A Human Antibody That Inhibits Factor IX/IXa Function Potently Inhibits Arterial Thrombosis Without Increasing Bleeding

Canio J. Refino, Surinder Jeet, Leo DeGuzman, Stuart Bunting, Daniel Kirchhofer

Abstract—10C12, a human antibody F(ab')2, which specifically binds to the γ-carboxyglutamic acid domain of factor IX/factor IXa (F.IX/IXa), interferes with all known coagulation processes in which F.IX/IXa is involved. In a rabbit model of carotid artery injury, intravenous administration of 10C12 or heparin decreased thrombosis dose dependently. The dose that resulted in a 90% reduction of thrombus mass (ED90) was a 30-μg/kg bolus of 10C12 or a 100-U/kg bolus plus 1.0 U·kg⁻¹·min⁻¹ infusion of heparin. Heparin, at and below the ED90, significantly prolonged coagulation times and cuticle bleeding times. In contrast, 10C12 had no effect on coagulation or bleeding times at doses up to 4 times the ED90. To further evaluate the effect of 10C12 on bleeding, it was compared with heparin in a novel model of blood loss. At the ED90 of heparin, blood loss induced by a standardized injury to the vasculature of the rabbit tibia increased to more than 2 times that of saline controls. In contrast, the dose of 10C12 required to produce a similar increase in blood loss was more than 30 times the ED90. The antithrombotic potency and relative safety of this fully human antibody suggests that it may have therapeutic value for treatment of thrombotic disorders. (Arterioscler-Thromb-Vasc-Biol. 2002;22:517-522.)

Key Words: thrombosis ■ factor IX ■ anticoagulants ■ antibody

The importance of factor IX/factor IXa (F.IX/IXa) in normal hemostasis is exemplified by the inherited hemophilia B trait, which is characterized by impaired blood clotting and spontaneous bleeding. Therefore, one may infer that an approach to treat thrombotic disorders by interfering with F.IXa function would have an unacceptable bleeding liability. Yet, Benedict et al were able to demonstrate that short-term inhibition of F.IXa function by active site–blocked F.IX (F.IXai) can be as safe as effective in preventing thrombus formation in a canine model of electric current-induced thrombosis. Subsequent in vivo studies with the prototypic inhibitor F.IXai further corroborated this paradigm, indicating that the specific targeting of F.IXa activity could represent a promising therapeutic strategy.

Activated platelets are probably the main assembly sites for the intrinsic Xase complex (F.VIIIa/F.IXa) as well as the prothrombinase complex (F.Va/F.Xa) in arterial thrombosis. F.IXa was shown to interact with the platelet membrane with high affinity and in a very specific manner involving the γ-carboxyglutamic acid (Gla) domain. Although the precise molecular structure of the membrane-binding site is unclear, it seems likely that one component is the phospholipid bilayer itself. Similar to the other Gla-containing coagulation factors, the phospholipid binding is mediated by a surface-exposed patch of three hydrophobic residues. Consistent with this view, these three hydrophobic amino acids (Leu6, Phe9, and Val10) are located within the 11-residue N-terminal portion of the F.IX-Gla domain identified as an important structural determinant for cell binding.

Based on the structural requirements for intrinsic Xase assembly, new approaches to specifically interfere with F.IXa activity are conceivable. To this end, we used a F.IX-Gla domain–directed phage display strategy to generate the antibody 10C12, a fully humanized F(ab'); that specifically binds to the Gla domain of F.IX/IXa. In addition to inhibiting platelet-dependent intrinsic Xase activity, 10C12 was found to block F.IX activation by F.XIa and by the tissue factor:F.VIIa (TF:F.VIIa) complex. In vivo, 10C12 exerted antithrombotic activities in guinea pig and rat models, with no or minimal effects on normal hemostasis. Similar findings were reported with another anti-F.IXa antibody, BC2, in rat models of arterial and venous thrombosis.

Although 10C12 was extremely potent in a guinea pig arterial thrombosis model, it was considerably less so in a rat, ferric chloride (FeCl3)-induced model of arterial thrombosis. Because of this discrepancy in potency, it remained unclear whether this antibody was an unusually potent antithrombotic with a very broad safety margin or not. The wide cross-reactivity of 10C12 with F.IX/IXa of many different species including rabbit allowed us to further investigate this question in a well characterized rabbit vascular injury model. Moreover, the relationship of antithrombotic potency and effects on normal hemostasis was examined in a new surgical blood loss model in rabbits. The results demonstrate that 10C12 is extremely potent in preventing thrombosis yet...
Materials

10C12, a fully humanized, Fr(ab’), monoclonal antibody, was expressed in *Escherichia coli* and purified as previously described.

The purified final pool contained 1.5 mg/mL of protein and ≤12.5 EU/mg protein of endotoxin. Unfractionated sodium heparin for injection (1000 U/mL) was purchased from Elkins-Sinn. Both reagents were diluted in an appropriate volume of sterile saline for injection (Baxter Health Care Corp) before administration to rabbits.

### Thrombosis Model

The model used in this study is a variation of a vascular damage model previously reported. That model was modified by the addition of a Lexan flow restrictor (1.2-mm internal diameter, Tramel Enterprises) placed proximal to the segment of artery that is damaged by an inflated balloon catheter (3F). Addition of the restrictor provided a defined diameter, which was used to calculate the shear rate adjacent to the damaged arterial segment. Shear rate (γ) was determined by using the equation γ = 4Q/r³, in which Q = blood flow in cm³/s and r = the radius of the lumen in cm. The protocol used in the current study was as follows. The left common carotid of an isoflurane-anesthetized rabbit was surgically isolated, and an ultrasonic flow probe (Transonics Systems) was placed proximal to the aorta. Treatment was initiated before the placement of the flow restrictor and 30 minutes before insertion of the balloon catheter. The deflated catheter was inserted into the lumen of the carotid via a small side branch, which was distal to the flow restrictor. The tip of the catheter was guided to the restricted region and the balloon inflated so as to gently dilate the vessel. The catheter was then forcefully pulled back 2 cm and the balloon deflated. This procedure was repeated a total of six times in a 2-minute period.

Flow measurements were recorded before and for 60 minutes after the balloon procedure. The incidence of occlusion (number of rabbits in a treatment group that had one or more periods of no arterial flow over the number of rabbits per treatment), time to occlusion (the time from completion of the balloon procedure until occlusion), and the total duration of occlusion was determined from these recordings. At 60 minutes, the artery was cross clamped, and any thrombus present in the damaged section was removed, blotted on filter paper, and weighed. A representative flow recording from a saline-treated rabbit is shown in Figure 1. Antithrombotic treatments were administered via the marginal ear vein as a single bolus (10C12) or as a bolus followed by a continuous infusion (saline and heparin). The treatment regimens evaluated are summarized in the Table. Blood samples were collected from a femoral artery catheter before treatment, and at 1, 30, and 60 minutes after the balloon procedure. Blood samples were collected and processed as previously described.

Prothrombin times (PT), activated partial thromboplastin times (APTT), and activated whole blood clotting times (ACT) were determined as described for rat plasma samples. Although there were differences in coagulation times between groups, within-group PT, APTT, and ACT remained essentially unchanged during the 60 minutes after the balloon injury. Therefore, changes from the pretreatment determination were expressed as a ratio of the 1-minute post-balloon procedure value over the pretreatment value. Cuticle transections were performed as described for rats and guinea pigs, and cuticle bleeding times were determined before treatment and 5 minutes after the balloon procedure. A post-occlusion pretreatment ratio was calculated from these two determinations.

### Blood Loss Model

#### Model Development and Validation

Rabbits were randomized to treatment with saline, 10C12, or heparin (N = 5 per group). Heparin and 10C12 doses that were comparable to

![Figure 1. Recording of carotid artery blood flow in a control (saline-treated) rabbit before and after vascular damage produced by multiple passes of an inflated balloon catheter. * represents times when blood samples were collected for coagulation assays. † represents times when cuticle bleeding time determinations were made.](image-url)

without impairing hemostasis, suggesting that this antibody may be an effective and safe anticoagulant for the treatment of acute thrombotic events.

### Methods

#### Materials

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the 90% effective dose (ED90) that was determined in the thrombosis model (ie, a 100-U/kg bolus plus a 1.0-U·kg⁻¹·min⁻¹ infusion of heparin or a single 30-µg/kg bolus of 10C12) were evaluated. However, unlike in the thrombosis model, the heparin and saline infusions were terminated after 30 minutes. Before treatment, the medial surface of the left tibia of an anesthetized rabbit was exposed. A single-speed rotary tool (28,000 rpm) fitted with a 0.0995-inch diameter drill bit (#39 bit, Small Parts Inc) was used to create a 2-mm deep puncture wound into the medullary canal at a point 3 cm distal to the insertion of the patellar ligament. The wound was cleaned of any loose tissue and preweighed gauze pads were used to collect the escaping blood. The pads were then reweighed to determine the volume of blood collected. Blood loss was determined for three consecutive 30-minute periods: (1) 0 to 30 minutes, before drug administration, (2) 30 to 60 minutes, immediately after drug administration, and (3) 60 to 90 minutes, after termination of the heparin and saline infusions. Samples for coagulation assays and a hematocrit were collected at 0, 15, 45, and 75 minutes.

**Dose Response of 10C12 and Heparin**

Once the model was validated with the above treatments, rabbits were randomly assigned to treatment with additional doses of 10C12 and heparin (N = 5 ea.). For 10C12, we tested doses as high as 1000 µg/kg (33.3 times the ED₉₀), a dose at which we had previously seen increased blood loss in transected rat tails. Because we had already demonstrated increased blood loss for the ED₉₀ of heparin in this model, we added the two lower dose regimens used in the efficacy study. In these experiments, blood loss was determined for 30 minutes before administration of the treatment and then for the following 30 minutes. To potentially increase the sensitivity of the assay to detect differences between treatment regimens, blood loss was expressed as the ratio of the post-treatment value over the pretreatment value. In the saline controls, the coefficient of variation decreased from 44% to 20% with this method of analysis.

**Statistical Analysis and Animal Use and Care**

If not otherwise specified, the data in the text, table and figures are expressed as mean±SEM. Because the data for thrombotic endpoints (time to occlusion, duration of occlusion, and thrombus mass) seemed to have a non-normal distribution, differences between treatments and controls were determined by using a Mann-Whitney test after determination of a significant difference between groups by using a Kruskal-Wallis test. A paired t test was used to determine the significance of the drop in hematocrit observed in the blood loss validation study. For all other endpoints, an unpaired t test (2-tail) after determination of a significant difference between groups by an analysis of variance was used. In all tests, \( P < 0.05 \) was considered significant. Protocols for the animal experiments were approved by the Animal Care and Use Committee of Genentech Inc. and were performed according to the guidelines of the USDA Animal Welfare Act. Male, New Zealand White rabbits, weighing 3.2 to 4.0 kg were used in both the thrombosis and bleeding experiments.

**Results**

**Characterization of Rabbit Thrombosis Model**

Placement of a Lexan flow restrictor on the carotid arteries of anesthetized rabbits resulted in a modest flow reduction (Figure 1) and an increase in shear stress. We calculated the median shear rate in these experiments to be 2710 s⁻¹ (range, 2259 to 3614 s⁻¹). Subsequent vascular damage of the artery produced by multiple passes of the balloon catheter resulted in rapid and sustained occlusive thrombosis as illustrated in Figure 1. In the saline controls, the incidence of occlusive thrombosis was 100% (17 of 17), and the mean time to occlusion was 3.4±0.8 minutes. Because all of the arteries remained occluded for the remainder of the 60-minute observation period, the duration of the occlusion was 56.6±0.8 minutes. The wet weight of the thrombus collected after 60 minutes was 29.6±3.7 mg. Saline treatment did not affect coagulation times (APTT, ACT, and PT) or cuticle bleeding time when compared with pretreatment values (Table).

**Effect of 10C12 or Heparin on Thrombosis, Coagulation, and Cuticle Bleeding Times**

Intravenous administration of 10C12 or heparin before vascular damage increased the time to occlusion and reduced the incidence and duration of occlusion in a dose-dependent fashion (Figure 2A). In the arteries that occluded, absence of blood flow persisted until the end of the experiment. Therefore, the duration of occlusion (not shown) for any group was the difference between the maximal time (60 minutes) and time to occlusion. Before statistical analysis, the thrombus mass in mg of each animal was converted to a percent of control as follows: (thrombus mass/mean of the control thrombus mass)×100. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) versus saline control.
the resulting thrombus mass significantly smaller than that of the saline controls (Figure 2B). At higher doses, 10C12 and heparin had more pronounced effects on incidence of, time to, and duration of occlusion. These more pronounced effects appeared to correlate with increasingly larger impacts on thrombus mass (Figure 2). This suggested that, in this model, thrombus mass reflects the inhibitors’ effects on multiple endpoints, such as incidence of, time to, and duration of occlusion. In addition, thrombus mass is likely to be more clinically relevant than any other single thrombotic endpoint determined in this model. Therefore, we chose to use changes in thrombus mass as the primary indicator of antithrombotic efficacy.

Similar to the effects on the other thrombotic endpoints, 10C12 and heparin decreased thrombus mass in a dose-dependent fashion (Figure 2B). 10C12, at a dose of 125 \( \mu \text{g/kg} \), completely prevented thrombus formation. At less than a quarter of this dose (30 \( \mu \text{g/kg} \)), 10C12 still reduced thrombus mass by approximately 90% (ED\(_{90}\)). Notably, neither this nor the higher dose had an effect on coagulation or cuticle bleeding (Table). In sharp contrast to 10C12, neither this nor the higher dose had an effect on coagulation/H\(_{9262}\) than a quarter of this dose (30 \( \mu \text{g/kg} \), 10C12). This diminution of blood loss probably reflects heparin’s rapid clearance from circulation because the APTT decreased substantially over the same time period (data not shown). Moreover, differences in blood loss between treatment groups were also evident in the hematocrit. The mean hematocrit for all rabbits before induction of bleeding was 36.6±0.6%. By the time the final blood sample was taken (75 minutes), hematocrit had decreased by 2.3±1.2%, 1.7±1.1%, and 5.1±1.2% in the saline, 10C12, and heparin treatment groups, respectively. In a paired \( t \) test (75-minute values versus presurgical values), the decreases in the saline and heparin treatment groups were significant (\( P=0.044 \) and \( P=0.026 \), respectively), whereas that of the 10C12 treated group was not (\( P=0.089 \)).

Effect of 10C12 and Heparin on Blood Loss in Rabbits

Model Development and Validation

The effects of 10C12, heparin, or saline on blood loss were determined in a novel model of surgically induced bleeding in rabbits. We developed this model because we believed it was important to evaluate the bleeding risk of 10C12 in a model that represented a more severe challenge to hemostasis than that of cuticle transaction. In preliminary experiments, doses of 10C12 (30–\( \mu \)g/kg bolus) and heparin (100–U/kg bolus plus 1–U·kg\(^{-1} \)·min\(^{-1} \) constant-rate infusion) comparable to those that inhibited thrombus mass by 90% (ED\(_{90}\)) in the thrombosis model were evaluated in the blood loss model. The dosing regimen in these studies differed from the thrombosis model in that the duration of the heparin and saline infusions was shortened to 30 minutes. Blood loss was measured for three consecutive 30-minute periods as described in Methods. The effects of these dose regimens on blood loss are shown in Figure 3. There was no significant difference in blood loss between the treatment groups before initiating treatment (0 to 30 minutes). Furthermore, there was no change in blood loss in the saline or 10C12 treatment groups after initiation of treatment (30 to 60 minutes). In contrast, heparin administration significantly increased blood loss, essentially doubling the pretreatment rate (Figure 3). Although blood loss tended to remain elevated in the 30-minute period after cessation of the heparin infusion, this rate was no longer statistically different from the saline controls. This diminution of blood loss probably reflects heparin’s rapid clearance from circulation.

Figure 3. Blood loss from a standardized puncture wound of rabbit tibias for three consecutive 30-minute periods. The treatment groups were saline (open bar), 10C12 administered as a single bolus of 30 \( \mu \text{g/kg} \) (hatched bar), and heparin administered as a bolus of 100 U/kg followed by a continuous infusion of 1 U·kg\(^{-1} \)·min\(^{-1} \) for 30 minutes (closed bar). N=5 per treatment. Times were 0 to 30 minutes (before treatment), 30 to 60 minutes (post-treatment but during the heparin infusion), 60 to 90 minutes (post-treatment but after termination of heparin infusion). *\( P<0.05 \) versus saline control.

Dose Response of 10C12 and Heparin

To more completely evaluate the bleeding risk of 10C12 or heparin, additional doses of each were tested in the model. The rationale for dose selection is discussed in Methods. In these experiments, blood loss was determined for 30 minutes before administration of the treatment and for the 30 minutes after drug administration. As in the validation study, 10C12 was administered as a bolus, whereas heparin was administered as a bolus plus a constant-rate infusion for 30 minutes. The data, summarized in Figure 4, show that similar to its effect on the cuticle bleeding time in the thrombosis model, heparin significantly increased bleeding at a dose that was half of the ED\(_{90}\); at this dose (50 U/kg + 0.5 U·kg\(^{-1} \)·min\(^{-1} \)), blood loss increased to 1.63±0.12 times the pretreatment value (\( P=0.01 \) versus saline treatment). At the ED\(_{90}\), heparin increased blood loss 2.03±0.20-fold (\( P=0.001 \)). 10C12 also increased blood loss in a dose-dependent fashion, but this effect was not significant until the dose was increased to >4 times the ED\(_{90}\). At this dose, 10C12 increased blood loss to 1.63±0.12 times the pretreatment level. However, 10C12 did not increase blood loss to the same extent as the ED\(_{90}\) of heparin until it was dosed at 1000 \( \mu \text{g/kg} \) or 33.3 times the ED\(_{90}\).

The doses of heparin or 10C12 used in this model that were comparable to those used in the thrombosis model (ie, same bolus or bolus plus infusion rate) produced ex vivo coagula-
remarkably, a single bolus dose of only 30 μg/kg prolonged the APTT (1.23±0.05 and 1.27±0.06-fold, respectively, \( P \leq 0.01 \) for both). However, they had no significant effect on the ACT (0.93±0.05 and 1.01±0.03-fold, respectively, \( P > 0.05 \) for both). In contrast, the lowest dose of heparin, which was ineffective in the thrombus model, had a pronounced effect on the APTT (2.0±0.16-fold). Consistent with its specificity for F.IX/IXa, 10C12 did not affect the ex vivo PT (data not shown).

**Discussion**

In vivo studies with the prototypic inhibitor F.IXai suggested that specific interference with the intrinsic Xase complex is an effective antithrombotic strategy.\(^2\)\-_\(^4\)\(^,\)\(^1\)\(^9\) Here, we provide further evidence that a fully human antibody, 10C12, directed against the Gla domain of F.IX/IXa potently prevents thrombosis in a vascular injury model without impairing normal hemostasis.

The 10C12 antibody was generated by using a phage display strategy and was characterized recently.\(^1\)\(^4\)\(^,\)\(^1\)\(^5\) In accord with the conserved F.IX-Gla domain sequences, 10C12 inhibited F.IX/IXa function in several species, including rabbit.\(^1\)\(^4\) Even though 10C12 was found to specifically prolong APTT in rabbit plasma in vitro, the doses that were effective in vivo were so low that neither APTT nor the ACT was prolonged. Remarkably, a single bolus dose of only 30 μg/kg reduced thrombus mass by 90% (ED\(_{90}\)). The dose-dependent effects on thrombus mass were mirrored in additional endpoints including incidence of occlusion, time to occlusion, and duration of occlusion. The comparable effective dose (ED\(_{90}\)) of heparin on thrombus mass was 100-U/kg bolus (plus 1-U·kg⁻¹·min⁻¹ infusion), but this dose produced only about 50% efficacy for the other endpoints measured. Most importantly, at and below this dose, heparin significantly affected normal hemostasis as measured by cuticle bleeding time, whereas 10C12 did not, even at the highest dose of 125 μg/kg.

The observed potency of 10C12 in this model (ED\(_{90}\), 30 μg/kg=0.3 nmol/kg) compares well with an earlier study in the guinea pig, in which an ED\(_{90}\) of 10 μg/kg (0.1 nmol/kg) for inhibition of arterial thrombosis was determined.\(^5\) A comparison with F.IXai in this guinea pig model showed that on a molar basis 10C12 was about 13 times more potent than F.IXai (ED\(_{90}\), 60 μg/kg≈1.3 nmol/kg).\(^4\)\(^,\)\(^1\)\(^5\) Therefore, these results suggested that the unusual potency of 10C12 might be linked to its mechanism of action, which differs from F.IXai in several aspects. In contrast to F.IXai, the 10C12 F(ab’)\(_2\) is a bivalent inhibitor and may show significantly increased affinity when F.IX/IXa are localized to the two-dimensional platelet membrane surface (avidity effects). Because the thrombi formed in the rabbit as well as the guinea pig thrombosis model are platelet-rich\(^4\)\-_\(^6\)\(^,\)\(^1\)\(^8\) andactivated platelets are the likely assembly sites for the intrinsic Xase complex,\(^5\) this potentially increased affinity may come into play. Additionally, 10C12 interferes with the activation of F.IX by both F.Xia and TF:VIIa.\(^1\)\(^5\) The latter mechanism could be particularly relevant, because experiments with specific inhibitors of the TF:VIIa complex suggested that, in the rabbit as well as the guinea pig thrombosis models, thrombus formation is triggered by exposure of TF.\(^4\)\-_\(^8\)

In contrast to the experiments in rabbits and guinea pigs, relatively high doses of 10C12 were required to produce comparable antithrombotic effects in a rat model of FeCl\(_3\) induced thrombosis.\(^1\)\(^5\) Similar observations were reported with another anti-F.IX/IXa antibody in a similar model.\(^1\)\(^7\)\-_\(^2\)\(^0\) These different dose requirements may be related to the model-specific characteristics of coagulation initiation and propagation. For example, heparin is also less potent in the rat model, because smaller decreases in thrombus mass were observed at even larger prolongations in the APTT than those attained in the rabbit model.\(^1\)\(^5\)\-_\(^1\)\(^7\)\-_\(^2\)\(^1\) Furthermore, preliminary studies show that neither anti-TF antibodies nor active site-blocked F.VIIa is inhibitory in the rat FeCl\(_3\) model (C.J. Refino, unpublished observations, 1999), suggesting that coagulation in this model is not TF-mediated. Because the potency of 10C12 observed in the rabbit and guinea pig models may have partially depended on its ability to block F.IX activation by TF:F.VIIa, then the likely absence of this pathway in the rat model may help to explain lower potency of 10C12 there.

Of greatest concern in using anticoagulants is their potential bleeding liability. This is even more pertinent for an antithrombotic approach that targets F.IX/IXa, because of the well known bleeding diathesis associated with inherited F.IX deficiency. In hemophilia B patients, there is generally a lack of excessive bleeding from minor cuts but prolonged hemorrhage can occur after trauma or surgical procedures.\(^1\) Although 10C12 had no effect on cuticle bleeding times in rabbits at doses up to 125 μg/kg or in guinea pigs or rats at doses up to 1000 μg/kg,\(^1\)\(^5\) increased blood loss, caused by rebleeding was observed in transected rat cuticles and tails.\(^1\)\(^5\) Although this rebleeding might have been a species-specific
phenomenon, the lack of prolongation of the cuticle bleeding time in multiple species after administration of doses as high as 1000 μg/kg might have resulted from a lack of sensitivity of this test to suppression of F.IXa generation or activity. This seems unlikely because prolonged cuticle bleeding times have been demonstrated in hemophilic dogs. Nonetheless, given the clinical profile of hemophilia, we believed it was important to evaluate the bleeding potential of 10C12 in a model that represented a more severe challenge to hemostasis than that of cuticle transection. To that end, we developed and characterized a model of blood loss after a standardized puncture into the marrow cavity of the rabbit tibia. Clearly, the challenge to hemostasis was severe, because blood loss was substantial enough to have an impact on the hematocrit. Furthermore, in control animals, blood loss was sustained and relatively stable for at least 90 minutes, thus facilitating evaluation of different antithrombotic regimens. In this new model, 10C12, at doses up to and including the ED90, had no significant effect on blood loss, whereas the equi-efficacious dose of heparin resulted in a 2-fold increase. Although 10C12 significantly increased blood loss at doses above the ED90, it did not produce blood loss comparable to the heparin ED90 until it was dosed at 1000 μg/kg or 33 times greater than its ED90. Interestingly, the APTT of the doses of 10C12 that produced bleeding were lower than any of the antithrombotically effective doses further indicates that this antibody has a much broader therapeutic window than heparin, which caused disturbances to normal hemostasis concomitant with its antithrombotic efficacy. Because 10C12 is a fully human F(ab')2, it may be of potential therapeutic value for ameliorating thrombotic disorders in humans.

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References

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