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The unique procoagulant adaptations of pseudonaja textilis venom factor V and factor X

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Chapter 6

Reversal Agents for the Direct Factor Xa Inhibitors: Biochemical Mechanisms of Current and Newly Emerging Therapies

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Abstract

The direct oral anticoagulants targeting coagulation factor Xa or thrombin are widely used as alternatives to vitamin K antagonists in the management of venous thromboembolism and nonvalvular atrial fibrillation. In case of bleeding or emergency surgery, reversal agents are helpful to counteract the anticoagulant therapy and restore hemostasis. While idarucizumab has been established as antidote for the direct thrombin inhibitor dabigatran, reversal strategies for the direct factor Xa inhibitors have been a focal point in clinical care over the past years. In the absence of specific reversal agents, the off-label use of (activated) prothrombin complex concentrate and recombinant factor VIIa has been suggested as effective treatment options during inhibitor-induced bleeding complications. Meanwhile, several specific reversal agents have been developed. In this review, an overview of the current state of non-specific and specific reversal agents for the direct factor Xa inhibitors is provided, focusing on the biochemistry and mechanism of action and the preclinical assessment of newly emerging therapies.

Introduction

The direct oral anticoagulants (DOAC) are a class of synthetic anticoagulant drugs that consist of a direct thrombin inhibitor (dabigatran) and several direct factor Xa (FXa) inhibitors (apixaban, rivaroxaban, edoxaban, and betrixaban). These drugs are characterized by a superior pharmacodynamic profile relative to the classic oral anticoagulant vitamin K antagonists, and their use is therefore considered as the preferred anticoagulant therapy[1]. The DOACs are prescribed for the prevention of venous thrombosis following surgery or during atrial fibrillation, and for the treatment of venous thromboembolism[2]. Unfortunately, as with all anticoagulant treatment options, bleeding complications associated with DOAC use remain the main concern in clinical practice. Therefore, rapid reversal of the anticoagulant DOAC activity may be required in case of life-threatening hemorrhage, major trauma, emergency surgery or DOAC overdose. DOAC reversal can be achieved by the use of non-specific or specific reversal agents. In the absence of specific reversal agents, non-specific plasma protein concentrates and recombinant factor VIIa have been suggested to overcome the anticoagulant effects of the DOACs. Specific reversal of the thrombin-targeting DOAC dabigatran is effectively achieved by administration of the US Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved monoclonal antibody fragment idarucizumab[3]. More recently, andexanet alfa, a specific antidote for the FXa-targeting DOACs was approved for the treatment of uncontrollable bleeds. In addition, several specific antidotes for the FXa-targeting DOACs are currently in preclinical development, which include the synthetic small molecule ciraparantag, a modified FXa-α2 macroglubulin complex and several prohemostatic factor X (FX)-based reversal agents. Since the direct FXa inhibitors apixaban and rivaroxaban are increasingly prescribed rather than the direct thrombin inhibitor dabigatran[4], the importance of an effective reversal agent for the direct FXa inhibitors is substantial. In this review, we will highlight the reversal of the FXa-targeting DOACs by specific and non-specific reversal agents, with a main focus on the biochemical mechanisms of the newly emerging reversal therapies that are in preclinical development.

Mechanistic Principles of the Direct Factor Xa Inhibitors

The FXa-targeting DOACs are small synthetic molecules with overall similar chemical structures that have identical mechanisms of action. Their anticoagulant effect stems from the fact that these inhibitors interact with the active site pocket of FXa, thereby effectively blocking the natural substrate prothrombin from accessing the active site **(Figure 1)** and preventing thrombin formation. The characteristic L-shape of these small molecule inhibitors mediates the high affinity interaction with the FXa active

site, resulting in a potent subnanomolar FXa inhibitory activity (K_i of 0.08-0.56 nM)[5-7]. Although the overall composition of the active site of serine proteases is generally conserved[8], the direct FXa inhibitors have an over 10,000-fold greater selectivity for FXa compared to other human serine proteases[9]. Binding to FXa is reversible and regulated via the S1 and S4 active site subpockets[5,6]. The *p*-methoxyphenyl P_1 moiety of apixaban and the chlorothiophene moiety of rivaroxaban are inserted deep into the S1 specificity pocket. Although the P_1 moiety of apixaban does not appear to directly interact with FXa, the choline substituent of rivaroxaban interacts with the aromatic ring of Tyr228 (chymotrypsinogen numbering) that is located at the bottom of the S1 subpocket and provides a high affinity interaction[5,6]. Apixaban interacts with the Gln192 backbone via its pyrazole N-2 and via a carboxyl interaction with Gly216[6]. Rivaroxaban forms two hydrogen bonds with Gly219 that serve an important role by directing its substituents into the S1 and S4 subpockets[5]. The S4 subsite of FXa is a narrow hydrophobic pocket defined by the aromatic rings of residues Tyr99, Phe174, and Trp215[10]. In this binding pocket, the P_4 ring of apixaban and the morpholinone ring of rivaroxaban are sandwiched between the residues Tyr99 and Phe174, thereby providing important binding stability and enabling high affinity binding[5,6]. While structural information on the interaction of the direct FXa inhibitors edoxaban or betrixaban with the FXa active site is lacking, these are considered to also engage the FXa S1 and S4 subsites[11].

The pharmacokinetic and pharmacodynamic profiles of the direct FXa inhibitors have several key advantages over that of the vitamin K antagonists. Their rapid onset makes them ideal for clinical use as no bridging therapies are needed and they sustain their anticoagulant effect up to 24 hours, with a half-life of 6-14 hours[12,13]. Unlike the vitamin K antagonists, the direct FXa inhibitors are characterized by linear and predictable pharmacodynamics, no known interactions with food components, and limited drug-drug interactions[14]. It is for these reasons that patients treated with a DOAC do not require routine monitoring.

Reversal of the Direct factor Xa Inhibitors

Bleeding complications remain a major concern of anticoagulant therapy. Compared to the conventional vitamin K antagonists, the direct FXa inhibitors are associated with comparable or slightly lower risks of major bleeding, stroke, and fatality. Still, major bleeding is observed in 1-5% of the patients receiving DOACs[15-17]. Since millions of people worldwide require anticoagulant treatment and this is projected to increase significantly in the coming decades, bleeding complications will remain a major healthcare burden[18,19]. In order to treat these DOAC-associated bleeding complications, the off-label use of several non-specific reversal agents used in hemophilia-associated and vitamin K antagonist-induced bleedings have been suggested as potential candidates for

reversal therapy, although supporting evidence is lacking or incomplete. In addition to these non-specific treatment options, the specific antidote for the direct FXa inhibitors andexanet alfa has recently been approved by the FDA and EMA and could significantly improve the management of major bleeding events[20,21]. Furthermore, various specific reversal agents with different modes of action are currently in preclinical development. In the following sections we will discuss the efficacy of the non-specific and specific reversal strategies and focus on their molecular details and mechanisms of action.

Non-Specific Reversal Strategies

Prothrombin Complex Concentrate

Prothrombin complex concentrates (PCC) are derived from human plasma and contain vitamin K-dependent coagulation factors which are partly purified via ion-exchange chromatography[22,23]. Strong ion exchangers produce 4-factor concentrates (4F-PCC), consisting of factor VII, factor IX, factor X, and prothrombin, while weak ion exchangers produce 3-factor concentrates (3F-PCC) that do not contain factor VII. Most PCC products have been supplemented with heparin to prevent factor activation[24] and may contain various concentrations of the vitamin K-dependent anticoagulants protein C and protein S. PCCs are used for the reversal of vitamin K antagonists by replenishing the lowered and defective circulating vitamin K-dependent factors if patients present with an urgent need to restore hemostasis[25]. In contrast, the direct FXa inhibitors do not affect the level or posttranslational modification of coagulation factors, thereby obviating the necessity for factor supplementation and replacement. PCC-mediated reversal of FXa inhibition has been studied in several animal models and in small-sized human studies. In rabbit models of apixaban or rivaroxaban-induced bleeding, 4F-PCC partially improved laboratory parameters but was not able to reduce blood loss[26,27]. In contrast, 4F-PCC significantly shortened the bleeding time of rivaroxaban-induced bleeding in a rat model and could reduce the prothrombin time (PT) in rivaroxaban-treated baboon plasma[28]. 4F-PCC was also able to dose-dependently reverse hematoma expansion in a murine intracerebral hemorrhage model following pretreatment with rivaroxaban[29]. In humans, the efficacy of PCC has mostly been assessed in healthy non-bleeding volunteers using surrogate laboratory tests. Similar to the animal models, these studies have led to variable reports on the efficacy of direct FXa reversal by PCC. While both 3F- and 4F-PCC administration was demonstrated to restore some parameters of coagulation (PT and endogenous thrombin potential (ETP) in thrombin generation [TG]) in rivaroxaban treated healthy individuals, other parameters such as the activated partial thromboplastin time (aPTT), anti-FXa activity, and the TG lag time were unaffected[30-33]. Similar results were obtained following 3F- and 4F-PCC administration to apixaban- or edoxaban-dosed healthy individuals[34-37]. A more

clinically relevant study in which bleeding following a punch biopsy in edoxaban-treated healthy individuals was assessed revealed that 4F-PCC reversed edoxaban's effect on the duration of bleeding and on the ETP, while the PT and total blood loss could only be partially restored[38]. It is important to consider that restoration of laboratory parameters has to be interpreted with caution as these do not accurately represent the clinical setting. To overcome this, the efficacy of PCC has been evaluated in the management of major bleeding events in patients using the direct FXa inhibitors. In clinical studies, 4F-PCC proved to be effective in 65-75% of the major bleeding events in patients treated with apixaban or rivaroxaban[39-42]. Collectively, the available clinical data indicates that both 3F- and 4F-PCC may provide an effective reversal agent for the majority of patients presenting with major bleeding events associated with direct FXa inhibitor therapy. However, larger studies would be required to evaluate the true potential of this non-specific reversal strategy in a real world patient population. When specific reversal agents are unavailable or unattainable, PCC is considered as the most effective reversal agent and has therefore been included as the first treatment of choice in life-threatening bleeding events[43].

Activated Prothrombin Complex Concentrate

Activated prothrombin complex concentrate (aPCC), or factor eight inhibitor bypassing activity (FEIBA), is a PCC product that contains small amounts of factor VIII and the activated factors factor VIIa, factor IXa, factor Xa, and thrombin. This product was originally used as bypassing agent for the treatment of bleeding complications in hemophilia patients with inhibitors[44-46]. Given that aPCC elevates the circulating levels of several coagulation factors thereby stimulating coagulation, it has the potential to be a reversal agent in DOAC-induced bleeding. Perzborn *et al.* reported that aPCC significantly reduced the bleeding time and partially restored the PT in rivaroxaban-induced bleeding in rat and baboon models[28]. Similarly, aPCC was found to dose-dependently reduce the bleeding time in edoxaban-treated rats[47]. Furthermore, *in vitro* and *ex vivo* studies have shown significant but variable improvements of surrogate endpoints, including the PT, aPTT, anti-Xa activity, and several TG parameters[30,48-51]. The administration of aPCC as reversal agent for the direct FXa inhibitors has also been evaluated in a few patient studies. In two small-scale studies, aPCC-mediated reversal of apixaban and rivaroxaban was associated with a reduction in intracranial hemorrhage (ICH) expansion[52,53]. In two larger studies, administration of aPCC led to an overall stable hemorrhage progression and a 14-29% mortality rate[54,55]. Collectively, aPCC is associated with reversal of direct FXa inhibitors in major bleeding events. Therefore, the off-label use of aPCC has been included in the guidelines for the management of uncontrolled bleeding in patients on DOACs[43]. However, as more (pre)clinical data

has become available pointing to a beneficial effect of PCCs in this setting, these are currently preferred over aPCC as non-specific reversal agent.

Recombinant Activated Factor VII

Recombinant human activated factor VII (rFVIIa), or NovoSeven (eptacog alfa), was initially developed for the treatment of hemophilia in patients presenting with inhibitors[56,57]. However, its off-label use has increased significantly for uncontrolled bleeding following trauma and surgery[58] and rFVIIa has been suggested as treatment option in DOAC-related bleeding. Evaluation of the efficacy of rFVIIa in animal models and healthy volunteers has shown variable results. In edoxaban-treated rats, rFVIIa dose-dependently reduced the bleeding time and restored the PT[47]. Comparable results were obtained for rivaroxaban reversal employing rat and baboon models[28]. Conversely, rFVIIa did not affect the PT or blood loss in an apixaban- and rivaroxaban-induced bleeding model in rabbits, while the aPTT and several rotational thrombelastography (ROTEM) parameters were at least partially restored[26,27]. These inconsistent and thus far unexplained effects reflect *in vitro* reversal studies, displaying mostly marginal effects of rFVIIa on the restoration of laboratory parameters[30,48,50,51,59] Thus far no reports on the efficacy of rFVIIa in major bleeding events that are associated with DOAC therapy are available. Therefore, the benefit of rFVIIa therapy in a non-hemophilia setting remains uncertain and as such, clinical guidelines state that rFVIIa might be considered for the reversal of the direct FXa inhibitors on an individual basis[60].

Mechanistic Principles of the Non-Specific Reversal Agents

Although the exact mechanism by which the non-specific reversal agents PCC, aPCC, and rFVIIa bypass the anticoagulant effect of the DOACs is currently not fully understood, it is generally acknowledged to result from an increase in factor concentrations that promote thrombin generation **(Figure 2)**. The latter is also at the basis of their factor VIII-bypassing activity that supports their use in hemophilia treatment. The plasma concentrates contain a variety of procoagulant clotting factors, with coagulation factors X and prothrombin being considered the main procoagulant components[61]. Turecek *et al.* revealed that aPCC was unable to generate thrombin in factor V (FV)-deficient plasma, indicating that prothrombinase activity is essential[62]. Furthermore, a highly purified complex of FXa and prothrombin was able to shorten the prolonged aPTT in hemophilia A plasma, in a similar manner as aPCC. The fact that both factors are essential was illustrated by TG assessment in which FXa addition induced a slight improvement in thrombin formation, while subsequent prothrombin supplementation dramatically increased the amount of thrombin formed[62,63]. Consequently, administration of FXa and prothrombin in equivalent amounts as obtained following aPCC administration normalized blood loss in a rabbit hemophilia model[62,63]. These results show that the administration of FXa and

its substrate prothrombin is sufficient to initiate blood coagulation. As FXa is capable of directly activating FV[64], a positive feedback loop is generated via assembly of the prothrombinase complex and conversion of prothrombin to thrombin[65]. Additionally, the thrombin formed will initiate the intrinsic pathway by activation of factors VIII and XI, thereby further propagating FXa and thrombin generation. While the latter mechanism describes the mode of action of aPCC, PCC is considered to mainly function by elevating the circulating levels of several coagulation factor substrates. In general, increasing the substrate concentration for the appropriate enzymes will enhance the catalytic turnover if the substrate concentration is limited. As such, PCC likely achieves its procoagulant effect by supplementation of the factors IX (substrate of factors VIIa and XIa) and X (substrate of factors VIIa and IXa) following PCC administration.

While the above mentioned mode of action predicts a procoagulant response under conditions where bypassing of FVIII is required, the question remains how this mechanism explains effectiveness in the presence of DOACs, given that the latter will target the newly formed FXa or thrombin. A possible explanation can be found in the reversible binding nature of these inhibitors. The direct inhibitors interact with the active site pocket of their respective targets and as a result they compete with the natural and irreversible inhibitor antithrombin that engages the FXa and thrombin active site through the S1 subsite[66,67]. In the absence of the DOACs, antithrombin rapidly inhibits free FXa or thrombin. However, Thalji *et al.* described that with the direct FXa inhibitor rivaroxaban present, the majority of FXa reversibly interacts with rivaroxaban rather than forming the slower but irreversible FXa-antithrombin complex[68]. As such, an equilibrium is formed between the FXa-antithrombin and the FXa-rivaroxaban complexes, in addition to the generation of a pool of free and uninhibited FXa. As the free FXa reaches steady-state, a persistent amount of free FXa is established under these conditions. While in most scenario's this pool of free FXa is likely functionally irrelevant, the enhanced factor levels and the subsequent increase in FXa formed ensuing supplementation with the non-specific reversal agents could significantly elevate the steady-state levels of free FXa. Thalji and colleagues suggest that these elevated levels of free FXa restore hemostasis in the presence of rivaroxaban[68].

These mechanistic principles might also provide an explanation for the reduced effectiveness of rFVIIa in DOAC-reversal. In hemophila, rFVIIa stimulates blood clot formation by FXa generation via tissue factor localized at the site of injury as well as on the surface of platelets independent of tissue factor[69]. While PCC and aPCC promote the generation of multiple active serine proteases, rFVIIa focuses largely on the formation of FXa. The rate of FXa and subsequent thrombin generation could therefore be lower and prove insufficient in the presence of the DOACs.

Specific Reversal Strategies

Andexanet Alfa

The specific antidote andexanet alfa (andexanet) is a universal reversal agent for the direct FXa inhibitors and is currently the only FDA- and EMA-approved treatment option for major bleeding events induced by these anticoagulants **(Figure 3)**. Andexanet is a recombinant modified human FXa variant that is expressed in its mature FXa form due to the replacement of the activation peptide by a double Arg-Lys-Arg linker (RKRRKR), which is intracellularly and proteolytically removed by proprotein convertases[21]. Furthermore, andexanet lacks the membrane-binding γ-carboxyglutamic acid (GLA) domain of native FXa which prevents its assembly into the prothrombinase complex. Additionally, andexanet is enzymatically inactive due to replacement of the catalytic triad residue serine 195 by alanine[21]. Despite this active site substitution, andexanet displays a similar apparent binding affinity for the direct FXa inhibitors compared to wild-type (wt-)FXa[21]. Consequently, andexanet will compete with endogenous FXa for the direct inhibitor binding and as such, when administrated in molar excess, will effectively bind and clear the circulation of inhibitors, thereby restoring thrombin generation via liberated endogenous FXa **(Figure 3)**. This mechanism has been proven effective against all currently approved direct FXa inhibitors, since andexanet dose-dependently reversed the anti-FXa activity in both purified and plasma systems[21,70]. Andexanet was also able to restore hemostasis in an inhibitor-infused rat model and was shown to reduce blood loss following liver laceration in a rabbit bleeding model[21,70,71]. Reversal of the direct FXa inhibitors was not associated with major adverse effects in monkeys, as no histopathological evidence of andexanet-related thrombosis was observed[71]. Based on these data, a randomized placebo-controlled study was performed in healthy volunteers treated with apixaban (ANNEXA-A) or rivaroxaban (ANNEXA-R)[72]. Within minutes after a bolus injection or bolus injection followed by a 1-2 hour infusion of andexanet, the anti-FXa activity was reversed by more than 90% and the ETP was restored in both apixaban- or rivaroxaban-treated volunteers. These parameters returned gradually to placebo levels within 1-2 hours as andexanet was eliminated from the circulation. Moreover, in a safety and dose-escalation study in healthy individuals, andexanet was well tolerated and thrombotic events were not observed, despite the detection of increased levels of D-dimer and prothrombin fragment 1.2[73].

The ANNEXA-4 study was designed to examine the use of andexanet in patients with potential life-threatening bleeding[20]. Recently, the study was completed and included 352 patients with major bleeding complications. While in 92% of the patients treatment with andexanet resulted in excellent or good efficacy as determined by anti-FXa activity, this primary outcome did not correlate with the overall hemostatic efficacy

(good efficacy occurring in 82% of the evaluated patients). Additionally, thrombotic events occurred in 10% of the patients within 30 days following andexanet treatment, which was relatively higher than idarucizumab-mediated reversal of dabigatran (4.8%)[3] and other anticoagulant reversal studies (3.9-7%)[25,74]. A possible explanation for the relative high percentage of thrombosis might be the interaction of andexanet with the natural anticoagulant tissue factor pathway inhibitor (TFPI). Ersayin *et al.* showed that andexanet demonstrates high affinity binding to TFPI, similar to GLA-domainless FXa[75]. The TFPI-neutralizing capacity of andexanet was further underscored by the observation that addition of supratherapeutic levels of andexanet to hemophilic or normal pooled plasma (in the absence of a FXa inhibitor) resulted a dose-dependent increase in the thrombin peak height in a TFPI-sensitive TG assay[75-77]. Importantly, a major limitation of the ANNEXA-4 study is the absence of a randomized comparison with PCC, the standard treatment option in the absence of a specific reversal agent. Interestingly, such a randomized comparison with PCC is also lacking for idaruzicumab. A randomized controlled trial evaluating andexanet with a PCC-control group is underway (NCT03661528).

Specific Reversal Strategies in Clinical Development

Ciraparantag

Ciraparantag (Aripazine, PER 977) is a synthetic small molecule that consists of two L-arginine units connected with a piperazine containing linker chain, which is considered a universal anticoagulant antidote capable of reversing the effects of low-molecular weight heparins and the direct thrombin and FXa inhibitors[78-80] **(Figure 4A)**. Data on this antidote is scarce and mainly derived from abstracts. As such, the mechanistic principles remain largely unknown. Yet, it has been stated that ciraparantag binds directly to the DOACs and heparins via non-covalent hydrogen bonding based on *in silico* modeling and dynamic light scattering data, while binding to any human coagulation factor or albumin was not observed[81]. Therefore, the mode of action of ciraparantag appears relatively similar to that of andexanet: interaction with and removal of the inhibitors from their intended target in order to generate free endogenous FXa. In line with this, ciraparantag was observed to completely reverse the anti-FXa activity in apixaban- and rivaroxaban-spiked human plasma[82]. Additionally, *in vivo* assessments employing rat tail transection and rat and rabbit liver laceration models revealed that ciraparantag significantly decreased apixaban-, rivaroxaban-, and edoxaban-induced bleeding[81-84]. Moreover, ciraparantag was well tolerated in rats and dogs up to doses of 20 mg/kg per day, and no toxicity was observed[85]. Data from a first-in-human study in 80 edoxaban-treated healthy volunteers who received an intravenous injection of 100 mg or 300 mg ciraparantag demonstrated a shortening of the whole blood clotting

time to within 10% of baseline levels and restoration of the fibrin clot diameter[78,86]. In addition, ciraparantag did not induce serious adverse effects or an increase in the procoagulant markers D-dimer or prothrombin fragment 1.2, nor were alterations in TFPI levels observed.

One of the main concerns when addressing the therapeutic potential of ciraparantag is the fact that no data concerning the direct binding of ciraparantag to the DOACs have been published thus far. This complicates our understanding of the mechanisms that are at the basis of the apparent selectivity of ciraparantag for heparins and DOACs and that prevent its interaction with other proteins and molecules. These concerns are also highlighted by studies in which ciraparantag was shown to be incapable of restoring the anti-FXa activity of either apixaban, rivaroxaban, or edoxaban in plasma or a purified system[87,88]. Furthermore, ciraparantag was found to be unable to restore the augmented clotting time induced by rivaroxaban and edoxaban, but could normalize the clot structure and fibrin fiber diameter[87]. More importantly, using isothermal titration calorimetry, direct binding of ciraparantag to either edoxaban or rivaroxaban could not be established[87]. In contrast, a direct interaction with factor IXa, but not FIX, was observed[87], corroborating earlier reports on ciraparantag-dependent stimulation of the FIXa-dependent activation of FX[88]. These results may suggest a role in FX activation for ciraparantag, as opposed to direct binding and interfering with anticoagulant molecules. It is essential that its exact mechanistic principles will be elucidated and potential off-target effects are fully characterized in order for ciraparantag to be used in clinical practice.

Specific Reversal Strategies in Preclinical Development

GLA-domainless Factor Xa in Complex with α2-macroglobulin

Similar to andexanet, Jourdi *et al.* have generated a specific antidote for the direct FXa inhibitors based on GLA-domainless FXa[89]. As andexanet might interact with several pro- and anticoagulant macromolecules such as factor Va (FVa), prothrombin, antithrombin, and most importantly TFPI[21,75,76,90], Jourdi *et al.* hypothesized that a complex between GLA-domainless FXa and α2-macroglobulin (GDFXa-α2M) would prevent these macromolecular interactions while retaining its ability to sequester the direct FXa inhibitors **(Figure 4B)**. Alpha2-macroglobulin (α2M) is a molecular trap inhibitor that targets a broad spectrum of proteases in the circulation without directly blocking the protease active site[91,92]. The inhibitor is composed of four identical subunits, each including a bait region targeted by the protease, although only two proteases are able to interact with the tetramer. Upon interaction and cleavage by the protease, α2M undergoes an irreversible conformational change which traps the

protein within a cage-like structure[91,92]. By making use of GLA-domainless FXa that retains its native active site, as opposed to catalytically inactive andexanet due to the Ser195Ala substitution, incubation with α2M results in cleavage of the α2M bait region, generating the irreversible GDFXa-α2M complex. Evaluation of the constants of apixaban and rivaroxaban inhibition revealed somewhat lower binding affinities for interaction with the GDFXa-α2M complex[89,93], although still in the low nanomolar range (1-2.5 nM) similar to wild-type FXa. Furthermore, GDFXa-α2M could fully reverse the effects of supraphysiological levels of rivaroxaban and apixaban in human plasma and restored blood loss and bleeding time in a rivaroxaban-treated mouse model following lateral tail vein transection[89]. Importantly, the GDFXa-α2M complex did not have any detectable effect on a clot waveform assay and was found to be resistant to heparin-catalyzed antithrombin and TFPI, indicating that the trapped FXa does not interact with any of the naturally occurring anticoagulation proteins. This was corroborated by the observation that the D-dimer and thrombin-antithrombin (TAT) complex levels in mice following GDFXa-α2M administration were comparable to vehicle-treated controls. Surprisingly, GDFXa-α2M also partially neutralized the anticoagulant effect of dabigatran[89]. Although the mechanism that lies at the basis of this observation has not been defined thus far, it could involve a non-specific dabigatran binding site in α2M. Together, the GDFXa-α2M complex provides an alternative for andexanet and could potentially be a safer option as it does not interact with relevant macromolecular molecules, i.e. prothrombin, antithrombin, and TFPI. However, since α2M is a large 720 kDa protein, large-scale recombinant production may prove difficult, requiring purification from human plasma.

Zymogen-like Factor Xa (FXa-Ile16Leu)

Besides specific bait antidotes that bind and sequester the direct FXa inhibitors, several prohemostatic bypassing agents have been developed that are able to enhance thrombin generation in the presence of the FXa-targeting inhibitors. One such reversal agent is a FXa variant comprising an Ile16Leu substitution (chymotrypsin numbering)[68,94] **(Figure 5A)**. Upon activation via proteolytic cleavage at the highly conserved Arg15-Ile16 site, serine proteases are converted to their protease state by insertion of the newly formed N-terminus in the active site, thereby forging an internal salt bridge of Ile16 with Asp194. This transition induces maturation of the serine protease domain, specifically following conformational rearrangement of the S1 specificity pocket and oxyanion hole[95]. Disrupting the zymogen to protease transition through modification of the N-terminus (Ile16Leu) induces a zymogen-like conformation in which the active site of FXa retains a partially immature state[94]. As a result, FXa-Ile16Leu displays an impaired chromogenic substrate conversion and is less sensitive to small molecule inhibitors. Although FXa-Ile16Leu also demonstrated weaker FVa binding and prothrombin

activation, saturating concentrations of the cofactor and anionic membranes partly restored thrombin conversion, suggesting that FVa acts as a stabilizing factor in the zymogen to protease transition of this variant. Further assessments revealed a 20- to 40-fold reduced sensitivity for the natural inhibitors antithrombin and TFPI, while, in contrast, prothrombinase-assembled FXa-Ile16Leu was similarly inhibited by antithrombin compared to wt-FXa[96]. Due to the reduced sensitivity, the FXa-Ile16Leu half-life was extended to ~40 min in plasma and approximately 5 min in mice relative to wt-FXa (half-life of ~1 min and <20 sec, respectively)[96-98]. These data indicate that FXa-Ile16Leu is protected from being targeted by natural anticoagulants in the circulation but still retains the ability to catalyze thrombin formation upon binding to FVa on an anionic membrane layer. As such, FXa-Ile16Leu was examined for its bypassing potential in various clinical bleeding settings and was found to be effective in hemophilia (in the presence and absence of inhibitors) and capable of reversing the effects of warfarin[96-99]. Moreover, it was hypothesized that the properties of FXa-Ile16Leu might enable this variant to bypass the direct oral anticoagulants. *In vitro* evaluation by TG and ROTEM assays demonstrated that FXa-Ile16Leu dose-dependently reversed the anti-FXa effects of rivaroxaban-spiked plasma and whole blood[68]. These results could be translated to murine *in vivo* models as mouse FXa-Ile16Leu completely restored hemostasis in several thrombosis and bleeding models in the presence of rivaroxaban[68]. Infusion of this FXa variant in mice did increase the circulating TAT levels, but only in the absence of rivaroxaban, while D-dimer levels remained unaltered[68]. The possible mechanism by which FXa-Ile16Leu reverses the effect of the direct FXa inhibitors might be similar to that of previously mentioned bypassing agents. In the presence of rivaroxaban, antithrombin and rivaroxaban are competing for FXa binding. As a consequence, an equilibrium is formed, characterized by a steady-state pool of free FXa[68]. Computional simulations revealed that the free FXa-Ile16Leu steady-state levels were substantially higher compared to those of wt-FXa. However, the net effect on thrombin generation was identical to wt-FXa due to the reduced catalytic activity of FXa-Ile16Leu. This suggests that besides FXa-Ile16Leu, wt-FXa could also be able to reverse the direct FXa inhibitor-induced anticoagulant state. Yet, FXa-Ile16Leu is dependent on the binding of FVa in order to rescue its catalytic activity, which could be beneficial in terms of safety and prothrombotic risks. As such, administration of FXa-Ile16Leu would only result in local clot formation at the sites of injury. Collectively, these studies highlight the potential efficacy of FXa-Ile16Leu as a rescue agent in a variety of bleeding disorders, including direct FXa inhibitor-induced bleeding complications. This prohemostatic agent therefore provides a rapid-onset alternative for the bait antidotes, thereby requiring significantly lower amounts of the reversal agent to control bleeding.

The clinical development of FXa-Ile16Leu is currently ongoing. Assessment of the pharmacokinetics and pharmacodynamics in rats and monkeys displayed a half-life of 2-3 min[100]. Furthermore, FXa-Ile16Leu administration resulted in a dose-dependent shortening of the aPTT in non-human primates, but simultaneously led to increased TAT levels. A first-in-human dose escalation study in 49 healthy volunteers revealed that FXa-Ile16Leu was well tolerated with no observed toxicity or serious adverse events[101]. The pharmacodynamics displayed shortening of the aPTT and TG lag time, as well as an increase in TG peak height, D-dimer, TAT, and prothrombin fragments 1.2 with all parameters returning to baseline within 24 hours. Finally, a clinical trial evaluating the safety and tolerability of FXa-Ile16Leu in subjects with spontaneous ICH is underway (NCT02687191). Whether this reversal agent will be further tested in the setting of the direct FXa inhibitors is unknown.

The 99-loop Factor X Variant (FX-C)

The 99-loop FX variant, also known as FX-C, is a chimeric FX protein comprising a structural modification derived from the so-called isoform FX variant that is expressed in the venom and liver of the eastern common brown snake (*Pseudonaja textilis)*[102] **(Figure 5B)**. Characterization of the isoform and venom FXa proteases revealed a high resistance for the direct FXa inhibitors rivaroxaban and apixaban. Sequence analysis showed that these FXa variants comprise an extended 99-loop (His91-Asp102) located N-terminal to the S4 subsite. Since the S4 pocket was demonstrated to be a primary binding site for the FXa inhbitors and stabilizes their positioning in the active site[5,6,102], this non-conserved structural element was hypothesized to be responsible for the observed resistance towards the direct FXa inhibitors. In order to further investigate the reduced sensitivity, the 99-loop extension of isoform FX was recombinantly inserted into human FX, thereby generating FX(a)-C. Insertion of this structural element significantly reduced the FXa sensitivity for apixaban and edoxaban (up to 700-fold), although this came at the cost of a 3-fold lower catalytic efficiency towards the natural substrate prothrombin[102]. To examine whether the reduced inhibitor sensitivity of FXa-C was sufficient to restore the thrombin formation, TG assays were employed in apixaban- and edoxaban-spiked plasma. These experiments revealed that the addition of zymogen FX-C resulted in a dose-dependent increase in thrombin generation. Moreover complete TG normalization was observed with the addition of 20-40 µg/mL of the FX-C variant. Interestingly, molecular dynamics simulations revealed that the extended 99-loop in FXa-C adopts a helical conformation which displays substantial mobility. This structural mobility results in the rapid displacement of the apixaban molecule, indicative of steric hindrance leading to impaired inhibitor binding. As such, significant levels of free FXa-C are formed that, despite its reduced prothrombin conversion rate, are able to restore thrombin

generation parameters to near-normal conditions, indicative of a restored hemostasis. The inhibitor-resistant variant FX-C is currently in preclinical and clinical development.

Phe174-substituted FX Variants

Arising from the FX-C study, a Phe174-substituted FXa variant displayed potential as bypassing agent for the direct FXa inhibitors **(Figure 5C)**. Molecular dynamics simulations of the human FXa-apixaban complex demonstrated a tight inhibitor interaction with the FXa S4 subsite pocket[102]. In this pocket, the P_4 moiety of apixaban is sandwiched between the aromatic side-chains of S4 subsite residues Tyr99 and Phe174. Replacing the S4 subsite residues Tyr99 and/or Phe174 with alanine disrupted apixaban binding, resulting in a markedly decreased FXa sensitivity for the inhibitor. While the Tyr99Ala substitution was accompanied by an almost complete diminished FXa activity, substitution of Phe174 retained the intrinsic clotting potential. As such, human FX variants were generated comprising an alanine (FX-F174A) or serine (FX-F174S) substitution at position 174[103] **(Figure 5C)**. These variants displayed a 10-fold reduced sensitivity towards apixaban and a 4-7-fold reduced sensitivity for inhibition by rivaroxaban and edoxaban. Similar effects were demonstrated in human plasma spiked with the direct FXa inhibitors, revealing a 6-17-fold loss of inhibitor sensitivity. Furthermore, addition of 30-45 µg/mL of these variants completely rescued the thrombin peak height in the presence of 1 µM apixaban and rivaroxaban, while an 80% improvement was found in identical conditions with edoxaban. Although further research is needed to fully elucidate the effects of the Phe174-substituted FX variants, these agents might prove to be a potential treatment option.

Concluding Remarks

Bleeding complications associated with DOAC-use remain a major concern, creating a critical need for rapid-onset reversal agents. Although a specific antidote is available for the direct thrombin inhibitor in idarucizumab, counteracting the effect of the direct FXa inhibitors has been a point of focus over the last years. In the absence of specific antidotes for the direct FXa inhibitors, off-label use of prohemostatic agents has been recommended as standard of care. In the meantime, considerable efforts have been made by researchers to fill the unmet need for a specific reversal agent, leading to the recent FDA and EMA approval of andexanet alfa. Furthermore, several specific reversal agents are currently in (pre)clinical development. Since the potential interaction of andexanet with TFPI might raise some concerns, other specific reversal agents that are currently in various stages of (pre)clinical development may prove to be alternative options for reversal. For instance, the Gla-domainless FXa-α2-macroglobulin complex did not display interactions with endogenous inhibitors.

Another potential option is ciraparantag, a small synthetic molecule that reportedly binds and sequesters the FXa-targeting inhibitors, although its mode of action seems unresolved at this point. Interestingly, several prohemostatic bypassing agents have been developed, including FXa-Ile16Leu, FX(a)-C, and the Phe174-substituted variants FX-F174A and FX-F174S. Their key advantage resides in the limited amount of protein that is needed to correct inhibitor-induced bleeding, in contrast to the stoichiometric bait antidotes. Concomitantly, these prohemostatic bypassing agents may increase the risk for thrombotic complications, and as such more caution might be required in clinical practice. Additionally, the protein modifications in the FX(a) variants may induce an antibody response, limiting the efficacy of repeated use. Further characterization of these specific agents is therefore imperative, but a future with specific, safe, and effective reversal agents for the direct FXa inhibitors seems imminent.

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Figures

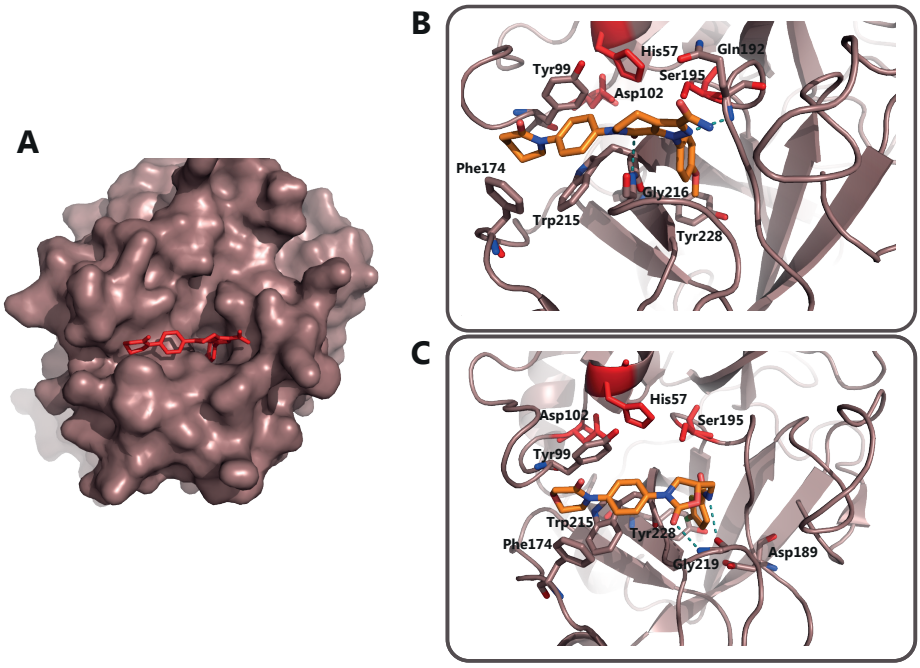

Figure 1. Binding mode of the direct factor Xa inhibitors. (A) Surface representation of the FXa X-ray structure in complex with the direct FXa inhibitor apixaban shown in red. **(B-C)** Close up view of apixaban **(B)** and rivaroxban **(C)** binding to FXa. Apixaban and rivaroxban are depicted in orange, with the oxygen atoms indicated in red and nitrogen atoms in blue. Essential amino acids in FXa are shown in stick configuration in which the catalytic triad residues His57, Asp102, and Ser195 are colored in red. All FXa residues are indicated in chymotrypsinogen numbering. Hydrogen bond interactions are shown in teal dashed lines.

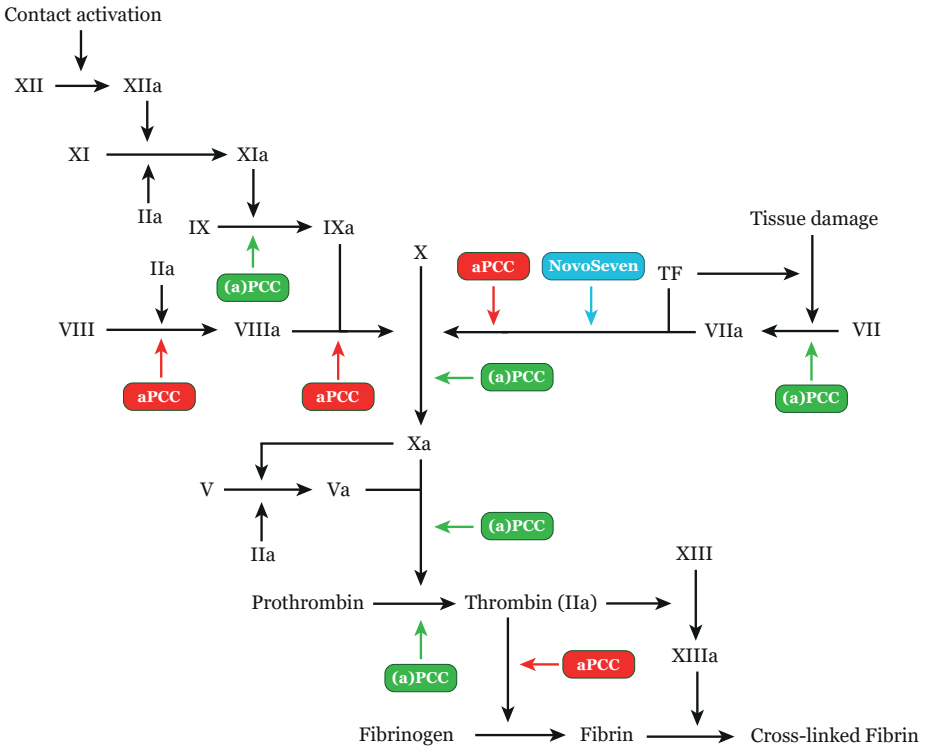

Figure 2. Schematic representation of the procoagulant actions exerted by non-specific reversal agents. The non-specific reversal agents promote various procoagulant reactions of the coagulation cascade in order to drive thrombin generation in the presence of the direct factor Xa inhibitors. The overlapping actions of the activated and non-activated prothrombin complex concentrates ((a)PCC) are indicated in green arrows. The distinct effects of aPCC are shown in red, and that of recombinant activated factor VIIa (rFVIIa) is shown in blue. TF indicates tissue factor.

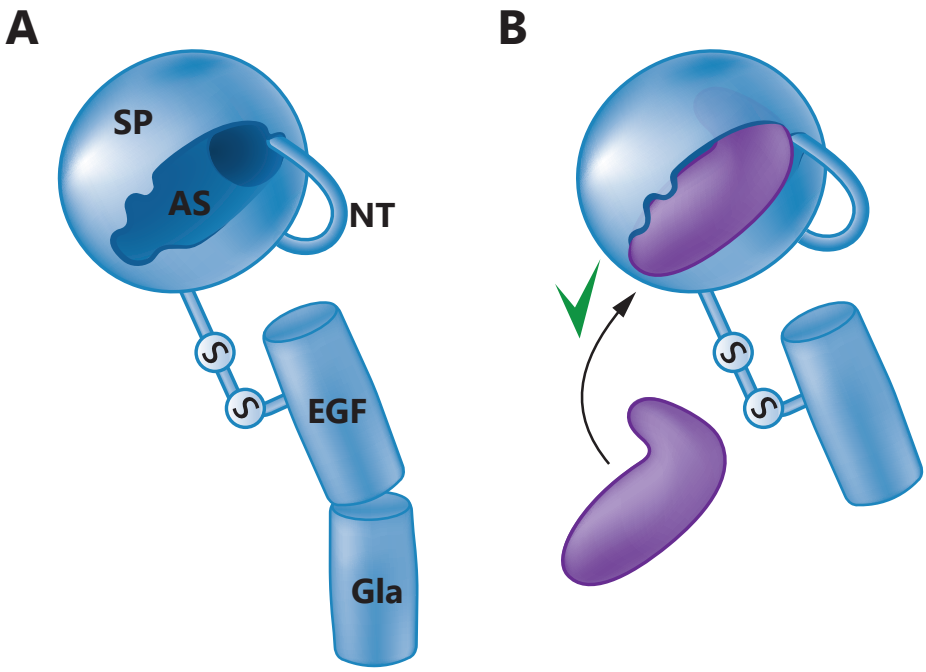

Figure 3. Schematic representation of human factor Xa and the reversal agent andexanet alfa. (A) The light chain of factor Xa (FXa) consisting of the γ-carboxyglutamic acid domain (GLA), the endothelial growth factor 1 and 2 domains (EGF), and the heavy chain comprising the serine protease domain (SP) and active site pocket (AS) are shown, linked by a disulfide bond (S-S). The heavy chain N-terminus (NT), formed upon proteolytic activation of factor X, is shown as an insertion into the active site pocket, representing the salt bridge between Ile16 and Asp194. **(B)** Schematic respresentation of andexanet alfa. Despite being enzymatically inactive due to a Ser195Ala substitution, andexanet alfa encompasses a high binding affinity for the direct FXa inhibitors, which are shown in purple. Removal of the Gla domain prevents incorporation of andexanet alfa into the prothrombinase complex.

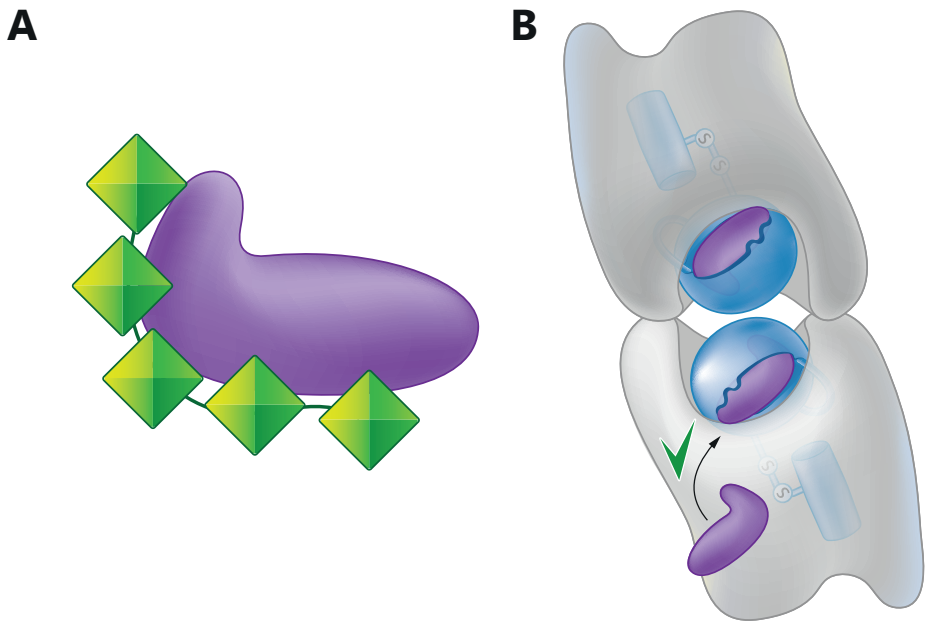

Figure 4. Schematic representation of the universal reversal agent ciraparantag and the GLA-domainless factor Xa in complex with α2-macroglobulin. (A) Ciraparantag (the green squares represent two L-arginine units at each end that are connected via a linker consisting of three units of which a piperazine comprises the middle unit) reportedly binds directly to the direct factor Xa inhibitors (shown in purple) via hydrogen-bonding, thereby sequestering the inhibitors from the circulation. **(B)** α2-macroglobulin consists of four identical subunits that allow for the interaction with two factor Xa (FXa) proteases. Despite being trapped, GLA-domainless FXa retains its ability to bind the direct FXa inhibitors (shown in purple), thereby clearing the inhibitors from the circulation. Complex formation with α2-macroglobulin further prevents macromolecular interactions with FXa.

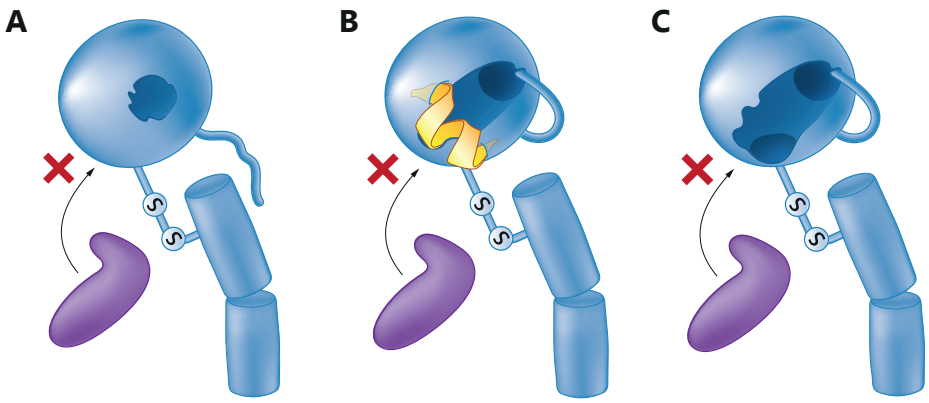

Figure 5. Schematic representation of the prohemostatic reversal agents in preclinical development. (A) Substitution of Ile16 for leucine (shown in red) disrupts the insertion of the newly formed N-terminus upon factor X activation, thereby destabilizing the formation of the characteristic internal salt bridge with Asp194. As a consequence, the active site of FXa-Ile16Leu is partly matured (indicated as a smaller active site), thereby inducing a zymogen-like state. This zymogen-like state prevents the inhibition of FXa-Ile16leu by the direct FXa inhibitors (shown in purple). Binding to factor Va rescues the maturation of the serine protease domain. **(B)** Factor Xa-C (FXa-C) is resistant to the direct factor Xa inhibitors (shown in purple) due to an insertion within the active site 99-loop. This structural element (highlighted in yellow) sterically hinders the binding of the direct FXa inhibitors to the active site. **(C)** The prohemostatic factor Xa (FXa) comprises an alanine or serine substitution of the phenylalanine 174 (shown in red). Altough the direct FXa inhibitors (shown in purple) are able to engage the active site, substitution of the phenylalanine 174 destabilizes inhibitor binding, resulting in a loss of affinity.

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