FXa-α_2-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 α_2-macroglobulin (GDFXa-α_2M) may neutralize the xabans without interfering with normal blood coagulation.

Purified α_2M was incubated with GDFXa to form GDFXa-α_2M. Affinity of apixaban and rivaroxaban for GDFXa-α_2M was only slightly decreased compared to FXa. Efficacy and harmlessness of GDFXa-α_2M were tested in vitro and in vivo. Stoichiometric excess of GDFXa-α_2M neutralized rivaroxaban and apixaban as attested by clot waveform assay and rotational thromboelastometry, whereas GDFXa-α_2M alone had no effect on these assays. Efficacy and pro-thrombotic potential of GDFXa-α_2M were also assessed in vivo. Half-life of GDFXa-α_2M in C57BL6 mice was 4.9 $\pm$ 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-α_2M significantly decreased the rivaroxaban-induced bleeding time ($p < 0.001$) and blood loss ($p < 0.01$). GDFXa-α_2M did not increase D-dimer or thrombin–antithrombin complex formation, suggesting a lack of pro-thrombotic potential. GDFXa-α_2M 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[1] 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.[6–9] 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.

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Pro-haemostatic agents (prothrombin complex concentrate activated or not) increase thrombosis risk, thus their use is currently restricted to life-threatening situations.[3,13,14] 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.[15,16] 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.[17,18] ANNEXA-4 clinical trial is currently in progress to confirm its efficacy and safety.[19] 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.[20,21] Besides antidotes, potent by-passing agents are also in the pipeline, such as the I16L FX variant, acting as a zymogen-like acquiring prothrombin activator potential through factor Va binding, or the DOAC resisting 99-loop FX variant.[22–24] Their key advantage resides in the limited amount needed to correct DOAC-induced bleeding, in contrast to stoichiometric lure or bait antidotes.

We hypothesized that Gla-domainless FXa (GDFXa) sequestered by α_2-macroglobulin (GDFXa-α_2M) 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.

Alpha$_2$M is a broad-spectrum molecular trap inhibitor[25–27] mainly targeting thrombin, FXa and plasmin in blood.[28–30] Native α_2M is a homotetrameric glycoprotein (MW 720 kDa; plasma concentration, 3.5 µM in adults). Each sub-unit includes a bait region targeted by numerous proteases and a cysteinyl-glutamyl thiol ester bond. Cleavage induces a major conformational change trapping the protease within a cage-like quaternary structure.[31–33] Native α_2M and α_2M 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 NH_2 ε-lysyl group of the protease covalently linking the entrapped protease.[34,35] Steric hindrance prevents macromolecules to interact with the entrapped protease, which nevertheless still cleaves small peptidyl substrates and is neutralized by peptidyl chloromethyl ketone.[29] Thus, it was reasonable to expect that entrapped GDFXa would still bind DOAC inhibiting FXa.

We prepared GDFXa-α_2M 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/EFS/064). Pooled normal PPP was purchased from Cryopep (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 H_2O 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. Enoxaparin (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 (Aclotine, Courtaboeuf, 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.[36] Chromogenic substrate N-α-benzyloxycarbonyl-d-Arg-Gly-Arg-pNA (S2765) and the inhibitors *D*-Phe-Pro-Arg-chloromethyl ketone (PPACK) were purchased from Cryopep, phenylmethylsulfonyl fluoride (PMSF) and methylamine (40% solution) from Sigma Aldrich (Steinheim, Germany). 1,5 Dansyl-Glu-Gly-Arg chloromethyl ketone (DEGRck) was purchased from Merck KGaA (Darmstadt, Germany).

GDFXa-α_2M Preparation and Characterization

Human α_2M was purified according to published protocols.[34,37,38] 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 80 mM barium chloride precipitation and the bulk of fibrinogen by 4% polyethylene glycol (PEG-6000). Supernatant was brought to 12% PEG-6000 and the resulting pellet was resuspended in 1/10 of the initial PPP volume in 20 mM sodium phosphate, pH 6.0. Alpha$_2$M was adsorbed onto iminodiacetic acid-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), α_2M was further purified by gel filtration on Superose 6B column (16 × 500 mm, 1 mL/min). Purified α_2M was concentrated by ultrafiltration up to 15 mg/mL estimated by immunonephelometry (BN II, Siemens). GDFXa-α_2M was formed by incubating GDFXa (2 µM) with

α_2M (8 µM) for 20 minutes at 37°C in TBS containing 5 mM $MnCl_2$.[29,30] Preparations were kept at 4°C until use. Complete sequestration of GDFXa by α_2M 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-α_2M refers to the concentration of active GDFXa sequestered. Inhibition constants (K_I) of apixaban and rivaroxaban for GDFXa-α_2M were determined as previously described.[39] Apparent values were corrected for the S2765 competition by taking into account its Michaelis constant for GDFXa-α_2M (60.3 ± 4.4 µM) estimated as previously reported for FXa.[40] For reference purpose, a small amount of purified α_2M was activated into the fast form by incubation with 200 mM methylamine (2 hours at room temperature) followed by extensive dialysis. Native α_2M, GDFXa-α_2M, and methylamine-treated α_2M were analysed by native polyacrylamide gel electrophoresis (Invitrogen NuPAGE 3–8% Tris-Acetate Gel, Thermo Fisher Scientific, France).

Clot Waveform and Rotational Thromboelastometry Assays

The kinetic of fibrin polymerization (clot waveform) was recorded by measuring A_{405} every 8 seconds at 37°C on a Tecan Infinite M200 Pro reader.[41] Coagulation was initiated in 80 µL PPP containing 5 pM TF and 4 µM phospholipid vesicles by adding 20 µL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 50 mM pH 7.35 containing 60 mg/mL bovine serum albumin and 100 mM $CaCl_2$. 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 A_{405}. Rotational thromboelastometry was performed on a ROTEM *delta* (Werfen, Baden-Dättwil, Switzerland). Coagulation was initiated through addition of 300 µL pre-warmed whole blood containing or not containing xaban and/or GDFXa-α_2M to 40 µL triggering solution ensuring final concentrations of 2.5 pM TF, 10 µM phospholipid vesicles and 20 mM $CaCl_2$. 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/6JRj male mice (25–30 g) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and all assays performed at the Animal Platform, CRP2–UMS 3612 CNRS – US25 Inserm-IRD (Université Paris Descartes). Mice were anaesthetized by intra-peritoneal injection of a ketamine (80 mg/kg) and xylazine (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-α_2M Half-Life in Mice

Half-life of GDFXa-α_2M was evaluated after a single 100 µL retro-orbital plexus injection of 150 nM GDFXa-α_2M (0.34 mg/kg). Following tail vein transection (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 estimate the in vivo half-life of GDFXa-α_2M. Persistence of GDFXa-α_2M in blood was evaluated through 100 µL injection in each retro-orbital plexus of 3.6 µM GDFXa-α_2M (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-α_2M activity was evaluated by measuring the rate of S2765 hydrolysis, as above.

Mouse Tail Vein Transection and Bleeding Model

Mouse bleeding model was adapted from published method.[42] Mice were force-fed with 10 mM HCl containing or not 50 mg/kg rivaroxaban. Two hours later, 100 µL GDFXa-α_2M (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 positioned 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 monitored. 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. A_{416} 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-α_2M

GDFXa-α_2M (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-α_2M was evaluated by clot waveform assay as described above. D-dimers and thrombin–antithrombin complexes (TAT) were measured by enzyme-linked immunosorbent 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 Prizm 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 antidote, D-dimers and TAT values were compared by unpaired two-tailed Mann–Whitney test. Statistical significance was accepted for p-value of < 0.05.

Results

GDFXa-α_2M Preparation and Characterization

Purified human α_2M appeared homogeneous by native polyacrylamide gel electrophoresis analysis (►**Fig. 1A**). Following incubation with GDFXa, the main species detectable migrated faster than native α_2M suggesting that in spite of the molar excess of α2M over GDFXa most α_2M had been activated to the fast form. Importantly, complete GDFXa sequestration by α_2M was achieved since hydrolysis of S2765 by GDFXa-α_2M 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-α_2M resisted TFPI inhibition: TFPI completely inhibited GDFXa but had no effect on GDFXa-α_2M amidolytic activity (►**Fig. 1C**). Active site titration confirmed that GDFXa fully sequestered by α_2M remains active albeit with a lower catalytic activity than that of free GDFXa (►**Fig. 1D**). Therefore, all reported concentrations of GDFXa-α_2M reflect the active site concentration of GDFXa within the complex and not total amount of α_2M. Adding 1 µM purified α_2M 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 upmost importance to avoid any traces of free GDFXa. Reproducible and satisfactory results were obtained by incubating α_2M with GDFXa at a 4/1 stoichiometric ratio. The inhibition constants (K_I) of apixaban and rivaroxaban for GDFXa-α_2M were slightly higher than those we recently reported for FXa[39] in strictly identical conditions ($K_I = 2.41 \pm 0.22$ nM [$R^2 = 0.84$] vs. 0.74 ± 0.03 nM and 1.29 ± 0.13 nM [$R^2 = 0.87$] vs. 0.47 ± 0.02 nM, respectively; ►**Fig. 2C**). Above all, the preparation of GDFXa-α_2M was devoid of pro- or anticoagulant activity as attested by clot waveform assay performed in PPP whether or not containing 1.7 µM GDFXa-α_2M.

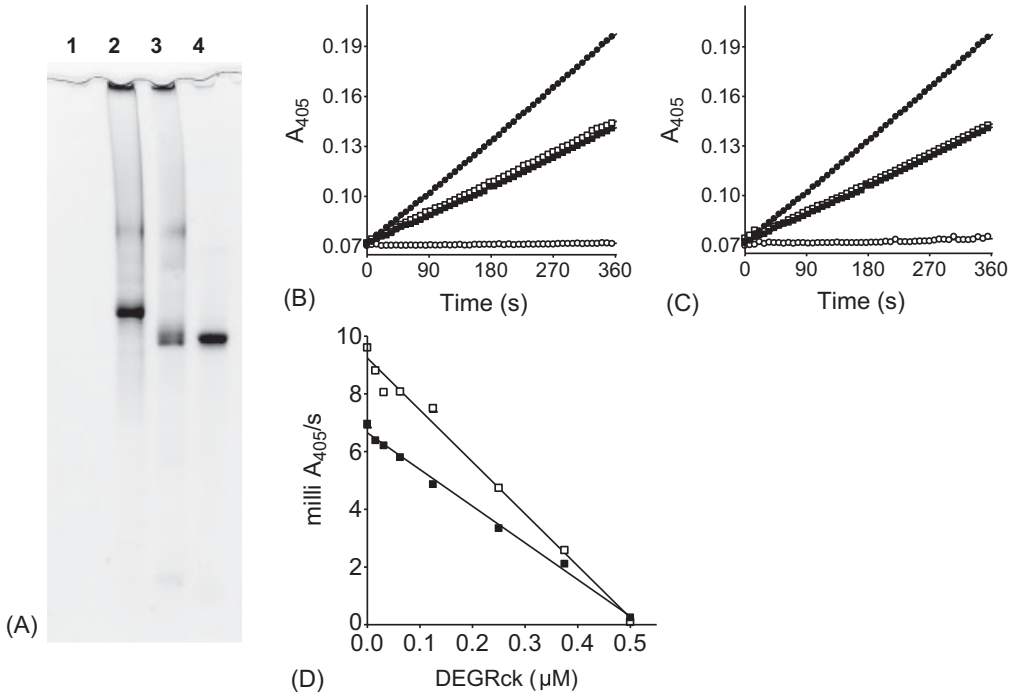

Fig. 1 Preparation of Gla-domainless FXa α_2-macroglobulin complex (GDFXa-α_2M). (**A**) Native polyacrylamide gel electrophoresis (PAGE) (gradient 3–8%): lane 1, GDFXa only (invisible in this gel system); lane 2, purified native α_2M; lane 3, GDFXa-α_2M complex; lane 4, fast form of α_2M (methylamine-treated). In spite of the 4/1 stoichiometric ratio, most if not all native α_2M shifted towards the fast form upon incubation with GDFXa. (**B** and **C**) Progress of S2765 hydrolysis by 1 nM GDFXa (closed circles) or GDFXa-α_2M (closed squares). Corresponding open symbols in **B** were obtained in the presence of 2.6 µM antithrombin and 1 IU anti-Xa/mL enoxaparin; corresponding open symbols in **C** were obtained in the presence of 145 nM tissue factor pathway inhibitor (TFPI). In both cases, GDFXa was fully inhibited, whereas GDFXa-α_2M was unaffected. (**D**) Active site titration of GDFXa (open squares) and GDFXa-α_2M (closed squares) by 1,5 Dansyl-Glu-Gly-Arg chloromethyl ketone (DEGRck). GDFXa or GDFXa-α_2M (0.5 µM) were incubated 30 minutes at 37°C with DEGRck at the indicated concentration and the residual catalytic activity was measured after 1/20 dilution using S2765. GDFXa cleaved 1.4-fold faster S2765 than GDFXa-α_2M.

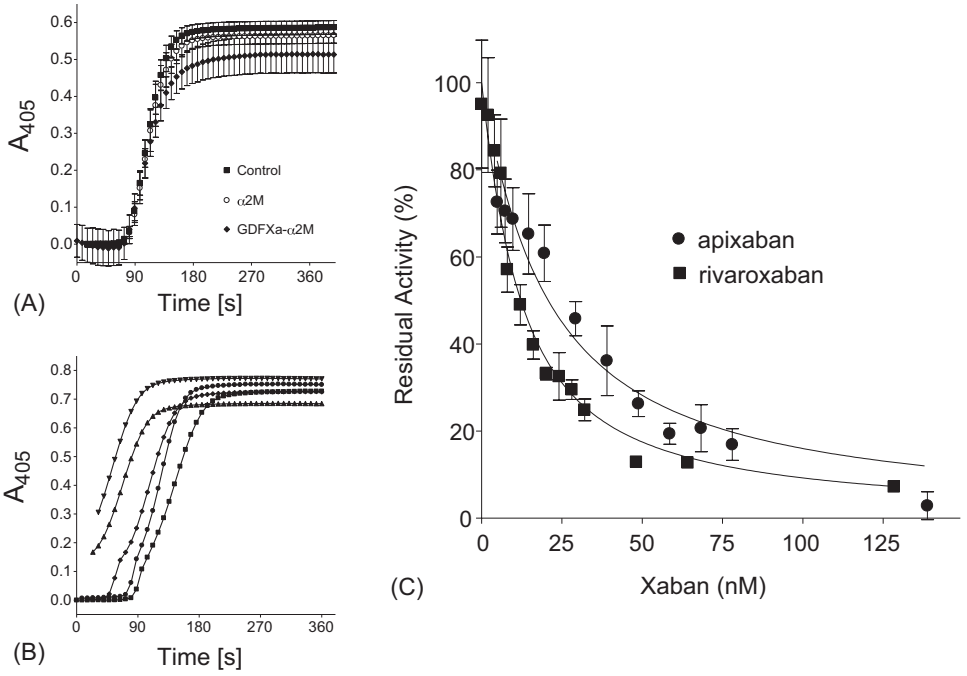

Fig. 2 Characterization of Gla-domainless FXa α_2-macroglobulin complex (GDFXa-α_2M). (**A**) Clot waveform triggered by adding 5 pM tissue factor (TF) and 4 µM phospholipid vesicles to platelet-poor plasma (PPP) (closed squares), containing 1 µM purified α_2M (open circles) or 1.7 µM GDFXa-α_2M (closed diamonds). Graph represents the A_{405} versus time (mean values ± standard error of the means [SEM] of 6 experiments). Lag time was defined as the time to reach 15% of the maximum. (**B**) Clot waveform triggered by adding 5 pM TF and 4 µM phospholipid vesicles to PPP alone (squares) or containing increasing amounts of GDFXa: 31.2 nM (circles), 62.5 nM (diamonds), 125 nM (upward triangles) and 250 nM (downward triangles). GDFXa was a potent pro-coagulant. (**C**) Graph represents the residual rate of 400 µM S2765 hydrolysis by 5 nM GDFXa-α_2M as a function of rivaroxaban (closed squares) or apixaban (closed circles); mean value ± SEM of three determinations. Solid lines were obtained by non-linear regression analysis using the tight-binding inhibition equation.[40] Values of 2.41 ± 0.22 nM ($R^2 = 0.84$) and 1.29 ± 0.13 nM ($R^2 = 0.87$) were obtained for apixaban and rivaroxaban, respectively.

In Vitro Neutralization of Xabans by GDFXa-α_2M

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-α_2M, we triggered clot waveform assays in PPP spiked with increasing amount of xabans in the presence or absence of 1.7 µM GDFXa-α_2M. Apixaban or rivaroxaban dose-dependently prolonged the lag time. GDFXa-α_2M fully neutralized xaban anticoagulant effect even at supra-therapeutic levels. Indeed, lag times in the presence of GDFXa-α_2M 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-α_2M 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-α_2M 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-α_2M in Mice

The reported half-life of FXa-α_2M in mice is 2 minutes[43] and that of subtilisin-α_2M in rats is 6 minutes.[44] 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-α_2M. However, uptake of protease-α_2M is saturable.[45–47] Thus, we evaluated the actual persistence of GDFXa-α_2M following injection at the high dose needed for stoichiometric xaban neutralization. When 0.5 mg/mice GDFXa-α_2M was injected, 50% of the catalytic activity of GDFXa-α_2M was still detectable 170 minutes following injection (►**Fig. 4B**). Consequently, we designed the in vivo experiments taking into account the actual persistence of GDFXa-α_2M in mice.

Neutralization of Xabans by GDFXa-α_2M in Mice

GDFXa-α_2M 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-α_2M (100 µL, 3.6 µM) were injected in each retro-orbital plexus. Primary endpoints were bleeding time and blood loss following lateral tail vein transection. 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-α_2M injection ($p > 0.05$). Overall, GDFXa-α_2M significantly decreased rivaroxaban induced bleeding time (from 4.92 ± 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-α_2M had no effect on bleeding time (2.90 ± 0.28 vs. 2.95 ± 0.51 minutes; $p > 0.05$) and

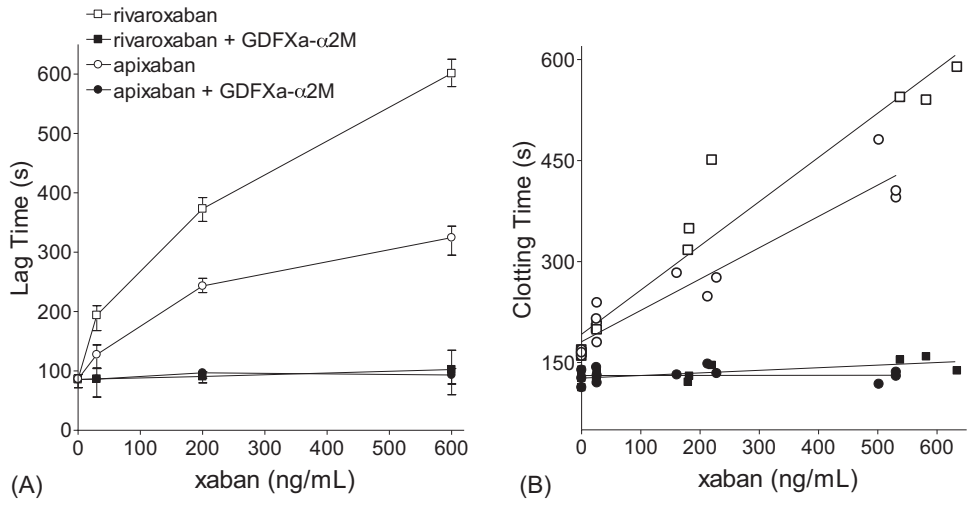

Fig. 3 Neutralization of xabans in vitro. (**A**) Graph represents the lag time of clot waveform triggered by adding 5 pM tissue factor (TF) and 4 µM phospholipid vesicles. Platelet-poor plasma (PPP) was spiked with increasing amounts (as indicated in the abscissa) of rivaroxaban (open squares) or apixaban (open circles). Corresponding closed symbols were obtained in these PPP containing in addition 1.7 µM Gla-domainless FXa α_2-macroglobulin complex (GDFXa-α_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-α_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-α_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.

blood loss (158 ± 99 vs. 163 ± 73 µL; $p > 0.05$). We concluded that GDFXa-α_2M effectively neutralized rivaroxaban anticoagulant effect in this in vivo model and that GDFXa-α_2M alone had no adverse effect on mouse haemostasis.

Evaluation of Pro-Coagulant Markers in Mice Receiving GDFXa-α_2M

The above data suggested that GDFXa-α_2M was devoid of in vivo adverse effect. To exclude the potential pro-thrombotic effect of GDFXa-α_2M in mice, we performed clot waveform assay in samples collected 30 minutes post-injection of vehicle or GDFXa-α_2M. Whether or not mice received GDFXa-α_2M, the lag time of clot waveform did not differ between groups ($p = 0.18$; ►**Fig. 6A**). Moreover, D-dimer and TAT levels were comparable ($p = 0.50$ and $p = 0.13$; ►**Fig. 6B** and **C**). Overall results suggested that GDFXa-α_2M was devoid of pro- as well as anticoagulant properties in vivo.

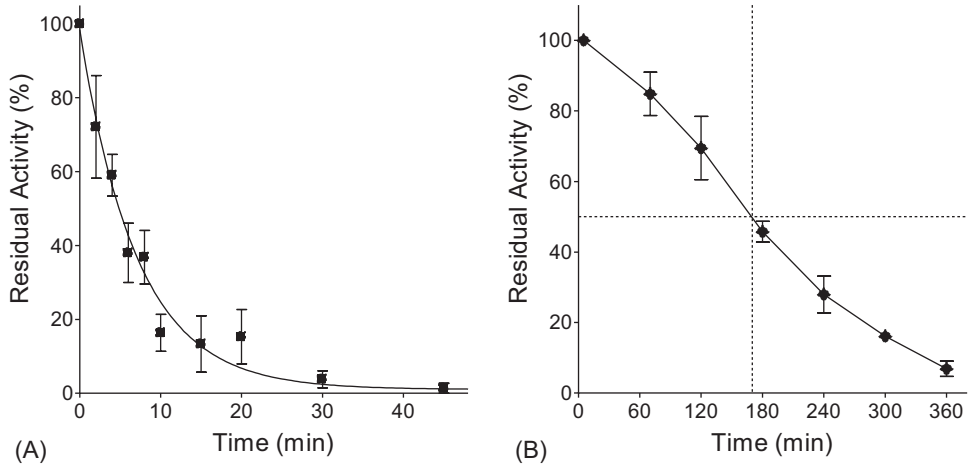

Fig. 4 Half-life and persistence of Gla-domainless FXa α_2-macroglobulin complex (GDFXa-α_2M) in mice. (**A**) Kinetic of the normalized residual rate of S2765 hydrolysis when 100 µL GDFXa-α_2M (150 nM) was injected in mice. Data points are mean value of three determinations (error bars min and max). Solid line was obtained by nonlinear regression analysis using a single exponential decay equation. Estimated half-life of GDFXa-α_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-α_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-α_2M catalytic activity persisted 170 minutes after injection.

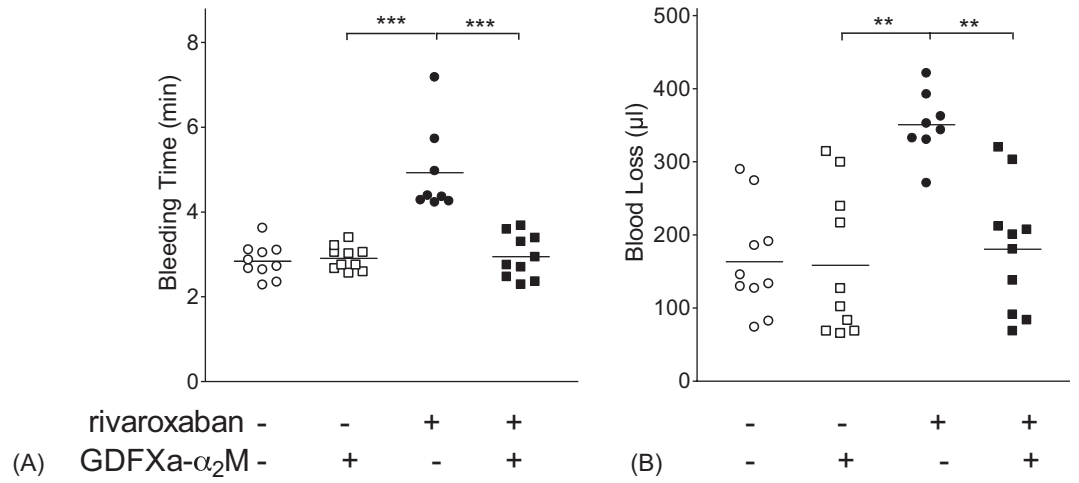

Fig. 5 Neutralization of rivaroxaban in mice. (**A**) Sum of secondary bleeding time (in minutes) following challenges at time 15, 30 and 45 minutes past initial tail vein transection. (**B**) Corresponding sum of secondary blood loss (in µL). Mice were force-fed with 10 mM HCl (open symbols) or rivaroxaban (closed symbols). Two hours later, 100 µL vehicle alone (circles) or containing 3.6 µM Gla-domainless FXa α_2-macroglobulin complex (GDFXa-α_2M) (squares) were injected in each retro-orbital plexus of the mice. Rivaroxaban significantly increased the bleeding time ($p < 0.001$) and blood loss ($p < 0.01$). This rivaroxaban-induced bleeding was significantly reduced by GDFXa-α_2M both for the bleeding time ($p < 0.001$) and blood loss ($p < 0.01$) becoming non-significantly different from the control or GDFXa-α_2M alone groups ($p > 0.05$). Drug/vehicle versus drug/antidote pair-wise comparisons estimated by Dunn tests ($^{**}p < 0.01$; $^{***}p < 0.001$).

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-α_2M 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-α_2M 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.[42] We evaluated GDFXa-α_2M efficacy as xabans neutralizing agent using this model. We confirmed as previously reported[15] 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-α_2M, values were comparable to those of the control group. Presumably rivaroxaban binding to GDFXa-α_2M neutralized its anticoagulant effect. That bleeding time and blood loss were comparable whether or not mice received GDFXa-α_2M suggested harmlessness in addition to effectiveness. In accord with this hypothesis, neither D-dimer nor TAT levels increased following GDFXa-α_2M injection.

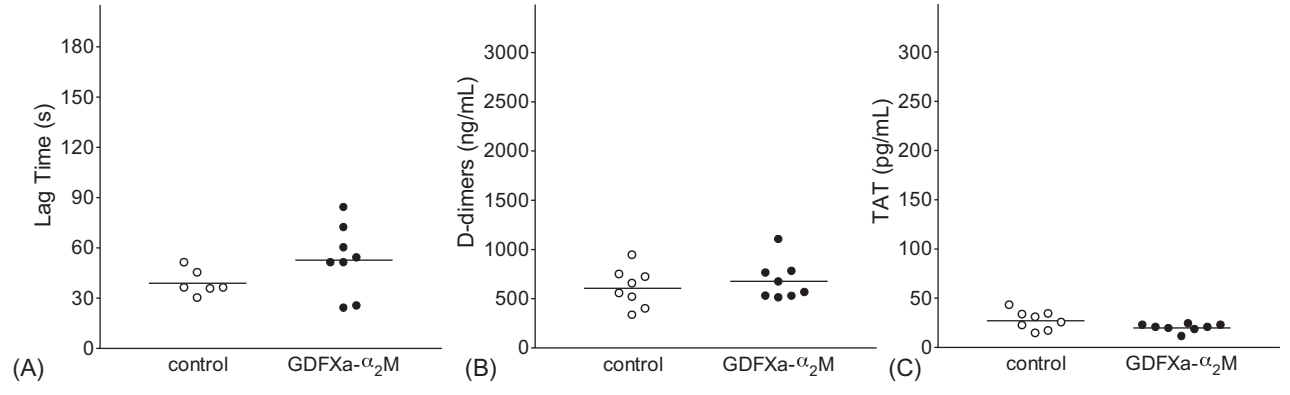

Fig. 6 Lack of Gla-domainless FXa α_2-macroglobulin complex (GDFXa-α_2M) pro-thrombotic potential in mice. Pro-thrombotic potential of GDFXa-α_2M was evaluated in platelet-poor plasma (PPP) from mice injected with vehicle alone (open circles) or containing 3.6 µM GDFXa-α_2M (closed circles). (**A**) Lag time (LT in seconds) of clot waveform. (**B**) Amount of D-dimers and (**C**) of thrombin–antithrombin (TAT) measured by enzyme-linked immunosorbent assay (ELISA). Determined by unpaired two-tailed Mann-Whitney test (vehicle vs. GDFXa-α_2M) no significant difference was observed between lag times ($p = 0.18$), D-dimers ($p = 0.50$) or TAT ($p = 0.13$) suggesting a lack of pro-thrombotic potential in mice for GDFXa-α_2M.

Ideally, an antidote should neutralize and eliminate its target in vivo rather than just sequestering it. Cleavage of the bait region in α_2M exposes previously concealed recognition sites for receptors on fibroblasts, macrophages and hepatocytes that uptake α_2M-protease from the circulation.[45–49] Clearance is indistinguishable whether or not the active site of the α_2M-linked protease is inhibited by low MW inhibitors.[48] Assuming that such depletion occurs with GDFXa-α_2M, it could literally deplete xaban from blood, bypassing the natural clearance mechanisms. On the other hand, we evidenced that high amount of GDFXa-α_2M had a relatively long persistence in blood, presumably due to saturation of the uptake mechanisms. GDFXa-α_2M would rapidly neutralize xabans but eliminate them only slowly. Accordingly, GDFXa-α_2M may constitute a fairly long-lasting antidote not requiring continuous perfusion or multiple injections. It is worth mentioning that α_2M and FXa are native blood components thus not immunogenic and that 1.7 µM GDFXa-α_2M neutralizing supra-therapeutic levels of xabans is below the normal plasma concentration of α_2M (3.5 µM in adults; 5.6 µM in newborn). Affinity of apixaban and rivaroxaban for GDFXa-α_2M 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.[50] However, GDFXa-α_2M ruined the thrombin generation assay because of its ability to cleave FluCa, precluding measurement of thrombin generation in its presence. Amount of GDFXa-α_2M added to neutralize xabans (1.7 µM) actually exceeded the thrombin potential (1.3 µM).

The prospect of using GDFXa-α_2M as an antidote to xaban has limitations. First, it is difficult to consider α_2M purified from human plasma for therapeutic use and large-scale production of recombinant α_2M may turn out difficult. Second, our GDFXa-α_2M preparation was a mixture of native α_2M, and activated α_2M having sequestered or not GDFXa. Adding micromolar quantities of native α_2M to PPP had no detectable effect on blood coagulation. Blood readily contains large amounts of α_2M which does not prevent xaban effects. We showed that active GDFXa is highly pro-coagulant and verified that GDFXa-α_2M preparation did not contain traces of GDFXa. We also verified that GDFXa-α_2M 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 α_2M such as methylamine-treated α_2M in addition to GDFXa-α_2M. Specifically, the catalytic activity of sequestered GDFXa is not affected by dabigatran, yet GDFXa-α_2M partially neutralizes the anticoagulant effect of dabigatran. Mechanism likely involves secondary binding sites latent in native α_2M. During blood coagulation, FXa-α_2M and thrombin-α_2M are formed. FXa-α_2M 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 α_2M content.

Overall, GDFXa-α_2M 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 α_2-macroglobulin and Gla-domainless FXa (GDFXa-α_2M) is an attractive neutralizing agent of xabans in vitro and in vivo.
- GDFXa-α_2M is effective as an antidote for rivaroxaban-induced bleeding in mice.
- GDFXa-α_2M 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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