A specific antidote for reversal of anticoagulation by direct and indirect inhibitors of coagulation factor Xa

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Inhibitors of coagulation factor Xa (fXa) have emerged as a new class of antithrombotics but lack effective antidotes for patients experiencing serious bleeding. We designed and expressed a modified form of fXa as an antidote for fXa inhibitors. This recombinant protein (r-Antidote, PRT064445) is catalytically inactive and lacks the membrane-binding γ-carboxyglutamic acid domain of native fXa but retains the ability of native fXa to bind direct fXa inhibitors as well as low molecular weight heparin–activated antithrombin III (ATIII). r-Antidote dose-dependently reversed the inhibition of fXa by direct fXa inhibitors and corrected the prolongation of *ex vivo* clotting times by such inhibitors. In rabbits treated with the direct fXa inhibitor rivaroxaban, r-Antidote restored hemostasis in a liver laceration model. The effect of r-Antidote was mediated by reducing plasma anti-fXa activity and the non–protein bound fraction of the fXa inhibitor in plasma. In rats, r-Antidote administration dose-dependently and completely corrected increases in blood loss resulting from ATIII-dependent anticoagulation by enoxaparin or fondaparinux. r-Antidote has the potential to be used as a universal antidote for a broad range of fXa inhibitors.

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Warfarin, a vitamin K antagonist, is widely used as an anticoagulant for the prevention and treatment of thrombotic diseases. However, it has substantial limitations, such as bleeding and poorly controlled levels of anticoagulation in patients. Thus, intensive effort has been focused on developing more specific inhibitors of the coagulation enzymes thrombin and fXa. Several of these new agents have been approved for the prevention of stroke in patients with atrial fibrillation, prevention of venous thromboembolism after orthopedic surgery and treatment of deep vein thrombosis or pulmonary embolism[1–4]. Randomized clinical trials have established that these new agents share the bleeding liability of warfarin and parenteral anticoagulants such as heparin. With a worldwide aging population leading to an increase in the prevalence of atrial fibrillation and a broader use of anticoagulants, the management of bleeding complications in frail elderly patients represents an unmet need in clinical practice.

Traditional anticoagulants have antidotes. Heparin can be neutralized by protamine, and warfarin anticoagulation can be reversed by vitamin K injections. Protamine can also partially reverse the effect of ATIII-dependent low–molecular weight heparins (LMWH) such as enoxaparin but has no corrective activity on shorter heparins (fondaparinux or idraparinux)[5–7]. Fresh frozen plasma, prothrombin-complex concentrate and recombinant factor VIIa have also been used as generic hemostatic agents in patients treated with anticoagulants who experience major trauma or severe hemorrhage[8–10]. However, the effectiveness of these nonspecific agents has not been established in blinded clinical trials.

As direct oral fXa inhibitors such as rivaroxaban and apixaban have the potential to become a mainstay of anticoagulant therapy, we have designed a recombinant protein as a universal antidote to this class of drugs. This specific antidote for fXa inhibitors is a truncated form of enzymatically inactive fXa. The modified recombinant protein (r-Antidote, PRT064445) lacks a membrane-binding γ-carboxyglutamic acid (GLA) domain and is catalytically inactive because of a mutation of the serine residue in the protease catalytic triad. We demonstrate that this fXa variant retains the capacity to bind direct fXa inhibitors. In addition, r-Antidote also binds to LMWH or pentasaccharide-activated ATIII and modulates the activity of these indirect fXa inhibitors. The recombinant protein was expressed in its mature, functional form and not as a zymogen. We report proof-of-concept results for this universal antidote for the reversal of anticoagulation by fXa inhibitors *in vitro* and in animal models of bleeding.

RESULTS

Expression and purification of r-Antidote

A schematic of the r-Antidote molecule (**Fig. 1a**) shows the three modifications to native fX, resulting in a protein that can bind to fXa inhibitors and counteract their activity but is no longer capable of assembly into the prothrombinase complex. We expressed r-Antidote in Chinese hamster ovary (CHO) cells and purified it from conditioned medium. The two-step purification procedure yielded purified proteins of the expected molecular weight for the light (~11 kDa) and heavy (~28 kDa) chains (**Fig. 1b**). Deletion of 34 residues in the light chain of native fX (residues 46–78) resulted in a lower molecular

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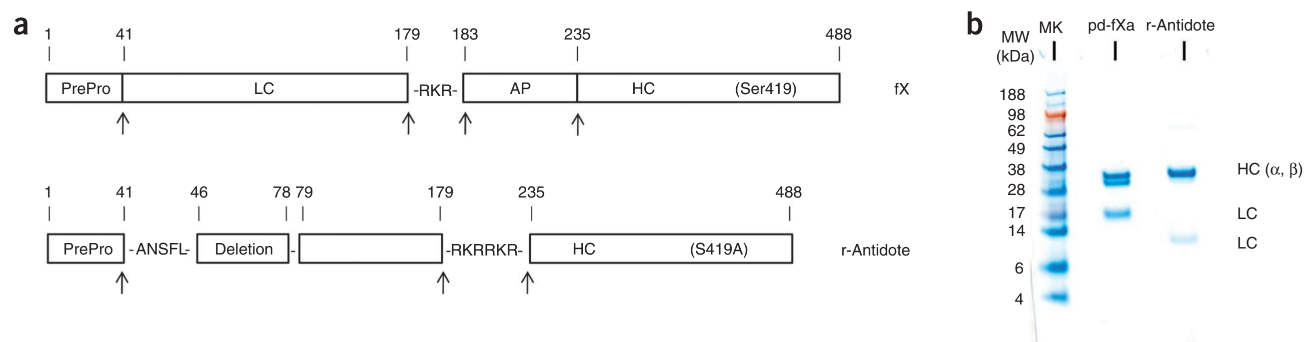

Figure 1 Design of r-Antidote and protein expression in CHO cells. (**a**) Schematic illustration of the domain structure of full-length human fX and r-Antidote (PRT064445) precursors. Using fX as a template, modifications were made in three regions to generate r-Antidote: deletion of a 34-residue fragment (residues 46–78) that contains the 11 GLA residues; replacement of the activation peptide (AP) with ArgLysArg (RKR) to form the RKRRKR linker that connects the light chain (LC) to the heavy chain (HC); and mutation of the active-site serine to alanine (S419A). Arrows indicate potential cleavage sites. (**b**) Reduced SDS-PAGE of purified r-Antidote showing bands of expected molecular weight (MW) for the light (~11 kDa) and heavy (~28 kDa) chains. The double bands of the heavy chain of plasma-derived fXa (pd-fXa) and r-Antidote correspond to the α- and β-isoforms. MK, molecular weight marker.

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weight for the r-Antidote light chain compared to plasma-derived fXa. The double bands observed for the heavy chain of both plasma-derived fXa and r-Antidote correspond to the α- and β-isoforms of the protein. The β-isoform results from additional proteolytic cleavage at the C terminus of the heavy chain[11,12]. We verified the identity of the heavy and light chains of the purified proteins by western blotting with human fX- and Xa-specific monoclonal antibodies (data not shown). N-terminal sequencing of both the light and heavy chains of purified r-Antidote yielded the expected amino acid sequences, indicating proper processing of the PrePro sequence at the N-terminus of the light chain and the RKRRKR linker at the N-terminus of the heavy chain (data not shown). To produce functional protein, proper processing requires that the N terminus of the heavy chain start at Ile235 and the RKRRKR linker be completely removed.

Reversal of direct fXa inhibitors by r-Antidote

We determined the potency (K_d) of r-Antidote binding to three direct fXa inhibitors (betrixaban, rivaroxaban and apixaban) and compared them to inhibition of purified human fXa using a peptidyl substrate (**Fig. 2a**). r-Antidote dose-dependently reversed the inhibitory activity of the small-molecule fXa inhibitors but did not produce any change in the rate of peptidyl substrate cleavage by fXa in the absence of inhibitor. This observation is consistent with the expected lack of catalytic activity of r-Antidote. Analysis of these kinetic data indicated that r-Antidote has subnanomolar affinities for these direct fXa inhibitors (**Table 1**). The relative potency of binding was in the same order of magnitude as the inhibition constants (K_i) reported in the literature for these inhibitors against plasma-derived fXa[13–15].

After testing in a purified enzyme system, we evaluated the ability of r-Antidote to reverse the effects of anticoagulation in plasma. We determined the residual inhibitory activity of rivaroxaban, as measured by anti-fXa activity, after incubation with varying amounts of r-Antidote. Inhibitory activity was dose-dependently and completely reversed by r-Antidote in both human and rat plasma (data not shown). We next used rivaroxaban as a prototype inhibitor to test the effects of r-Antidote on markers of anticoagulation as measured

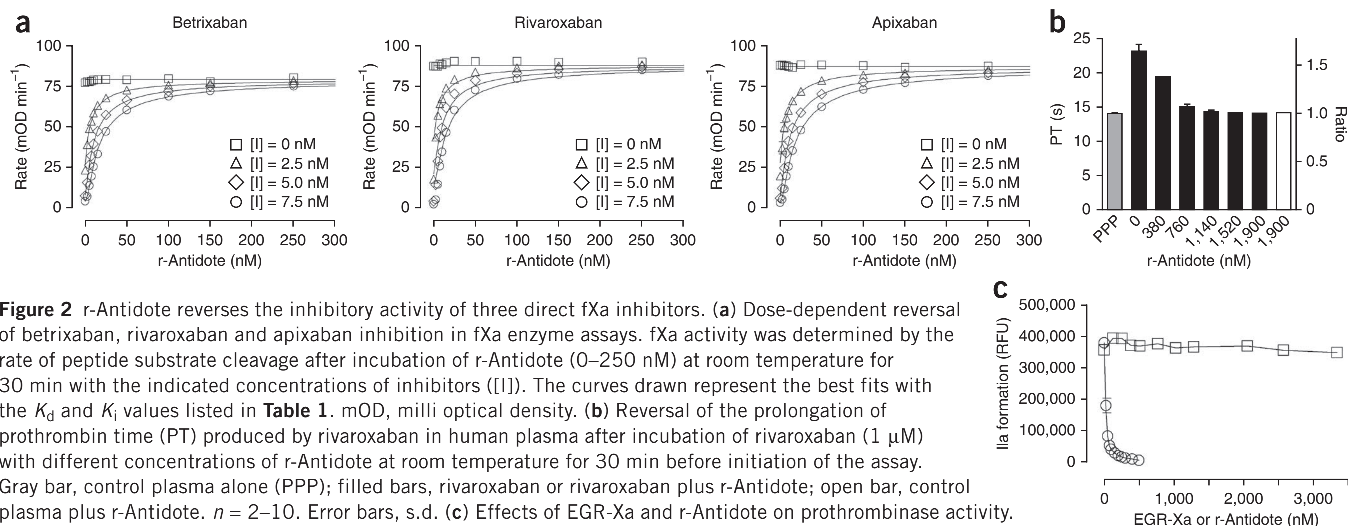

Figure 2 r-Antidote reverses the inhibitory activity of three direct fXa inhibitors. (**a**) Dose-dependent reversal of betrixaban, rivaroxaban and apixaban inhibition in fXa enzyme assays. fXa activity was determined by the rate of peptide substrate cleavage after incubation of r-Antidote (0–250 nM) at room temperature for 30 min with the indicated concentrations of inhibitors ([I]). The curves drawn represent the best fits with the K_d and K_i values listed in **Table 1**. mOD, milli optical density. (**b**) Reversal of the prolongation of prothrombin time (PT) produced by rivaroxaban in human plasma after incubation of rivaroxaban (1 μM) with different concentrations of r-Antidote at room temperature for 30 min before initiation of the assay. Gray bar, control plasma alone (PPP); filled bars, rivaroxaban or rivaroxaban plus r-Antidote; open bar, control plasma plus r-Antidote. n = 2–10. Error bars, s.d. (**c**) Effects of EGR-Xa and r-Antidote on prothrombinase activity. Human plasma was incubated with increasing concentrations of r-Antidote (squares) or EGR-Xa (circles) for 30 min at room temperature. Thrombin generation (IIa formation) was then initiated by the addition of Ca^{2+} and tissue factor, and cleavage of the thrombin-specific substrate Z-GlyGlyArg-aminomethylcoumarin (Z-GGR-AMC; relative fluorescence units, RFU) was measured at 37 °C for 10 min.

Table 1 Affinity of r-Antidote for direct fXa inhibitors

Inhibitor	r-Antidote K_d (nM)	fXa K_i (nM)	Ref.
Betrixaban	0.53 ± 0.01	0.117	13
Rivaroxaban	1.53 ± 0.22	0.400	14
Apixaban	0.58 ± 0.02	0.100	15

K_d (mean ± s.d.) values were calculated by nonlinear fitting of the kinetic data in **Figure 2a** with the reported K_i value for each inhibitor.

by *ex vivo* clotting assays. r-Antidote reversed the anticoagulant effect of rivaroxaban in human plasma (**Fig. 2b**). At a near-peak therapeutic concentration, rivaroxaban (1 μM) caused a prolongation of prothrombin time. This prolongation was corrected to baseline by an approximately equal molar concentration of r-Antidote. Although r-Antidote bound and neutralized the anticoagulant effect of rivaroxaban in a dose-dependent manner, r-Antidote by itself did not change prothrombin time at the highest concentration tested (**Fig. 2b**). Thus, r-Antidote alone did not produce detectable amounts of procoagulant or anticoagulant activity as measured by this clotting assay.

As an additional test of the procoagulant or anticoagulant activity of r-Antidote, we used a thrombin generation assay, which tests fXa activity in the presence of cofactor factor Va and phospholipids in the prothrombinase complex. r-Antidote lacks the GLA domain and should have a substantially reduced capacity to incorporate into the prothrombinase complex. In contrast, active site–inhibited fXa with an intact GLA domain is expected to be a potent anticoagulant, as it can compete with endogenous fXa for assembly into the prothrombinase complex. We compared the effect of r-Antidote on prothrombinase activity with that of EGR-Xa, an active site–inhibited full-length human fXa. We measured prothrombinase activity by thrombin generation in human plasma with increasing concentrations of r-Antidote (0–3.3 μM) or EGR-Xa (0–0.5 μM). Formation of thrombin (as measured by relative fluorescence units) in the presence of r-Antidote remained essentially unchanged up to a concentration of 3.3 μM, whereas EGR-Xa showed potent inhibition in the same assay (half-maximal inhibitory concentration of 26 nM) (**Fig. 2c**). These results are consistent with an earlier report documenting inhibition of prothrombinase activity by active site–inhibited native fXa[16].

We next tested the ability of r-Antidote to reverse the anticoagulant effect of three direct fXa inhibitors *in vivo* in a rat model. Infusion of rivaroxaban (0.25 mg per kg body weight h^{-1}) over a 30-min period produced a twofold increase in the level of anticoagulation as measured by whole-blood INR (international normalized ratio) (**Fig. 3a**). After rivaroxaban infusion was stopped, rats were treated with vehicle or were given an intravenous (i.v.) bolus injection of r-Antidote (4 mg per rat) followed by sustained r-Antidote infusion (4 mg h^{-1}). In the vehicle-treated group, INR values decreased gradually as a result of clearance of the circulating inhibitor. In contrast, in the r-Antidote-treated group, the raised INR was rapidly and completely reversed to baseline values, and this reversal was sustained.

We also evaluated the effect of r-Antidote administration on the pharmacokinetics of rivaroxaban (**Supplementary Fig. 1**). After i.v. administration of r-Antidote, the total rivaroxaban plasma concentration increased immediately as a result of the redistribution of rivaroxaban from extravascular compartments. Despite the increase in total rivaroxaban concentration, the amount of free, non–protein bound rivaroxaban (the fraction responsible for anticoagulant activity) decreased to very low levels. Thus, a decrease in the free fraction of rivaroxaban correlated with the correction of whole-blood INR values. The plasma molar ratios of r-Antidote to rivaroxaban (total) during the experimental time course were 2.1 (35 min), 1.7 (60 min) and 1.3 (90 min). As the plasma molar ratio of r-Antidote to fXa inhibitor decreased during the time course, some reestablishment of anticoagulation was observed in the rats.

We next performed similar experiments testing the effects of r-Antidote after betrixaban and apixaban infusion into rats (**Fig. 3b**,**c**). As above, we collected serial blood samples to measure whole-blood INR values, plasma concentrations of r-Antidote and the total and free plasma fractions of the inhibitors. Both betrixaban and apixaban–mediated whole-blood INR increases were similarly reversed by r-Antidote. After i.v. infusion of the three fXa inhibitors (each administered individually) for 30 min, the total plasma concentrations of rivaroxaban, betrixaban and apixaban were 1.4 ± 0.4 μM (mean ± s.d.), 0.2 ± 0.01 μM and 1.4 ± 0.3 μM, respectively, and the percentages of unbound inhibitor were 2.2% ± 0.8% (mean ± s.d.), 40% ± 7.2% and 1.5% ± 0.3%, respectively. After administration of r-Antidote, the total plasma concentrations of the inhibitors increased to 1.9 ± 0.09 μM, 2.0 ± 0.4 μM and 4.2 ± 0.7 μM, respectively, and the percentage of unbound inhibitor declined to 0%, 0.3% ± 0.1% and 0.05% ± 0.02%, respectively. Thus, for each of the three inhibitors, correction of prothrombin time by r-Antidote to near-normal values was associated with a reduction in the free fraction of the inhibitor.

r-Antidote restores hemostasis in animal models of blood loss

To evaluate whether effects of r-Antidote in reversing coagulation as measured by INR translates into a cessation of bleeding, we first tested the ability of r-Antidote to restore normal hemostasis in a mouse

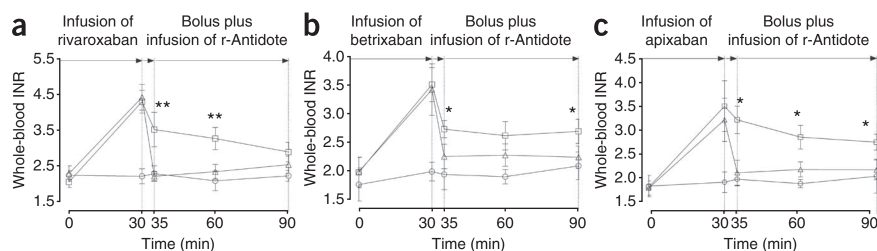

Figure 3 Sustained reversal of whole-blood INR by r-Antidote in rats treated with direct fXa inhibitors. (**a**) Whole-blood INR values (mean ± s.d.) in rats infused with rivaroxaban (0.25 mg per kg body weight h^{-1}) or vehicle for 30 min and then treated with either vehicle or r-Antidote by i.v. bolus (4 mg) over 5 min plus infusion (4 mg h^{-1}) for up to 90 min. Circles, vehicle + vehicle; squares, rivaroxaban + vehicle; triangles, rivaroxaban + r-Antidote. $^{**}P \leq 0.01$ compared to the r-Antidote treatment group determined by unpaired two-tailed *t* test. (**b**) Whole-blood INR values (mean ± s.d.) in rats infused with betrixaban (1 mg per kg body weight h^{-1}) or vehicle and then treated with either vehicle or r-Antidote by i.v. bolus (6 mg) over 5 min plus infusion (9 mg h^{-1}) for up to 90 min. Circles, vehicle + vehicle; squares, betrixaban + vehicle; triangles, betrixaban + r-Antidote. $^{*}P \leq 0.02$ compared to the r-Antidote treatment group determined by unpaired two-tailed *t* test. (**c**) Whole-blood INR values (mean ± s.d.) in rats infused with apixaban (0.5 mg per kg body weight h^{-1}) or vehicle and then treated with either vehicle or r-Antidote by i.v. bolus (6 mg) over 5 min plus infusion (6 mg h^{-1}) for up to 90 min. Circles, vehicle + vehicle; squares, apixaban + vehicle; triangles, apixaban + r-Antidote. $^{*}P \leq 0.01$ compared to the r-Antidote treatment group determined by unpaired two-tailed *t* test.

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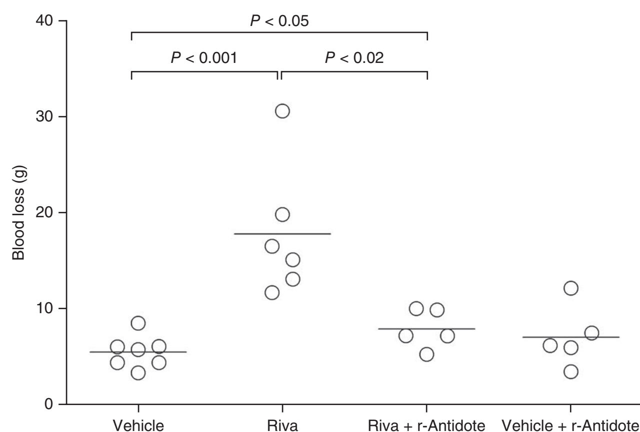

Figure 4 Mitigation of blood loss caused by rivaroxaban-induced anticoagulation with r-Antidote in a rabbit liver laceration model. In this model, rabbits were dosed with rivaroxaban (Riva; 1 mg per kg body weight) or vehicle by i.v. bolus over 2 min and then administered r-Antidote or vehicle as an i.v. bolus over 5 min. The *P* values shown were calculated by unpaired two-tailed *t* test.

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model of blood loss. Oral administration of rivaroxaban alone at a high dose (50 mg per kg body weight) produced variable blood loss in mice (**Supplementary Fig. 2**; 216 ± 222 μl (mean ± s.d.) as compared to 86 ± 79 μl for vehicle treated controls ($P = 0.14$)). Aspirin (ASA) monotherapy at an oral dose of ~100 mg per kg body weight d^{-1} for 5 d did not result in a statistically significant increase in blood loss (118 ± 71 μl compared to 86 ± 79 μl for vehicle-treated controls, $P = 0.48$). However, combination of the 5-day ASA treatment with a single oral dose of rivaroxaban at 50 mg per kg body weight increased blood loss by 3.4-fold (403 ± 107 μl) compared to treatment with ASA alone. An i.v. injection of r-Antidote (0.96 mg per mouse) 2 h after administration of the oral dose of rivaroxaban reduced the increased blood loss by ~84% (163 ± 82 μl) relative to rivaroxaban and ASA treatment.

The effect of r-Antidote on circulating rivaroxaban concentrations was similar to our previous observations in the rat. After r-Antidote administration, the total rivaroxaban concentration in plasma was increased by 7.8-fold (data not shown). In parallel to the reduction in blood loss, rivaroxaban anticoagulant activity was reduced by >80% as measured by plasma anti-fXa activity (data not shown).

As tail transection in mice or rats may not be representative of anticoagulant-induced bleeding after major trauma and as fXa inhibitors are often used as monotherapy in the absence of aspirin, we further investigated the potential for restoration of hemostasis by r-Antidote in a rabbit model of liver laceration. We administered vehicle or 1 mg per kg body weight rivaroxaban by i.v. bolus injection to anesthetized rabbits. After 30 min to allow for biodistribution of the inhibitor, we administered vehicle or r-Antidote (75 mg per rabbit) as a bolus injection. We then lacerated two liver lobes and collected the lost blood on a preweighed gauze over 15 min. Blood loss caused by rivaroxaban anticoagulation represented approximately 10% of the total blood volume of the rabbits (**Fig. 4**). As in the rodent models, we measured total and free rivaroxaban plasma concentrations, plasma anti-fXa activity, prothrombin time and activated partial thromboplastin time (aPTT).

After its administration, the circulating concentration of rivaroxaban was 1.65 ± 0.5 μM at 30 min, resulting in a 2.3-fold and a 1.9-fold prolongation of prothrombin time and aPTT, respectively, and a 3.2-fold increase in blood loss compared to vehicle treatment. Administration of r-Antidote reduced blood loss by >85% and decreased peak anti-fXa activity by 98%, prothrombin time by 74% and aPTT by 66% and altered the non–protein bound fraction of rivaroxaban in plasma from 26% ± 0.9% to 0.5% ± 0.3%. r-Antidote treatment alone had no effect on blood loss. Similarly to the rodent model (**Fig. 3**), the correction of rabbit visceral blood loss by r-Antidote correlated with a reduction in anti-fXa activity and in the free fraction of direct fXa inhibitor.

Reversal of ATIII-dependent fXa inhibitors by r-Antidote

r-Antidote, with a binding site mimicking that of native fXa, should have the capacity to interact with ATIII. Enoxaparin (LMWH) and fondaparinux (a pentasaccharide) increase the affinity of ATIII for fXa. Therefore, we tested the effect of r-Antidote on the anticoagulant

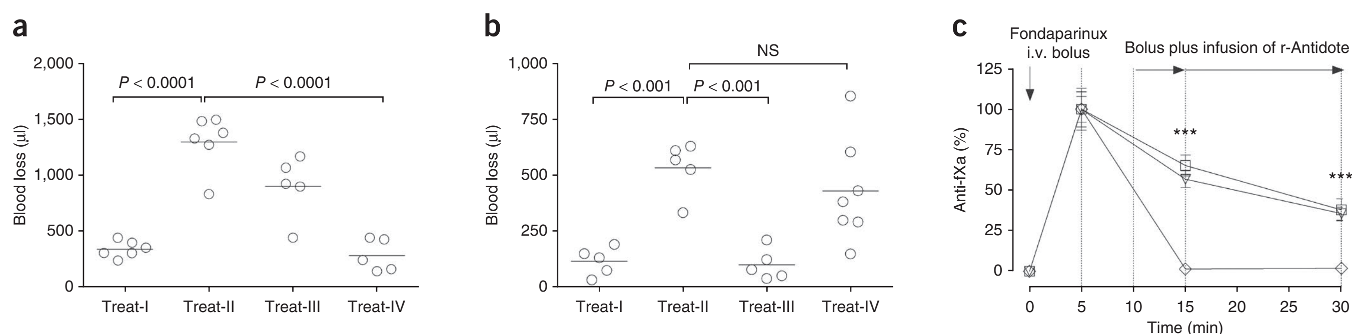

Figure 5 Reversal of ATIII-dependent fXa inhibitors by r-Antidote in the rat tail transection model. (**a**) Dose titration of r-Antidote for the mitigation of blood loss caused by enoxaparin-induced anticoagulation in the indicated groups. Treat-I, vehicle treatment alone; Treat-II, enoxaparin (4.5 mg per kg body weight) dosed by i.v. bolus over 5 min followed by treatment with vehicle; Treat-III, enoxaparin (4.5 mg per kg body weight) dosed by i.v. bolus over 5 min followed by treatment with 2 mg bolus plus 2 mg h^{-1} infusion of r-Antidote; Treat-IV, enoxaparin (4.5 mg per kg body weight) dosed by i.v. bolus over 5 min followed by treatment with 4 mg bolus plus 4 mg h^{-1} infusion of r-Antidote. (**b**) Mitigation of blood loss caused by fondaparinux-induced anticoagulation with r-Antidote in the indicated groups. Treat-I, vehicle treatment alone; Treat-II, fondaparinux (25 mg per kg body weight) dosed as i.v. bolus over 5 min followed by treatment with vehicle; Treat-III, fondaparinux (25 mg per kg body weight) dosed as i.v. bolus over 5 min followed by treatment with r-Antidote (6 mg bolus plus 6 mg h^{-1} infusion); Treat-IV, fondaparinux (25 mg per kg body weight) followed by treatment with protamine dosed as an i.v. bolus at 0.9 mg per rat. NS, not significant. (**c**) The anti-fXa activity in fondaparinux-anticoagulated rats (shown in **b**) at different time points for the Treat-II (square), Treat-III (diamond) and Treat-IV (inverted triangle) groups. Anti-fXa activity was normalized to the activity of the Treat-II group at the 5-min time point. ***$P \leq 0.0001$ comparing the Treat-III group to the Treat-II group. All *P* values were calculated by unpaired two-tailed *t* test.

activities of enoxaparin and fondaparinux. The anti-fXa activities of therapeutic amounts of enoxaparin and fondaparinux were dose-dependently reversed after incubation with r-Antidote in either human or rat plasma (**Supplementary Fig. 3a**,**b**). To further investigate the specific interaction between r-Antidote and ATIII, we measured the kinetics of inhibition in a reconstituted system with purified human proteins (fXa and ATIII) and fondaparinux. r-Antidote reduced the rate of fXa inhibition by the ATIII-fondaparinux complex in a dose-dependent manner (**Supplementary Fig. 4a**). Nonlinear fitting of the experimental data with a single exponential decay function yielded an apparent association rate constant (k_{obs}) for the reaction. In the absence of fondaparinux, r-Antidote had a minimal effect on the inhibition of fXa by ATIII (data not shown). r-Antidote had an enhanced binding affinity to the ATIII-fondaparinux complex as compared to ATIII alone (**Supplementary Fig 4b**). The dissociation constant for the r-Antidote–ATIII-fondaparinux complex was 53 nM. A similar high-affinity interaction between an active site–modified human fXa mutant and ATIII-pentasaccharide complex has been previously reported[17].

We next tested the ability of r-Antidote to restore hemostasis in a rat tail transection model after treatment with enoxaparin or fondaparinux. Unlike rivaroxaban, administration of enoxaparin or fondaparinux without concomitant administration of an antiplatelet agent was sufficient to establish a reproducible pattern of bleeding in the rats. We evaluated two different doses of r-Antidote for reversal of blood loss after the same enoxaparin dose (**Fig. 5a**). r-Antidote treatment at 4 mg per rat bolus plus 4 mg h^{-1} infusion completely corrected the increase in blood loss to baseline, whereas a lower dose of r-Antidote (2 mg per rat bolus plus 2 mg h^{-1} infusion) resulted in a partial (42%) correction in blood loss. In parallel to the reduction in blood loss, anti-fXa activity was reduced in a dose-dependent manner (data not shown).

As LMWH, such as enoxaparin, have both anti-fXa and anti-thrombin inhibitory activities, we investigated fondaparinux, a fXa-specific indirect inhibitor in the rat tail transection model. Supratherapeutic doses of fondaparinux (25 mg per kg body weight) were required to produce a statistically significant increase in blood loss (**Fig. 5b**). Administration of r-Antidote produced a complete cessation of bleeding. In parallel, r-Antidote also substantially reduced the anti-fXa activity of fondaparinux (**Fig. 5c**). Protamine is not expected to reverse the activity of fondaparinux. Accordingly, the tested dose of protamine (0.9 mg per rat) had no statistically significant effect on fondaparinux-induced anticoagulation ($P = 0.39$) (**Fig. 5b**).

DISCUSSION

As part of the prothrombinase complex, fXa is the key enzyme responsible for thrombin generation in the vasculature. Although fXa in solution has low catalytic activity toward cleaving its macromolecular substrate prothrombin, this procoagulant activity is increased 300,000-fold when it is assembled with fVa in the presence of Ca^{2+} into the prothrombinase complex[18]. As thrombin is the major enzyme responsible for both clot formation and platelet aggregation, reduction in thrombin generation and activity leads to therapeutic benefit in thrombotic diseases, and fXa inhibitors have been approved as a new generation of anticoagulants[1,2].

The structural modifications that we made to fX to generate the r-Antidote molecule were designed to retain the active site–binding pocket to allow for the binding of small-molecule fXa inhibitors. To eliminate the possibility of thrombin generation caused by the proteolytic cleavage of prothrombin, we disrupted the catalytic triad by mutation of the serine residue. Because active site–mutated full-length fXa has the potential to bind fVa and act as a competitive inhibitor of the prothrombinase complex, we also deleted the membrane-binding GLA domain. The recombinant protein was expressed in its functional form and could thus be purified from harvested cell culture fluid without additional activation steps by fVIIa or fIXa.

We demonstrated both *in vitro* and *in vivo* that r-Antidote does not interfere with normal fXa function in hemostasis and does not have anticoagulant activity, as demonstrated by its lack of interference in tissue factor–initiated thrombin generation assays and in traditional clotting assays. However, on the basis of kinetic measurements, r-Antidote retains a binding site that is capable of binding direct fXa inhibitors with affinities comparable to those of native fXa. r-Antidote also binds to pentasaccharide-activated ATIII, as shown in assays using purified proteins. It should be noted that r-Antidote has potential interactions with other proteins that may not have been uncovered in the *in vitro* experimental systems or the animal models studied here. On the basis of reported interactions of native fX and Xa, r-Antidote might interact with circulating ATIII (even in the absence of heparin-like moieties), tissue factor pathway inhibitor, factor V or protein S or protein Z inhibitor. Although we did not detect effects of r-Antidote on signaling by protease-activated receptor 2 in cell lines expressing this receptor (S. Delaney, Portola Pharmaceuticals, personal communication), the possibility exists that the *in vitro* system used is not sensitive enough to detect changes that may be important in primary cells.

To bypass the need for its activation through intrinsic or extrinsic pathways of coagulation, we expressed r-Antidote in its mature, functional form in CHO cells. However, the use of a recombinant human protein variant, albeit with a conserved N-terminal amino acid sequence of the native protein, has the potential for immunogenicity. Studies in which r-Antidote is administered repeatedly to patients with long-term follow up will be needed to address concerns regarding the generation of r-Antidote–specific antibodies or crossreactive antibodies to native fX or fXa.

The anticoagulant activity of LMWH and fondaparinux is mediated by ATIII, which is an irreversible inhibitor of serine proteases of the coagulation cascade. Under physiologic conditions, inhibition of fXa by ATIII proceeds at a slow rate. However, as our results and those in the literature show, the rate of inhibition is greatly enhanced in the presence of heparin-like polysaccharides[19,20]. Although protamine is used for the neutralization of unfractionated heparin, it only partially reverses the anticoagulant activity of LMWH and has no effect on fondaparinux[5,21–24]. Additionally, concerns about the potential side effects of protamine, such as allergic reactions, have limited its use in the clinical setting. Synthetic small-molecule salicylamide derivatives have been proposed for the neutralization of heparin and LMWH[25]. Modified ATIII mutants have also been used as reversal agents for ATIII-dependent anticoagulants[26]. None of these agents is currently indicated for use in patients.

The mechanism of action of r-Antidote is different from the approaches discussed above. Because r-Antidote still has the fXa active site, albeit without the catalytic serine residue, the protein can compete with fXa to bind to ATIII in the ATIII-heparin complex. Administration of r-Antidote reduces the effective concentration of the ATIII-heparin complex, reversing the anticoagulant effect of ATIII-dependent fXa inhibitors. An advantage of targeting the complex over either ATIII or the anticoagulant saccharide is that the complex is the rate-limiting component in the inhibition pathway,

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implying that lower circulating concentrations of r-Antidote would be needed to sustain reversal of anticoagulation.

Recent trials in large numbers of patients using direct fXa inhibitors for the prevention of deep vein thrombosis and atrial fibrillation have demonstrated efficacy in these clinical settings but have also shown that bleeding is the major safety concern with these promising new agents[27]. A recent report of the reversal of rivaroxaban anticoagulation in healthy volunteers using prothrombin-complex concentrate used surrogate markers to monitor the reversal of anticoagulation and did not demonstrate a reduction in bleeding[28].

We presume that after dosing into an animal previously treated with a direct fXa inhibitor, r-Antidote binds immediately to the inhibitor and thereby reduces the non–protein bound free fraction of the inhibitor. Thus, the anticoagulant effect caused by a direct fXa inhibitor is neutralized by administration of r-Antidote. We demonstrated the rapid onset of action for r-Antidote to bind and neutralize this class of inhibitors in animals treated with rivaroxaban, betrixaban and apixaban. The effect of r-Antidote in reducing the free plasma concentrations of direct fXa inhibitors is analogous to that of DigiFab, which reduces the free concentration of digoxin and reverses digoxin toxicity[29]. However, it is difficult to extrapolate animal model data to estimate what dose of r-Antidote would be needed in humans. By necessity, the animal models are stringent and use very high doses of fXa inhibitors to produce reproducible blood loss. Also, the extent of reversal of anticoagulation in patients that is necessary to restore hemostasis will be dependent on circulating concentrations of fXa inhibitor, as well as other factors (for example, renal clearance and concomitant medications).

We have established the ability of r-Antidote to reverse the anticoagulant activity of fXa inhibitors and restore hemostasis in anticoagulated animals. The capacity of r-Antidote to reverse both direct and indirect fXa inhibitors demonstrates its potential as an effective universal antidote for current and emerging anticoagulants for patients suffering major bleeding or requiring elective surgery.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

AUTHOR CONTRIBUTIONS

G. Lu, S.J.H. and U.S. conceived of the study, designed experiments and wrote the manuscript. G. Lu, F.R.D., M.J.K., K.A., G. Lee, P.L., A.H. and M.I. designed and conducted experiments. P.B.C. discussed and interpreted data with G. Lu, M.J.K. and P.L. D.R.P. provided expertise, designed experiments and interpreted data.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Design, expression and purification of r-Antidote. Full-length human fX complementary DNA (cDNA) was obtained by PCR using Human Fetal Liver Quick-Clone cDNA as a template (BD/Clontech). Modifications to native fX to generate r-Antidote are shown in **Figure 1a** and are as follows: (i) a fragment of 34 residues in the GLA domain containing the 11 GLA residues was deleted by ligating the native fX PrePro peptide and N-terminal residues (ANSFL) to the remaining fX light chain; (ii) the active-site serine (Ser419) in the heavy chain was changed to alanine (S419A); and (iii) the C terminus of the light chain was linked to the N terminus of the fXa heavy chain using the linker peptide RKRRKR to replace the activation peptide of native fX, enabling expression of the mature and functional form r-Antidote protein without further proteolytic activation steps[16].

The verified cDNA sequence was cloned into an expression vector containing the promoter for human elongation factor 1α (EF1-α) and dihydrofolate reductase (DHFR) as the selection marker. CHO-DUX B11 cells were transfected with the expression vector using a cationic liposome (Lipofectamine 2000, Invitrogen). Stable pools were established in tetrahydrofolate (HT)-deficient medium by stepwise methotrexate (MTX) selection at 50, 250 and 500 nM (refs. 30,31). Stable clones were selected by subcloning with 500 nM MTX. Protein expression levels were assayed by ELISA using paired antibodies to human fX and fXa (FX-EIA, Enzyme Research Laboratories, 1:100 dilution). The stable clones were adapted to the serum-free suspension medium CDM4CHO (HyClone) or ProCHO5 (Lonza). Protein expression was carried out in either WAVE bags (GE) with CDM4CHO or bioreactors with ProCHO5 medium.

Conditioned medium was clarified by prefiltration and concentrated by ultrafiltration using a 5-kDa molecular weight cutoff filter (Millipore). r-Antidote was purified by either a two-step procedure consisting of a Q-Sepharose FF ion exchange column (GE Healthcare) followed by a soybean trypsin inhibitor affinity column or conventional multiple-step ion exchange and hydrophobic interaction columns. Protein concentration was quantified by absorbance at 280 nm using the extinction coefficient E_{280} = 1.16 ml mg^{-1} cm^{-1}; this extinction coefficient was used for human plasma fX and fXa[32]. The purity of the protein was assessed by SDS-PAGE with Coomassie blue staining. The identity of the purified protein was further verified by N-terminal sequencing and immunoblotting. Light- and heavy-chain antibodies were from Enzyme Research Laboratories (Mab HFX-LC200 diluted 1:2,000 and Mab HFX-HC 210 diluted 1:5,000, respectively). Peroxidase-conjugated affiniPure rabbit antibody to mouse IgG was from Jackson Labs (catalog number 315-035-003).

fXa enzyme activity assay. To measure the inhibition of fXa activity by direct fXa inhibitors and the reversal of its inhibitory effect by r-Antidote, purified human plasma fXa (3 nM) (Haematologic Technologies), varying concentrations of inhibitor (0, 2.5, 5.0 and 7.5 nM) and r-Antidote were added to the assay buffer (20 mM Tris, 150 mM NaCl, 5 mM Ca^{2+} and 0.1% BSA, pH 7.4). After incubation at room temperature for 30 min, 100 μM Spectrozyme-fXa (American Diagnostica) was added to the mixture, and the initial rate of substrate cleavage was monitored continuously for 5 min at 405 nm in a 96-well plate reader (Molecular Devices). The initial velocity of product formation as a function of inhibitor and r-Antidote concentrations was analyzed by Dynafit (Biokin) to estimate the binding affinity of r-Antidote to each inhibitor. Betrixaban was synthesized at Portola Pharmaceuticals. Rivaroxaban was from J Star Research, and apixaban was from American Custom Chemicals Corporation.

Anti-fXa activity. Anti-fXa activity was measured by an adapted assay using reagents from a commercial Heparin kit (Coamatic, DiaPharma). Pooled platelet-poor plasma from healthy human donors or animals (mouse or rat) was prepared in 0.32% citrate (pH 7.0) for the generation of standard curves and sample dilutions.

Bovine fXa and S2732 fXa substrate from the Heparin kit were reconstituted according to the manufacturer's instructions. The reaction mixture contained 75 μl citrated plasma sample or standard and 50 μl S2732 fXa substrate. After preincubation at room temperature for 30 min, 25 μl bovine fXa was added to the mixture, and residual fXa activity was determined by measuring cleavage of the substrate at room temperature for 5 min. The reaction was quenched by adding 20% acetic acid (50 μl). The standard curves were constructed with known concentrations of each individual anticoagulant.

Interaction of r-Antidote with the ATIII-fondaparinux complex. Kinetic analysis of fXa inhibition by ATIII and the ATIII-fondaparinux complex was carried out under pseudo–first order reaction conditions in which the ATIII concentration exceeded the fXa concentration in the fXa plus ATIII reaction. In reactions containing fondaparinux, a limiting amount of fondaparinux was mixed with saturating ATIII concentrations. The concentrations used for both ATIII and fondaparinux were greater than the reported K_d for complex formation (K_d = 32 nM)[33]. The reaction mixtures contained human fXa (20 nM), human ATIII (Haematologic Technologies) (200 nM) and fondaparinux (GlaxoSmithKline) (100 nM), along with increasing concentrations of r-Antidote in assay buffer. After initiation of the reaction, 10 μl of reaction mixture was removed at various time points and assayed for residual fXa activity by measuring Spectrozyme-fXa cleavage. fXa activity was normalized by comparing it to the activity at t = 0 before the addition of either fondaparinux or ATIII (E/E_o).

Thrombin generation assay in human plasma. Pooled human plasma in 0.32% citrate (75 μl) was mixed with $CaCl_2$ and the Z-GGR-AMC (Bachem) fluorogenic thrombin substrate. Innovin (Dade Behring) was used as the source of tissue factor to initiate the generation of thrombin. The reaction mixture (final volume, 100 μl) contained 15 mM Ca^{2+}, 100 μM Z-GGR-AMC and 0.1 nM tissue factor. Thrombin formation was monitored continuously at 37 °C in a 96-well fluorescence plate reader (Molecular Devices) measuring the RFU. r-Antidote and EGR-Xa, an active site–inhibited human plasma-derived fXa (Haematologic Technologies), when present, were preincubated with plasma for 30 min at room temperature before the initiation of thrombin generation. Tris-buffered saline (20 mM Tris and 150 mM NaCl, pH 7.4) was used to adjust the final volume of the reaction mixture.

Clotting assays. For human *in vitro* and rat *ex vivo* experiments, prothrombin time was measured using an MLA Electra 800 automatic coagulation timer. Innovin was automatically dispensed to plasma samples (100 μl) according to the manufacturer's instructions. fXa inhibitor and r-Antidote, when present, were preincubated with human plasma at room temperature for 30 min before initiation of the clotting measurements. For rabbit experiments, prothrombin time and aPTT were measured using a Beckman Coulter ACL Elite instrument with HemosIL reagents (Instrumentation Laboratories). Whole-blood INR values for the animal samples (described below) were measured by Hemchron Jr. Signature Cartridges (International Technidyne Corporation) according to the manufacturer's instructions. Baseline values in the rat blood corresponded to an INR of ~2, rather than 1, as the cartridges used for measurements are calibrated for human use.

Reversal of the pharmacodynamic effect of fXa inhibitors in anesthetized rats. Rats (male, Sprague Dawley, Charles River) were anesthetized with intraperitoneal administration of a ketamine cocktail and a jugular and femoral vein catheterized for fXa inhibitor and r-Antidote administration and serial blood sampling. Blood sampling catheter patency was maintained by slow infusion of normal saline. Serial blood samples were obtained during the experimental time course. Total fXa inhibitor concentration was measured by HPLC with tandem mass spectrometry. The free fraction of fXa inhibitor not bound to plasma proteins was determined by ultrafiltration using a Microcon device followed by HPLC tandem mass spectrometry quantification. r-Antidote concentration was measured by ELISA as described above.

Reduction of blood loss in the rabbit liver laceration model. We used a modified rabbit (male, New Zealand white, Charles River) liver laceration model[34] to demonstrate the effects of r-Antidote to reverse rivaroxaban-induced anticoagulation. Rivaroxaban (1 mg per kg body weight) or vehicle was dosed to anesthetized rabbits by i.v. bolus over 2 min through a marginal ear vein catheter. After 30 min, r-Antidote or vehicle was administered as a bolus i.v. injection over 5 min through a contralateral ear vein catheter, followed by laceration of two liver lobes with a scalpel blade (five times in each lobe, 1-cm long and 3-mm deep incisions), and lost blood was collected on preweighed gauze over 15 min.

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Serial blood samples were collected at 0, 30, 35, and 50 min after administration of rivaroxaban. Measurements included reduction in blood loss, decrease in unbound rivaroxaban in the plasma using the equilibrium dialysis method, anti-fXa activity, prothrombin time and aPTT.

Restoration of hemostasis in the rat tail transection blood loss model. A rat tail transection model was used to study the effect of r-Antidote on blood loss induced by anticoagulation with enoxaparin and fondaparinux[35]. Rats were anesthetized and catheterized for serial blood sampling and administration of anticoagulant and r-Antidote. Rats were prophylactically administered r-Antidote before the initiation of blood loss, and the tail was subsequently transected with a scalpel blade and immersed in normal saline at 37 °C, followed by a 15-min r-Antidote infusion and continuous collection of blood. The collected blood samples were processed by freezing at −80 °C to lyse erythrocytes. Hemoglobin concentration and corresponding blood volume were quantified spectrophotometrically (absorbance at 490 nm) from a standard curve constructed with known volumes of blood. Blood samples were collected at 0, 5, 15, 30 min for anti-fXa activity and r-Antidote concentration measurements.

Mouse tail transection blood loss model. Mice (male, C57BL/6, Charles River) were first treated with aspirin at ~100 mg per kg body weight d^{-1} for 5 d in drinking water. A single, oral administration of rivaroxaban (50 mg per kg body weight) or vehicle (0.5% methylcellulose) increased blood loss 3.4-fold compared to the ASA treatment group. Mice were anesthetized, and r-Antidote (0.96 mg per mouse, i.v. bolus) or vehicle (formulation buffer, 200 µl per mouse) was administered 2 h after the oral dose of rivaroxaban and prophylactically before the initiation of blood loss (15 min by tail transection, similarly to the rat model). Cardiac blood samples at the end of the experiment were used for measurements of rivaroxaban, r-Antidote plasma concentrations, whole-blood INR values and anti-fXa activity.

All procedures were approved by Portola Pharmaceuticals' Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Blood was collected from the antecubital vein of healthy volunteers who gave written informed consent to the blood donation protocol approved by the Human Subjects Committee of Portola Pharmaceuticals Inc.

Statistical analyses. Data are reported as the mean ± s.d. Graphpad Prism 4.03 was used to calculate *P* values (two tailed unpaired *t* test).

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