vein Transsection and Bleeding Model
Mouse bleeding model was adapted from published
method. Mice were force-fed with 10 mM HCl containing
or not 50 mg/kg rivaroxaban. Two hours later, 100 μL
GDFXa–α2M (3.6 μM) or its vehicle were injected in each
retro-orbital plexus. Mouse tail was soaked in a mixture of
NaCl (0.15 M) and EDTA (2 mM) at 37°C. Mouse was posi-
tioned on its right side and tail introduced in a homemade
device enabling positioning at precisely its 2.5-mm diameter
section. Using a mechanical linear guide, the left lateral tail
vein was transected by a 0.5-mm deep incision. Mouse tail
was replaced into soaking mixture and initial bleeding time
monitored. Fifteen, 30, and 45 minutes post-injury, wound
was challenged by gently wiping it twice with a 37°C saline-
wetted gauze swab in the distal direction. Following each
challenge, mouse tail was placed into a new collection tube
containing the soaking mixture and re-bleeding was mon-
tored. Red blood cells in each collection tube were collected
(1,500 × g, 10 minutes, 20°C) and lysed in 20 mM Tris pH 7.5.
A416 was transcribed into microlitre blood loss in reference to
a titration curve. Secondary bleeding time and blood loss
were defined as the sum of bleeding time and blood loss
following the three challenges.

Evaluation of Pro-Coagulant Markers in Mice
Receiving GDFXa–α2M
GDFXa–α2M (3.6 μM) or its vehicle were injected in each
retro-orbital plexus and blood collected 30 minutes later by
cardiac puncture, as above. Pro-thrombotic potential of
GDFXa–α2M was evaluated by clot waveform assay as
described above. D-dimers and thrombin–antithrombin
complexes (TAT) were measured by enzyme-linked immu-
nosorbent assay (ELISA) according to the manufacturer’s
instructions using Mouse D-Dimers (D2D) ELISA Kit (Cusabio,
anticorps-enligne.fr) and TAT complexes Mouse ELISA
Kit (Abcam, Paris, France), respectively.
Statistical Analyses
All statistical analyses were computed using the GraphPad Prism software. Half-life and \( K_i \) values were expressed as mean ± standard deviation of three determinations. Coefficients of determination \( (R^2) \) were given by linear or non-linear regression analyses. Clotting time, lag time ratio, bleeding time and blood loss values were compared all together by Kruskal–Wallis test followed by Dunn tests performed for pair-wise comparisons. Vehicle versus antithrombin and 1 IU anti-Xa/mL enoxaparin was added was indistinguishable whether or not a mixture of 2.6 µM antithrombin and 1 IU anti-Xa/mL enoxaparin was added (Tables). Vehicle versus anti-Xa and 1 IU anti-Xa/mL enoxaparin mixture. The same was true when enoxaparin was substituted with 1.4 mg/mL fondaparinux. We also verified that GDFXa-α2M resisted TFPI inhibition: TFPI completely inhibited GDFXa but had no effect on GDFXa-α2M amidolytic activity (Fig. 1C). Active site titration confirmed that GDFXa fully sequestered by α2M remains active albeit with a lower catalytic activity than that of free GDFXa (Fig. 1D). Therefore, all reported concentrations of GDFXa-α2M reflect the active site concentration of GDFXa within the complex and not total amount of α2M. Adding 1 µM purified α2M to PPP had no detectable effect on clot waveform assay (Fig. 2A). On the contrary, active GDFXa dose-dependently decreased the lag time, thus had pro-coagulant potential (Fig. 2B). It was therefore of utmost importance to avoid any traces of free GDFXa. Reproducible and satisfactory results were obtained by incubating α2M with GDFXa at a 4/1 stoichiometric ratio. The inhibition constants \( (K_i) \) of apixaban and rivaroxaban for GDFXa-α2M were slightly higher than those we recently reported for FXa\(^{39}\) in strictly identical conditions \( (K_i = 2.41 ± 0.22 \text{ nM} \quad R^2 = 0.84) \) vs. \( 0.74 ± 0.03 \text{ nM} \) and \( 1.29 ± 0.13 \text{ nM} \quad R^2 = 0.87 \) vs. \( 0.47 ± 0.02 \text{ nM} \), respectively; Fig. 2C). Above all, the preparation of GDFXa-α2M was devoid of pro- or anticoagulant activity as attested by clot waveform assay performed in PPP whether or not containing 1.7 µM GDFXa-α2M.

Results
GDFXa-α2M Preparation and Characterization
Purified human α2M appeared homogeneous by native polyacrylamide gel electrophoresis analysis (Fig. 1A). Following incubation with GDFXa, the main species detectable migrated faster than native α2M suggesting that in spite of the molar excess of α2M over GDFXa most α2M had been activated to the fast form. Importantly, complete GDFXa sequestration by α2M was achieved since hydrolysis of S2765 by GDFXa-α2M was indistinguishable whether or not a mixture of 2.6 µM antithrombin and 1 IU anti-Xa/mL enoxaparin was added (Fig. 1B). On the contrary, rate of S2765 hydrolysis by GDFXa alone was fully inhibited by the antithrombin enoxaparin mixture. The same was true when enoxaparin was substituted with 1.4 mg/mL fondaparinux. We also verified that GDFXa-α2M resisted TFPI inhibition: TFPI completely inhibited GDFXa but had no effect on GDFXa-α2M amidolytic activity (Fig. 1C). Active site titration confirmed that GDFXa fully sequestered by α2M remains active albeit with a lower catalytic activity than that of free GDFXa (Fig. 1D). Therefore, all reported concentrations of GDFXa-α2M reflect the active site concentration of GDFXa within the complex and not total amount of α2M. Adding 1 µM purified α2M to PPP had no detectable effect on clot waveform assay (Fig. 2A). On the contrary, active GDFXa dose-dependently decreased the lag time, thus had pro-coagulant potential (Fig. 2B). It was therefore of utmost importance to avoid any traces of free GDFXa. Reproducible and satisfactory results were obtained by incubating α2M with GDFXa at a 4/1 stoichiometric ratio. The inhibition constants \( (K_i) \) of apixaban and rivaroxaban for GDFXa-α2M were slightly higher than those we recently reported for FXa\(^{39}\) in strictly identical conditions \( (K_i = 2.41 ± 0.22 \text{ nM} \quad R^2 = 0.84) \) vs. \( 0.74 ± 0.03 \text{ nM} \) and \( 1.29 ± 0.13 \text{ nM} \quad R^2 = 0.87 \) vs. \( 0.47 ± 0.02 \text{ nM} \), respectively; Fig. 2C). Above all, the preparation of GDFXa-α2M was devoid of pro- or anticoagulant activity as attested by clot waveform assay performed in PPP whether or not containing 1.7 µM GDFXa-α2M.
In Vitro Neutralization of Xabans by GDFXa-α2M
We used clot waveform assay as a coagulation-based assay allowing detection of as little as 5 to 10 ng/mL xaban in PPP. To evaluate xaban neutralization by GDFXa-α2M, we triggered clot waveform assays in PPP spiked with increasing amounts of xabans in the presence or absence of 1.7 μM GDFXa-α2M. Apixaban or rivaroxaban dose-dependently prolonged the lag time. GDFXa-α2M fully neutralized xaban anticoagulant effect even at supra-therapeutic levels. Indeed, lag times in the presence of GDFXa-α2M were comparable to control, irrespective of the xaban concentration (→ Fig. 3A).

ROTEM triggered by low TF is a whole blood coagulation-based assay potentially allowing xaban detection. Apixaban or rivaroxaban dose-dependently prolonged the clotting time. Adding 1.7 μM GDFXa-α2M fully reversed xaban anticoagulant effect: the clotting times were comparable to those obtained in the absence of xaban, irrespective of the drug concentration of up to 600 ng/mL (→ Fig. 3B).

Overall, GDFXa-α2M neutralized supra-therapeutic amounts of apixaban and rivaroxaban in blood and PPP, whereas it was without effect in the absence of xabans.

Half-Life and Persistence of GDFXa-α2M in Mice
The reported half-life of FXa-α2M in mice is 2 minutes and that of subtilisin-α2M in rats is 6 minutes. In accord with these data, we estimated a half-life of 4.9 ± 1.1 minutes ($R^2 = 0.90$; → Fig. 4A) after injection of 10 μg/mice GDFXa-α2M. However, uptake of protease-α2M is saturable.

Thus, we evaluated the actual persistence of GDFXa-α2M following injection at the high dose needed for stoichiometric xaban neutralization. When 0.5 mg/mice GDFXa-α2M was injected, 50% of the catalytic activity of GDFXa-α2M was still detectable 170 minutes following injection (→ Fig. 4B). Consequently, we designed the in vivo experiments taking into account the actual persistence of GDFXa-α2M in mice.

Neutralization of Xabans by GDFXa-α2M in Mice
GDFXa-α2M efficacy for xaban neutralization was evaluated in vivo using a mouse bleeding model. Mice were force-fed with rivaroxaban or placebo and 2 hours later, vehicle or GDFXa-α2M (100 μL, 3.6 μM) were injected in each retroorbital plexus. Primary endpoints were bleeding time and blood loss following lateral tail vein transsection. Initial bleeding time and blood loss were comparable whether or not mice were force-fed with rivaroxaban ($p > 0.05$). However, following challenges, rivaroxaban increased secondary bleeding time ($p < 0.001$) as well as blood loss ($p < 0.01$), whereas both were comparable to placebo following GDFXa-α2M injection ($p > 0.05$). Overall, GDFXa-α2M significantly decreased rivaroxaban induced bleeding time (from 4.9 ± 1.0 to 2.84 ± 0.4 minutes; $p < 0.001$; → Fig. 5A) and blood loss (from 351 ± 45 to 180 ± 87 μL; $p < 0.01$; → Fig. 5B). In the absence of rivaroxaban, GDFXa-α2M had no effect on bleeding time (2.90 ± 0.28 vs. 2.95 ± 0.51 minutes; $p > 0.05$) and...
blood loss (158 ± 99 vs. 163 ± 73 µL; \( p > 0.05 \)). We concluded that GDFXa-\( \alpha \)-2M effectively neutralized rivaroxaban anticoagulant effect in this in vivo model and that GDFXa-\( \alpha \)-2M alone had no adverse effect on mouse haemostasis.

**Evaluation of Pro-Coagulant Markers in Mice Receiving GDFXa-\( \alpha \)-2M**

The above data suggested that GDFXa-\( \alpha \)-2M was devoid of in vivo adverse effect. To exclude the potential pro-thrombotic effect of GDFXa-\( \alpha \)-2M in mice, we performed clot waveform assay in samples collected 30 minutes post-injection of vehicle or GDFXa-\( \alpha \)-2M. Whether or not mice received GDFXa-\( \alpha \)-2M, the lag time of clot waveform did not differ between groups (\( p = 0.18; \text{ Fig. 6A} \)). Moreover, D-dimer and TAT levels were comparable (\( p = 0.50 \) and \( p = 0.13; \text{ Fig. 6B and C} \)). Overall results suggested that GDFXa-\( \alpha \)-2M was devoid of pro- as well as anticoagulant properties in vivo.

![Fig. 3](image-url) **Neutralization of xabans in vitro.** (A) Graph represents the lag time of clot waveform triggered by adding 5 pM tissue factor (TF) and 4 µM phospholipid vesicles. Platelet-poor plasma (PPP) was spiked with increasing amounts (as indicated in the abscissa) of rivaroxaban (open squares) or apixaban (open circles). Corresponding closed symbols were obtained in these PPP containing in addition 1.7 µM Gla-domainless FXa \( \alpha \)-2-macroglobulin complex (GDFXa-\( \alpha \)-2M). Solid lines link the mean value of three determinations (error bars min and max). (B) Graph represents the clotting time (CT in seconds) of rotational thromboelastometry assays triggered by adding 2.5 pM TF and 10 µM phospholipid vesicles to blood spiked with rivaroxaban (squares) or apixaban (circles) and containing or not 1.7 µM GDFXa-\( \alpha \)-2M (closed or open symbols, respectively). Xaban concentrations indicated in the abscissa are the actual concentrations measured in PPP by anti-Xa activity. Solid lines were obtained by linear regression analysis. Overall, 1.7 µM GDFXa-\( \alpha \)-2M fully neutralized supra-therapeutic amounts of xaban (600 ng/mL) without affecting (in their absence) the lag time in PPP or the clotting time in the whole blood.

![Fig. 4](image-url) **Half-life and persistence of Gla-domainless FXa \( \alpha \)-2-macroglobulin complex (GDFXa-\( \alpha \)-2M) in mice.** (A) Kinetic of the normalized residual rate of S2765 hydrolysis when 100 µL GDFXa-\( \alpha \)-2M (150 nM) was injected in mice. Data points are mean value of three determinations (error bars min and max). Solid line was obtained by nonlinear regression analysis using a single exponential decay equation. Estimated half-life of GDFXa-\( \alpha \)-2M in mice was 4.9 ± 1.1 minutes (\( R^2 = 0.90 \)). (B) Kinetic of the normalized residual rate of S2765 hydrolysis when 100 µL GDFXa-\( \alpha \)-2M (3.6 µM) were injected in each retro-orbital plexus in mice. Data points are mean value of three determinations; solid line links the mean values (error bars min and max). Probably due to saturation of the uptake mechanisms, 50% of GDFXa-\( \alpha \)-2M catalytic activity persisted 170 minutes after injection.
Our goal was designing a neutralizing agent for xabans in vitro and in vivo. Our specification was that agent should neutralize xabans without otherwise affecting haemostasis. GDFXa-α2M was an attractive candidate because the active site of FXa within the complex would be preserved and bind xabans, whereas steric hindrance would prevent interaction with macromolecules (prothrombin, factor Va, antithrombin or TFPI). We herein documented that 1.7 µM GDFXa-α2M neutralized supra-therapeutic amounts of apixaban or rivaroxaban while devoid by itself of pro- or anticoagulant properties.

Mouse tail vein transection is a pre-clinical model sensitive to pharmacological intervention in haemophilia A mice. We evaluated GDFXa-α2M efficacy as xabans neutralizing agent using this model. We confirmed as previously reported that oral administration of rivaroxaban produced variable initial blood loss. In contrast, secondary bleeding time and blood loss were significantly increased in mice treated with rivaroxaban alone, whereas in the groups having received in addition GDFXa-α2M, values were comparable to those of the control group. Presumably rivaroxaban binding to GDFXa-α2M neutralized its anticoagulant effect. That bleeding time and blood loss were comparable whether or not mice received GDFXa-α2M suggested harmlessness in addition to effectiveness. In accord with this hypothesis, neither D-dimer nor TAT levels increased following GDFXa-α2M injection.

Discussion

Our goal was designing a neutralizing agent for xabans in vitro and in vivo. Our specification was that agent should neutralize xabans without otherwise affecting haemostasis. GDFXa-α2M was an attractive candidate because the active site of FXa within the complex would be preserved and bind xabans, whereas steric hindrance would prevent interaction with macromolecules (prothrombin, factor Va, antithrombin or TFPI). We herein documented that 1.7 µM GDFXa-α2M neutralized supra-therapeutic amounts of apixaban or rivaroxaban while devoid by itself of pro- or anticoagulant properties.

Mouse tail vein transection is a pre-clinical model sensitive to pharmacological intervention in haemophilia A mice. We evaluated GDFXa-α2M efficacy as xabans neutralizing agent using this model. We confirmed as previously reported that oral administration of rivaroxaban produced variable initial blood loss. In contrast, secondary bleeding time and blood loss were significantly increased in mice treated with rivaroxaban alone, whereas in the groups having received in addition GDFXa-α2M, values were comparable to those of the control group. Presumably rivaroxaban binding to GDFXa-α2M neutralized its anticoagulant effect. That bleeding time and blood loss were comparable whether or not mice received GDFXa-α2M suggested harmlessness in addition to effectiveness. In accord with this hypothesis, neither D-dimer nor TAT levels increased following GDFXa-α2M injection.
Ideally, an antidote should neutralize and eliminate its target in vivo rather than just sequestering it. Cleavage of the bait region in α2M exposes previously concealed recognition sites for receptors on fibroblasts, macrophages and hepatocytes that uptake α2M-protease from the circulation. Clearing is indistinguishable whether or not the active site of the α2M-linked protease is inhibited by low MW inhibitors. Assuming that such depletion occurs with GDFXa-α2M, it could literally deplete xaban from blood, bypassing the natural clearance mechanisms. On the other hand, we evidenced that high amount of GDFXa-α2M had a relatively long persistence in blood, presumably due to saturation of the uptake mechanisms. GDFXa-α2M would rapidly neutralize xabans but eliminate them only slowly. Accordingly, GDFXa-α2M may constitute a fairly long-lasting antidote not requiring continuous perfusion or multiple injections. It is worth mentioning that α2M and FXa are native blood components thus not immunogenic and that 1.7 µM GDFXa-α2M neutralizing supra-therapeutic levels of xabans is below the normal plasma concentration of α2M (3.5 µM in adults; 5.6 µM in newborns). Affinity of apixaban and rivaroxaban for GDFXa-α2M was just slightly less than that for FXa, supporting the hypothesis of stoichiometric interaction with the xabans.

Thrombin generation assay is affected by xabans and thus would allow evaluating their neutralization. However, GDFXa-α2M ruined the thrombin generation assay because of its ability to cleave FluCa, precluding measurement of thrombin generation in its presence. Amount of GDFXa-α2M added to neutralize xabans (1.7 µM) actually exceeded the thrombin potential (1.3 µM).

The prospect of using GDFXa-α2M as an antidote to xaban has limitations. First, it is difficult to consider α2M purified from human plasma for therapeutic use and large-scale production of recombinant α2M may turn out difficult. Second, our GDFXa-α2M preparation was a mixture of native α2M, and activated α2M having sequestered or not GDFXa. Adding micromolar quantities of native α2M to PPP had no detectable effect on blood coagulation. Blood readily contains large amounts of α2M which does not prevent xaban effects. We showed that active GDFXa is highly pro-coagulant and verified that GDFXa-α2M preparation did not contain traces of GDFXa. We also verified that GDFXa-α2M preparation itself was devoid of adverse effect in PPP (neither pro- nor anticoagulant). It can be concluded that sequestered GDFXa neutralized the xabans. We unexpectedly observed that partial neutralization of dabigatran as well as of heparin or its derivative occurs with activated forms of α2M such as methylamine-treated α2M in addition to GDFXa-α2M. Specifically, the catalytic activity of sequestered GDFXa is not affected by dabigatran, yet GDFXa-α2M partially neutralizes the anticoagulant effect of dabigatran. Mechanism likely involves secondary binding sites latent in native α2M. During blood coagulation, FXa-α2M and thrombin-α2M are formed. FXa-α2M formation with its 170 nM FXa potential is minor with respect to therapeutic xaban concentration. Again xabans are efficient coagulation inhibitors in spite of blood α2M content.

Overall, GDFXa-α2M represented an in vitro and in vivo attractive neutralizing agent of xabans, neither pro- nor anticoagulant.

What is known about this topic?
- Bleeding complications may occur with direct oral anticoagulants (DOACs) as with any anticoagulant drug.
- No antidote is yet approved for the DOAC targeting FXa (xabans). This is specially threatening in clinical scenarios such as massive haemorrhage, trauma, stroke requiring thrombolysis or urgent surgery.

What does this paper add?
- A complex between α2-macroglobulin and Gla-domainless FXa (GDFXa-α2M) is an attractive neutralizing agent of xabans in vitro and in vivo.
- GDFXa-α2M is effective as an antidote for rivaroxaban-induced bleeding in mice.
- GDFXa-α2M is devoid of pro-thrombotic potential in mice.

Authors’ Contributions
B.L.B. conceived the study and together with G.J. designed and performed research, analysed data and wrote the manuscript; I.G.-T., V.S., S.G. and P.G. critically discussed the data, revised the manuscript and gave final approval.

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Conflict of Interest
I.G.-T. received honoraria for participating in expert meetings on apixaban (Bristol-Myers Squibb/Pfizer). P.G. received honoraria for participating in expert meetings on enoxaparin (Sanofi) and together with I.G.-T. and V.S. on rivaroxaban (Bayer Healthcare AG). The other authors declare no conflict of interest.

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