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ORIGINAL ARTICLE

Novel inhibitor ZED3197 as potential drug candidate in anticoagulation targeting coagulation FXIIIa (F13a)

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Abstract

Background: Factor XIII (FXIII) is the final enzyme of the coagulation cascade. While the other enzymatic coagulation factors are proteases, FXIII belongs to the transglutaminase family. FXIIIa covalently crosslinks the fibrin clot and represents a promising target for drug development to facilitate fibrinolysis. However, no FXIII-inhibiting compound has entered clinical trials. Here, we introduce the features of a peptidomimetic inhibitor of FXIIIa (ZED3197) as a potential drug candidate.

Methods: The potency of ZED3197 against FXIIIa and the selectivity against other human transglutaminases were characterized using transamidation and isopeptidase assays. The inhibition of fibrin crosslinking was evaluated by biochemical methods and thromboelastometry. Further, the pharmacology of the compound was explored in a rabbit model of venous stasis and reperfusion.

Results: ZED3197 proved to be a potent and selective inhibitor of human FXIIIa. Further, the compound showed broad inhibitory activity against cellular FXIIIA from various animal species. Rotational thromboelastometry in whole human blood indicated that the inhibitor, in a dose-dependent manner, prolonged clot formation, reduced clot firmness, and facilitated clot lysis without affecting the clotting time, indicating minimal impact on hemostasis. In vivo, the novel FXIIIa inhibitor effectively decreased the weight of clots and facilitated flow restoration without prolongation of the bleeding time.

Conclusions: ZED3197 is the first drug-like potent compound targeting FXIIIa, a yet untapped target in anticoagulation. Due to the function of FXIII downstream of thrombin the approach provides minimal impact on hemostasis. In vivo data imply that the inhibitor dissociates an antithrombotic effect from increased bleeding tendency.

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KEYWORDS
anticoagulation, drug discovery, factor XIII, fibrinolysis, transglutaminase

1 | INTRODUCTION

Substantial effort has been dedicated to the development of antithrombotics targeting coagulation factors or platelet activation. Coagulation factor XIII (FXIII, F13) is a promising yet widely untapped and challenging target for drug development.[1-3] Whereas the other enzymes within the coagulation cascade are serine proteases, the FXIIIA subunit belongs to the transglutaminase family (EC 2.3.2.13: protein-glutamine γ-glutamyltransferase) consisting of eight human isoenzymes (FXIIIA and TG1-TG7).[4,5] The most characteristic catalytic function for transglutaminases is the formation of isopeptide bonds between the side chains of susceptible protein bound glutamine and lysine residues in a tightly controlled manner.

FXIII plays a key role in clot formation, maturation, and composition.[6,7] FXIII recognizes fibrin as substrate and covalently crosslinks fibrin γ-chains and, in an ordered sequence, fibrin α-chains, providing mechanical stability to the fibrin fibers. In parallel, FXIII-catalyzed covalent incorporation of antifibrinolytic proteins such as α_2-antiplasmin renders the clot biochemically stable.[8] In blood, the noncovalent FXIII-A_2B_2 heterotetramer (pFXIII)[9] is bound to fibrinogen.[10,11] For activation, thrombin cleaves the N-terminal activation peptide from FXIIIA subunits. Subsequent binding of calcium ions promotes dissociation of the carrier B-subunits yielding active FXIIIa, which most likely is monomeric as suggested by the crystal structure of calcium activated recombinant FXIIIa solved in complex with the irreversible peptidic inhibitor ZED1301[2] supported by analytical ultracentrifugation[12] and atomic force microscopy.[13] Besides the insight into a calcium-induced conformational shift and the catalytic mechanism the X-ray data provided the blueprint for our inhibitor discovery program.

The clot-modulating function and the positioning downstream of thrombin make FXIII a promising target for drug development.[8] Specific inhibitors targeting FXIII would not interfere with thrombin generation, fibrin formation, or with platelet activation. Blocking thrombin—directly or via upstream FXa—by the currently available anticoagulants is characterized by an enhanced bleeding risk, thus excluding many patients from beneficial treatment.[14] Considering this well-known medical need, novel therapeutic approaches with minimal or no bleeding risk are desperately needed.[15] Even if the development of FXIII inhibitors may provide one such option, noticeably few such compounds have been identified or synthesized by the pharmaceutical industry so far.

Finney et al[16] reported that the 66 amino acid polypeptide "tridegin" from the salivary gland of the giant Amazon leech *Haementeria ghilianii* is a potent FXIII inhibitor. Further, in the late 1980s, a series of small molecules irreversibly inhibiting FXIIIa were explored in animal models of thrombosis in the presence of t-PA facilitating increased clot lysis in vivo.[17,18] Due to the lack of selectivity and potency along with short plasma half-lives of only a few minutes, these inhibitors were solely considered as pharmacological tools but not as prospective drug candidates.[18] The pharmacokinetic profile of an irreversibly acting inhibitor carrying a thiadiazole warhead was studied in rabbits in order to support and facilitate the design and selection of drug candidates.[19] Further, medicinal chemists reported cyclopropenone derivatives from fungi and synthetic analogues as potent FXIIIa inhibitors.[20] In both cases, from a drug discovery perspective, the low potency of the compounds disqualifies them for further development. In accordance with this assumption no (pre)clinical studies have been reported.

Essentials

- Plasma transglutaminase FXIII represents a promising yet untapped target for drug development.
- The comprehensive characterization of the direct-acting FXIIIa inhibitor ZED3197 is presented.
- Biochemical assays and in vivo data proved the potency and selectivity of the novel compound.
- ZED3197 is a potential drug candidate possibly dissociating an antithrombotic effect from bleeding.

Potent, drug-like FXIII-inhibitors are a prerequisite that is still lacking for further exploring the therapeutic concept. Here we report the comprehensive in vitro characterization of a novel peptidomimetic FXIIIa-blocker (ZED3197) used subsequently in vivo for target validation in a rabbit model of venous stasis and reperfusion.

2 | METHODS

2.1 | Structure, mode of inhibition, and synthesis of ZED3197

The peptidomimetic blocker ZED3197 (Figure 1A) is a modified hexapeptide derived from lead optimization of ZED1301.[2] The compound contains a Michael acceptor warhead covalently blocking the active site cysteine (Figure 1B). The synthesis of the molecule and the determination of physicochemical parameters are detailed in Data S1 in supporting information.

2.2 | FXIII activity assays

2.2.1 | Isopeptidase assay for determining inhibitor potency

FXIIIa activity has been determined using substrate A101 (Zedira), which is based on the N-terminal dodecapeptide of α_2-antiplasmin. FXIIIa catalyzes by its isopeptidase activity the release of dark quencher dinitrophenyl at the original substrate glutamine

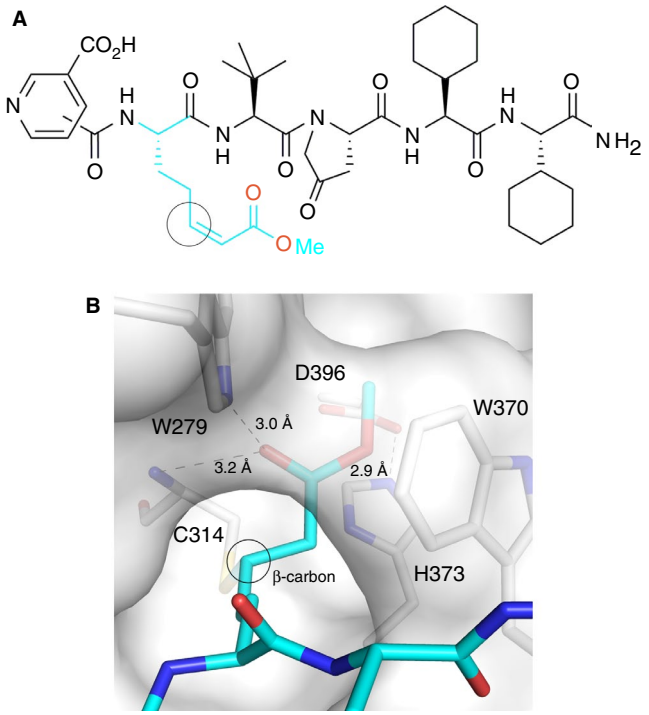

FIGURE 1 A, Structure of ZED3197 is the peptidomimetic compound carrying a Michael acceptor warhead (cyan). The backbone provides both, potency and selectivity to the target FXIIIa. The molecule consists of artificial amino acids known from approved antiviral drugs, e.g. telaprevir[36] and boceprevir[37]. Rather unique is the 4-oxo-proline moiety providing conformational constraint. Further, the compound possesses cinchomeronic acid as N-terminal heterocyclic cap. B, Illustration of the warhead bound to the active site of FXIIIa The Michael acceptor warhead (trans-α,β-unsaturated methyl ester, cyan) replaces the actual substrate glutamine side chain. Embedded in a suitable peptidic/peptidomimetic backbone the warhead addresses specifically the catalytic center of active FXIIIa (surface in gray). The thiolate-histidine imidazolium ion pair essentially forms the catalytic triade (Cys314-His373-Asp396). The cysteinyl Sγ atom moiety (yellow) attacks the complementary electrophilic β-carbon of the unsaturated ester. The reaction leads to the covalent, irreversible inhibition of FXIIIa as shown. The Michael acceptor warhead is accommodated by Trp279 and Trp370 forming the hydrophobic tunnel for the lysine co-substrate characteristic for transglutaminases. Distances of H-bonds (dashed lines) are indicated. Substantial efforts to co-crystallize ZED3197 failed. We suspect the constraint oxo-proline to hamper co-crystallization using the established procedure with calcium activated FXIIIa°. For illustration purposes, the crystal structure of the lead compound ZED1301 (PDB ID: 4KTY) was used instead, carrying the identical warhead.

position resulting in fluorescence increase (based on the N-terminal 2-aminobenzoyl fluorescent dye).[21]

Briefly, 12 µL recombinant human FXIII-A_2 (25 µg/mL, T027, Zedira) or FXIII-A_2B_2 derived from human plasma (T007) (50 µg/mL) and 3 µL human α-thrombin (0.5 U/mL, T056, Zedira) were mixed with 270 µL assay buffer (50 mmol/L Tris–HCl, 10 mmol/L $CaCl_2$, 150 mmol/L NaCl, 5.56 mmol/L glycine methyl ester, 5 mmol/L DTT, pH 7.5) containing 55 µmol/L A101 substrate. The mixture was incubated for 20 minutes at room temperature to activate FXIII. Fifteen µL of inhibitor solution (serial dilution from 1.25 µmol/L to 1.25 nmol/L) dissolved in DMSO/assay buffer were added, mixed and the kinetic measurement started after 3 minutes. Fluorescence emission was monitored at 418 nm (λ_{ex} = 313 nm) and 37°C for 30 minutes using a CLARIOstar fluorescence micro plate reader (BMG Labtech). For measurements without inhibitor, 15 µL of assay buffer/2% (v/v) DMSO were added. All measurements were performed in triplicate. The respective IC_{50} values were calculated by nonlinear regression using the MARS software package (BMG Labtech).

The inhibition of FXIIIa from animal species was performed accordingly using 36 µg/mL mouse FXIII-A_2 (T061, Zedira), 27 µg/mL rat FXIII-A_2 (T065), 11 µg/mL pig FXIII-A_2 (T066), 32 µg/mL dog FXIII-A_2 (T062), and 22 µg/mL cynomolgus monkey FXIII-A_2 (T161), all produced recombinantly.

2.2.2 | Transamidation assay for determining inhibitor selectivity

The most relevant off-targets are the transglutaminase isoenzymes especially tissue transglutaminase (TG2) because the enzyme is ubiquitously expressed throughout the human body. To determine selectivity, the fluorescence increase upon transglutaminase-catalyzed incorporation of dansylcadaverine into the universal substrate N,N-dimethylcasein was used.[22]

Briefly, 15 µL of recombinant transglutaminase enzyme[5] (15 µg/mL hTG1 [T035], 69 µg/mL hTG2 [T022], 29 µg/mL hTG6 [T021], 18 µg/mL hTG7 [T011], all from Zedira) were mixed with 270 µL assay buffer containing dansylcadaverine and N,N-dimethyl casein. In the case of FXIII, 12 µL FXIII-A_2 (25 µg/mL) and 3 µL human α-thrombin (0.5 U/mL) were mixed with 270 µL assay buffer. The mixture was incubated for 20 minutes at room temperature to activate FXIII. In the case of TG3, 78 µg hTG3 (T024, Zedira) were activated using 14 µg dispase II (Roche) in the presence of 1.4 mmol/L $CaCl_2$ and incubated for 30 minutes at 25°C. The activated hTG3 was subsequently assayed as described above. Fifteen µL of inhibitor solution dissolved in DMSO/assay buffer were added, mixed, and the kinetic measurement started after 3 minutes. Fluorescence emission was continuously monitored for 30 minutes at 500 nm (λ_{ex} = 330 nm) and 37°C using the CLARIOstar fluorescence plate reader. All measurements were performed in triplicate. The respective IC_{50} values were calculated by nonlinear regression using the MARS software package (BMG Labtech).

2.3 | Thromboelastometry

Thromboelastometry (TEM) is a viscoelastic method for the assessment of blood coagulation.[23] Clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF), and lysis index at 60 minutes (LI60) were obtained using fresh whole blood in the ROTEM® delta device according to the manufacturer's instructions.

The potency of ZED3197 (serial dilution covering 20.0-0.08 μmol/L final concentration) in the presence of 0.02 μg/mL (final concentration) tissue plasminogen activator (t-PA; P016, Zedira) was investigated. Briefly, 20 μL star-TEM® (0.2 mol/L $CaCl_2$), 20 μL r ex-TEM® (recombinant tissue factor, phospholipids, heparin inhibitor), 10 μL inhibitor stock solution or respective control, combined with 10 μL t-PA stock solution, and 300 μL fresh citrated whole blood (from healthy consenting donors) were mixed in a disposable cuvette and subsequently measured.

2.4 | Rabbit model of venous stasis and reperfusion

Purpose-bred animals were identified upon arrival in the test facility (IPST) according to the respective guidelines. Male New Zealand White rabbits (1.3-2.8 kg) were anesthetized for the duration of the procedure. The rabbit's right jugular vein was exposed and any collateral veins to the venous stasis segment were ligated. In order to prevent embolization, a polyester suture thread was inserted from upstream into the lumen of the designated stasis segment prior to the ligations to allow thrombus formation around the thread. An ultrasound probe was placed perivascularly on the right jugular vein downstream to the venous stasis segment, and blood flow was recorded continuously (3 mm probe, Transonic Systems Inc.). Blood samples were taken from the right femoral artery. Test compound ZED3197 (n = 7) or negative control (n = 6) were administered at the selected concentration and flow rate through an IV bolus or infusion via the right femoral vein (see Figure S2 in supporting information for detailed treatment schedule and sampling timeline). The negative control animals received 2 × PBS/5% glucose containing in mmol/L: NaCl 273.8, NaH_2PO_4 × 2 H_2O 14.2, KCl 5.4 and KH_2PO_4 2.9, pH 7.4 ± 0.05/5% (w/v) glucose. Fifteen minutes (counting from the end of the injection) after the slow bolus injection (approximately 60 seconds injection time) of inhibitor or vehicle, the right jugular vein was clamped, starting with the downstream clamp, followed 10 seconds later by the upstream one. One hundred fifty μL of blood were collected from the femoral artery and supplemented with 45 μL of 0.25 mol/L calcium chloride. Next, 25 μL of human α-thrombin (2.5 U/mL, Sigma-Aldrich) was added to the blood mixture to induce coagulation. Immediately after mixing the blood, the clotting blood was administered in the isolated part of the right jugular vein.

After a venous stasis period of 15 minutes, the vessel clamps were removed to restore blood flow from the jugular vein. The inhibitor was infused during this venous stasis period. Blood flow was recorded with the transonic flow probe for a period of 2 hours while the compound was infused at the selected concentration (compare to Table S1 in supporting information). Two hours after reperfusion, the venous stasis segment was removed, opened longitudinally, and emptied into a petri dish containing 5% sodium citrate solution. Any existing thrombi were removed and blotted on a filter paper. The thrombi were measured, weighed, and the appearance was judged. A bleeding time was performed 30 minutes after beginning of reperfusion using an ITC Surgicutt™ Bleeding Time Device (International Technidyne SU50I via Fisher Scientific). Bleeding time was assessed with a filter paper by carefully collecting blood from the wound rim until no red staining of the filter paper could be observed. For each measure, a different nonstained part of the filter paper was used. The maximum bleeding time was defined as 300 seconds.

For each blood sample time point, plasma samples were generated using 150 mmol/L sodium citrate as anticoagulant. Samples were stored at −20°C until further analysis: determination of ZED3197 concentration by HPLC and determination of residual FXIII activity after thrombin activation was made using the isopeptidase assay described above. In addition, one blood sample was taken at 60 minutes after inhibitor administration for thromboelastography (TEG). The TEG 5000 traces were recorded on the fresh whole blood sample for a period of 60 minutes according to the manufacturer. The read-outs are similar to the TEM and key parameters were combined to give the coagulation index (CI). Subsequent to the observation period of about 150 minutes after reperfusion, the animals were euthanized following an intracardiac blood draw by administering an overdose of pentobarbital.

3 | RESULTS

3.1 | ZED3197; mode-of-inhibition, physicochemical features, and in vitro FXIII inhibition

FXIII belongs to the transglutaminase family and the unique crosslinking reaction determines the mechanical and biochemical stability of blood clots formed. Accordingly, FXIII was considered for decades as a target for drug development.[1] ZED3197 is a novel synthetic FXIIIa-blocker carrying a mechanism-based warhead (Figure 1A). The compound resulted from a structure-assisted drug design project inspired on the X-ray data obtained from the calcium activated FXIIIa-ZED1301 complex.[2] The peptidomimetic backbone was optimized with respect to potency, selectivity, and stability. However, the drug discovery project is beyond the scope of this paper and will be reported elsewhere. The backbone of ZED3197 positions the warhead into the catalytic center of FXIIIa. Only complementary orientation of the electrophilic Michael acceptor allows irreversible inactivation of the active site cysteine (Figure 1B). Cysteine 314 is exceptionally nucleophilic due to the assisting histidine.[24] Importantly, the intrinsic reactivity of the warhead is low, preventing reaction with biological thiols such as glutathione.

The key physicochemical features of ZED3197 are displayed in Table 1. The molecular weight calculates to 838 g/mol. The compound shows good solubility in saline, even better solubility in saline/glucose formulation, and a drug-like distribution-coefficient (logD).[25] In vitro the compound was stable in saline (>97%) as well as in human and rabbit plasma (>95%) over a period of 2 hours. As shown in Table 2a, ZED3197 inhibits human plasma derived FXIII-A_2B_2 and the recombinant cellular form (FXIII-A_2) with a similar IC_{50} value (inhibitor concentration to block 50% of enzymatic activity in a given experimental setting) of 10 and 14 nmol/L, respectively, using the isopeptidase assay.[21] In addition, ZED3197 strongly blocks FXIII-A_2 from mouse, rat, rabbit,

TABLE 1 Key physicochemical and stability features of ZED3197

Molar weight	838 g/mol
Solubility in PBS (pH 7.4)	2.5 mg/mL (3.0 mmol/L)
Solubility in 2× PBS / 5% (w/v) glucose	6.0 mg/mL (7.2 mmol/L)
logD (octanol/PBS pH 7.4)	−0.02
Stability in PBS (pH 7.4)	>97% (120 min)
Stability in plasma (human)	>95% (120 min)
Stability in plasma (rabbit)	>95% (120 min)

dog, and cynomolgus monkey (IC_{50} of 8-30 nmol/L). Apparently the compound is a significantly weaker inhibitor of FXIII-A_2 from pig (IC_{50} value of ~370 nmol/L). In the classical transamidation assay using methylated casein as universal glutamine donor substrate (Table 2b), ZED3197 blocked FXIII of all animals including pig (IC_{50} values of 6-19 nmol/L) with similar high efficacy as that for human FXIII-A_2 (20 nmol/L). The apparently weak inhibition of pig FXIII observed in the isopeptidase assay can probably be explained by high affinity of the assay substrate to the pig active site. The lysine at the C-terminus of the A101 dodecapeptide is derived from human α_2-antiplasmin sequence. Remarkably, only in pig this crucial amino acid is converted to glutamate. We did not follow the hypothesis but assigned the apparent lack of potency being assay-specific. Therefore, all species tested qualify for pharmacological studies or toxicological assessment. The selectivity profile of ZED3197 against human transglutaminases TG1, TG2, TG3, and TG7[5] varied from 20-fold to 3000-fold in the transamidation assay (Table 2b) suggesting sufficient selectivity against the most relevant off-targets. In contrast, the compound efficiently inhibits human neuronal transglutaminase (TG6) indicating a similar architecture of the active site to that of FXIII. Unfortunately, by now a TG6 crystal structure is missing to prove this hypothesis. For the purpose of comparison we synthesized the "L-682.777" compound (Zedira product T101) originally developed by Merck & Co.[26] This irreversible-acting small molecule blocked both FXIIIa and tissue transglutaminase with an IC_{50} of ~250 nmol/L in the isopeptidase assay. In summary, ZED3197 proved not only more potent than T101 by more than one order of magnitude but also provided selectivity due to the peptidomimetic backbone.

3.2 | Inhibition of fibrin crosslinking; biochemical studies and thromboelastometry in whole human blood

The most characteristic reaction catalyzed by FXIIIa is the crosslinking of fibrin. Within minutes isopeptide bonds are formed between two antiparallel oriented fibrin γ-chains of neighboring D-domains. Specifically, one of the glutamine residues (ie Q398/Q399) of one fibrin γ-chain is transferred to lysine K406 of the abutting γ-chain and vice versa.[4] Further, α_2-antiplasmin is covalently incorporated into the nascent clot. Fibrin clots formed by the addition of thrombin to human fibrinogen spiked with cFXIII, at physiological concentrations, in the presence of 10 µmol/L ZED3197 were readily dissolved when human plasmin was added. Size exclusion chromatography (Figure S1 in supporting information) and subsequent SDS-PAGE

TABLE 2 (a) Inhibition of FXIII derived from different species by ZED3197 in isopeptidase and transamidation assay, (b) selectivity against human transglutaminases (recombinant) based on the transamidation assay

2a

Transglutaminase	Species	Isopeptidase assay IC_{50} [nmol/L]	Transamidation assay IC_{50} [nmol/L]
FXIII-A_2	Human	16 ± 0.6	24 ± 1.5
FXIII-A_2	Mouse	19 ± 0.6	15 ± 1.2
FXIII-A_2	Rat	8 ± 0.1	17 ± 0.6
FXIII-A_2	Rabbit	20 ± 1.0	7 ± 1.7
FXIII-A_2	Dog	28 ± 0.6	24 ± 0.6
FXIII-A_2	Pig	365 ± 8.0	16 ± 1.5
FXIII-A_2	Cynomolgus	15 ± 0.1	19 ± 0.6

2b

Transglutaminase	Transamidation assay IC_{50} [nmol/L]	Selectivity
TG1	11035 ± 1003	463
TG2	445 ± 20	19
TG3	66511 ± 4544	2791
TG6	17 ± 0.6	1
TG7	1330 ± 102	56

and Western-blotting using monoclonal antibodies directed against the fibrin γ-chain or recognizing the crosslinking site revealed mainly non-crosslinked fibrin degradation products (FDPs). In contrast, in the absence of the inhibitor the larger crosslinked fibrin degradation products (xFDPs) were formed, as expected.

Rotational TEM has been described as a reliable method to detect FXIII in whole human blood in sharp contrast to standard clotting tests.[23,27] TEM uses changes in viscoelasticity along hemostasis, frequently used in the perioperative setting. Even if the method is not meant to quantify FXIII activity, we found TEM being appropriate to monitor the inhibition of plasma transglutaminase. A characteristic thromboelastogram on whole human blood from a healthy volunteer spiked with ZED3197 is shown in Figure 2A. Hemostasis is induced by recombinant tissue factor/phospholipids after recalcification. The clot formation and lysis is monitored over time based on elasticity. Figure 2B depicts the reduction of the maximum amplitude by ZED3197. In a dose-dependent manner (serial dilution 20-0.08 µmol/L compared to control), the inhibitor reduced MCF from usually about 60-65 mm down to a lower limit of 40-45 mm. The absolute numbers depend on the individual blood donor and are therefore compared to control (MCFc). The lower limit of viscosity does not rely on FXIII activity but corresponds to the contribution of aggregating platelets and to fibrin formation,[23] indicating that neither thrombin generation nor platelet activation was disturbed by FXIIIa inhibitors. The EC_{50} (half maximum effective concentration) value for maximum clot firmness reduction compared to control is estimated to be 1.7 ± 0.2 µmol/L. The lysis index at 60 minutes compared to control (LI60c) in the presence of tPA (0.02 µg/mL) also follows a saturation curve but with a remarkably lower EC_{50} of about 0.7 ± 0.2 µmol/L for ZED3197 (Figure 2). It seems conceivable that the inhibitor blocks more efficiently the enzymatic incorporation of α_2-antiplasmin than the

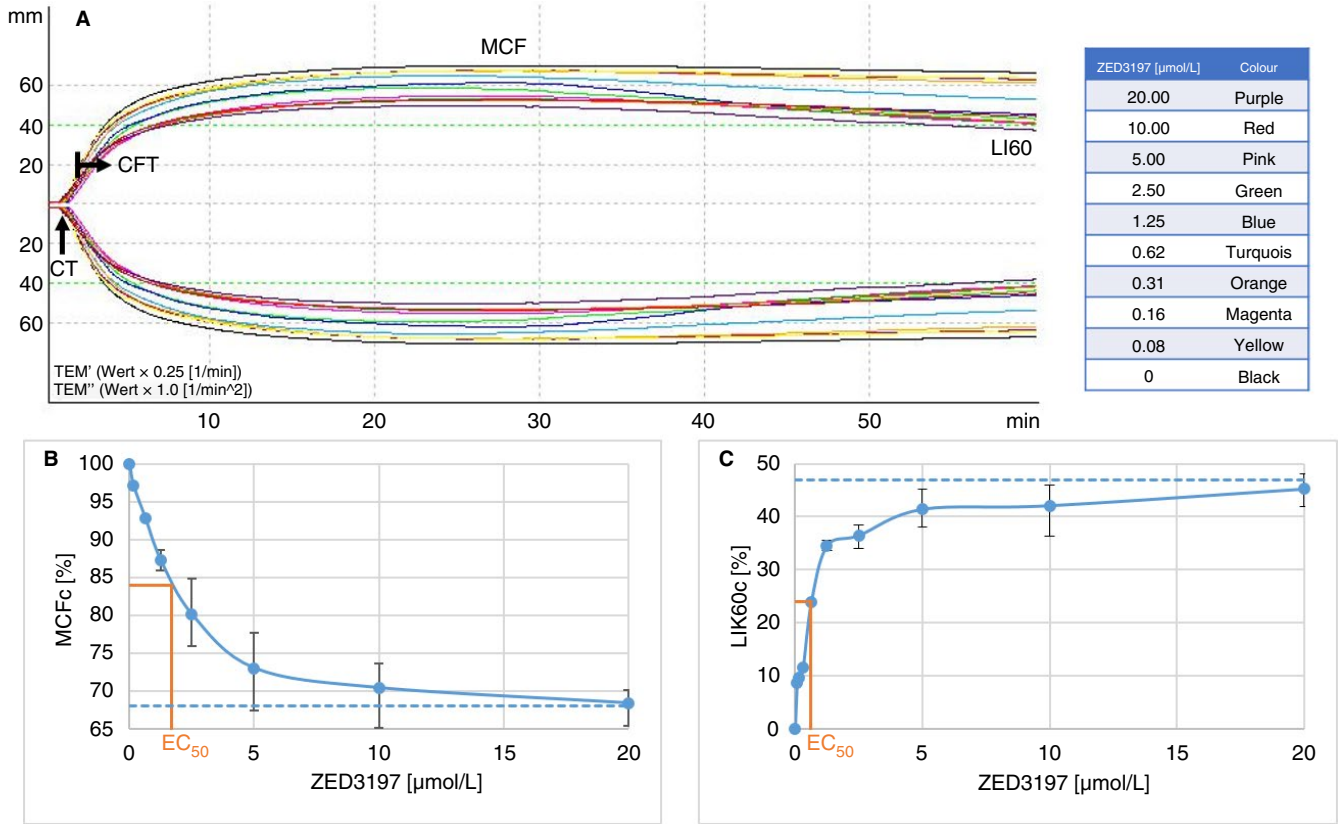

FIGURE 2 A, Thromboelastogram of whole human blood spiked with ZED3197. Thromboelastometry is an established visco-elastic method to monitor key coagulation parameters. Thromboelastograms measured with the ROTEM® device in the presence of ZED3197 (serial dilution 20 µM – 0.08 µM) compared to control are displayed in different colors. While ZED3197 had no influence on the clotting time (CT), e.g. the start of fibrin formation, it slightly delayed the clot formation time (CFT, time to reach firmness of 20 mm). Most suitable parameters to determine efficacy of the FXIIIa inhibitor were the reduction of maximum clot firmness (MCF, Fig. 2b) and the increase of clot lysis at 60 minutes in the presence of 0.02 µg/ml t-PA (LI60, Fig. 2c). B, Reduction of maximum clot firmness compared to inhibitor-free control (MCFc). The maximum clot firmness is a key parameter determined by the ROTEM device. It displays the highest viscosity of the respective blood clot formed induced by the StarTEM reagent in the presence or absence (control) of the ZED3197 compound. The graph shows the dose-dependent decrease of viscosity. The half-maximum effective concentration (EC_{50}) calculated to 1.7 ± 0.2 µM. Please notice that ~32% of clot firmness is due to the FXIII activity while the remaining ~68% are contributed by fibrin and activated platelets. C, Lysis index at 60 minutes in the presence of 0.02 µg/ml t-PA compared to control (LI60c) The lysis index displays the decrease of viscosity triggered by fibrinolysis of the respective clot at 60 minutes. The graph shows the dose-dependent increase of lysis and the calculated EC_{50} value (0.7 ± 0.2 µM) compared to control in the absence of ZED3197.

cross-linking of the "prelocalized" fibrin fibers.[28] The assumption is supported by FXIIIa inhibitor studies performed by Merck Sharp & Dohme scientists. They described differences in effective concentration to inhibit fibrin γ-chain crosslinking, α_2-antiplasmin incorporation and α-chain polymer formation.[18] Further, according to the thromboelastogram, the clot formation time is slightly prolonged, indicating the distinct function of FXIII in clot accretion and propagation. In sharp contrast, the clotting time is not influenced by ZED3197, further supporting that neither thrombin generation nor fibrin formation induced by thromboplastin is perturbed. The unique mode of inhibition becomes obvious when comparing with other anticoagulants like dabigatran. We refer to the thromboelastogram of the direct-acting thrombin inhibitor presented in Figure S3 in supporting information. To further validate our findings, efficacy was compared to the "L-682.777" Merck compound (T101, *vide supra*) again. TEM revealed the same mode of action modulating clot firmness and facilitating fibrinolysis. In this experimental setting, ZED3197 showed to be ~15-fold more potent than T101 (data not shown). Based on the EC_{50} values and the dose-response curves shown in Figure 2B,C, a target concentration of >5 µmol/L was set for animal studies. Exceeding this cut-off value, nearly maximum clot lysis was achieved, considered as most relevant read-out for efficacy. In conclusion, TEM data showed that ZED3197 sufficiently blocks the fibrin-bound FXIII activity in fresh whole human blood, as the "target tissue."

3.3 | In vivo proof-of-principle study in rabbit venous stasis and reperfusion model

The "Wessler" rabbit model of venous stasis and reperfusion[29] was chosen for the proof-of-principle study to elucidate the impact of FXIII inhibition. Initial single-dose pharmacokinetic studies revealed attainment of sufficient plasma levels of ~10 µmol/L of ZED3197 but a short half-life of 5-10 minutes. This issue was overcome by a bolus injection followed by continuous infusion of the compound. According to the protocol detailed in Figure S2, plasma samples were collected and used for FXIII inhibition assays and exposure level analysis. The concentration of ZED3197 determined at the initiation of stasis (15 minutes) was 11.8 ± 1.2 µmol/L and remained at a steady level of 13.6 ± 2.7 µmol/L over the course of the study (30-135 minutes). These concentrations were well above the target concentration of >5 µmol/L. Accordingly, plasma samples taken throughout the study showed FXIII inhibition >95% using the sensitive isopeptidase assay.[21]

Within the proof-of-principle study the primary readout was vessel patency after termination of the stasis. Blood flow of control (2 × PBS/5% glucose vehicle) and ZED3197 treated animals were at the same level upon removal of the clamps. During the course of the experiment blood flow continuously increased for ZED3197 treated animals, while a decrease for control animals was observed (Figure 3). During the reperfusion period, ZED3197 caused an average baseline blood flow recovery of 22%, compared to 6% for the negative control. The mean area under the curve (AUC) of the jugular flow rate normalized to baseline, between time points 35 and 135 minutes, confirmed those results (253 for control versus 662 for ZED3197, Figure 3 upper left graph).

At the end of the experiment, the jugular segment was opened in order to remove and evaluate the formed thrombi. The mean (n = 7) thrombus weight upon ZED3197 treatment was two times lower (P = .0265) compared to the PBS control group (29 ± 6 mg versus 65 ± 13 mg) at comparable thrombus lengths (11 ± 1 mm versus 13 ± 2 mm). Overall the thrombi of rabbits infused with ZED3197 were softer than those exposed only to vehicle, and adhered less to the suture thread which was placed in the jugular vein segment to prevent thrombus embolization during the course of the observation. These data suggest that the inhibitor did not influence the gross fibrin network but clot compaction and clot composition in accordance to the literature.[7] Most remarkably, a template bleeding time was also performed on all animals showing no difference between ZED3197 animals (135 seconds) and controls (134 seconds; Figure 4B).

After 60 minutes of infusion, a blood sample was also taken for TEG analysis at the CRO site. An unpaired Student's *t*-test confirmed that the coagulation index obtained for ZED3197 rabbits (−6.9) was significantly lower than that of the negative control-treated rabbits (−0.6). This hypocoagulable state in the treated group primary assigned to the reduction of clot strength is comparable to the TEM data shown in Figure 2B.

In summary, the data obtained in the rabbit Wessler model in vivo demonstrated ZED3197 being a potent anticoagulant providing >95% inhibition of FXIII activity throughout the study. With respect to target validation, the compound facilitated fibrinolysis without

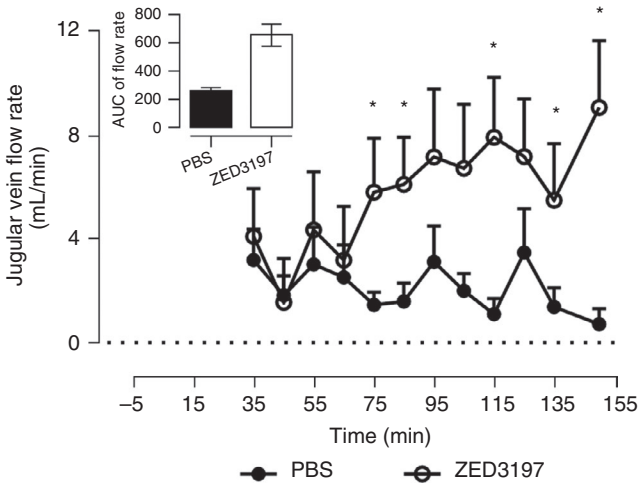

FIGURE 3 Vessel patency expressed as blood flow normalized to baseline according to the study protocol (Figure S2 and table S1 supplemental information) Blood flow is expressed in mL/min. Significantly higher flow rates after ZED3197 infusion compared to PBS control animals are marked by an asterisk ($P \leq 0.05$). Data is depicted as mean ± S.E.M. (n = 6-7). Upper left graph: Area under the curve of flow rate Mean areas under the curve (AUC) of the jugular flow rate normalized to baseline between time points 35 and 135 minutes. Data is depicted as mean ± S.E.M. (n = 6-7).

additional pharmacological intervention and showed no impact on the bleeding time.

4 | DISCUSSION

Anticoagulation therapy aims to attenuate thrombosis without interfering with hemostasis. Even if the direct acting oral anticoagulants have a favorable risk-benefit profile compared to vitamin K antagonists, bleeding remains a major issue.[30] There is still an urgent medical need for safer treatment options. A huge effort is currently being directed toward factor XI and XII as targets for new anticoagulants. These factors upstream to thrombin are considered to be mediators of thrombosis but to play a minor part in hemostasis.[15] It remains to be shown whether clinical trials in certain indications currently under way and future real world data prove superiority compared to the respective standard of care.

FXIII is a unique factor in determining clot stability and half-life downstream to thrombin. However, very few in vivo pharmacological studies using small molecule inhibitors targeting FXIII have been performed so far.[17,18] This is somewhat surprising because direct-acting blockers of coagulation factors are highly attractive, both from a clinical and commercial perspective. An explanation might be that transglutaminases in general are not easily druggable and far away from mainstream targets in drug development; FXIII, in particular, is widely "neglected" and "challenging" as a drug target.[3,31]

To our knowledge ZED3197 is the first drug-like inhibitor of FXIIIa. The peptidomimetic backbone provides affinity and positions the mild electrophilic warhead in perfect orientation to the active site cysteine. The mechanism-based inhibitor efficiently inactivates FXIIIa despite the low intrinsic reactivity of the Michael acceptor warhead—a prerequisite to avoid side effects and toxicity. In vitro, ZED3197 blocks human FXIII in the low nanomolar range. Fibrin clots generated in the presence of the inhibitor are readily hydrolyzed by plasmin yielding non-crosslinked FDPs. The potency of ZED3197 was assessed in more detail by TEM in fresh whole human blood, representing the "target tissue." The inhibitor, in a dose-dependent manner, reduces clot firmness, slightly delays clot formation, and enhances clot lysis in accordance to studies performed with an antibody targeting FXIII.[27] A concentration of >5 µmol/L yielded sufficient efficacy in TEM, for example acceptable reduction of MCF and nearly maximum clot lysis, corresponding to about 40 times the calculated FXIIIA concentration of ~0.125 µmol/L in plasma.[31] In sharp contrast, neither thrombin generation nor platelet activation was disturbed as indicated by the unchanged clotting time. The safety of the compound was further assessed with respect to key off-targets in coagulation and fibrinolysis. Neither thrombin nor plasmin is inhibited by ZED3197 at 100 µmol/L concentration supporting a wide safety margin (data not shown).

In preparation for future in vivo studies ZED3197 also shows excellent potency against FXIII obtained from animals (eg, mouse, rat, rabbit, dog, pig, and cynomolgus monkey)—an important prerequisite for efficacy testing or toxicology studies. Finally, the pharmacology of ZED3197 was evaluated in the "Wessler" model of venous stasis and reperfusion. After removal of the clamps, the blood flow was restored in the presence of the FXIIIa inhibitor. In the control group the vein remained widely occluded. The weight of the blood clots was reduced in the ZED3197 group and the clots appeared to be much "softer." Further the TEG coagulation index decreased while the ear bleeding time was not prolonged. The unchanged bleeding time (about 130 seconds) in the model suggested substantially lower bleeding tendency compared to therapeutic doses of low molecular weight (LMW) heparin (bleeding time >300 seconds), according to historical data in rabbits reported by the CRO. It should be noted that within all the animal studies in the present work no acute adverse or toxic effects were obvious. These in vivo data implied ZED3197, without additional pharmacological intervention with for example t-PA as suggested in former studies,[17,18] to be a potent profibrinolytic compound in this animal model.

FXIII inhibition provides a promising therapeutic approach in hypercoagulable patients, for example in the intensive care setting. Avoiding the formation of stable (micro)thrombi in the vascular system in sensitive organs should be of utmost importance. The

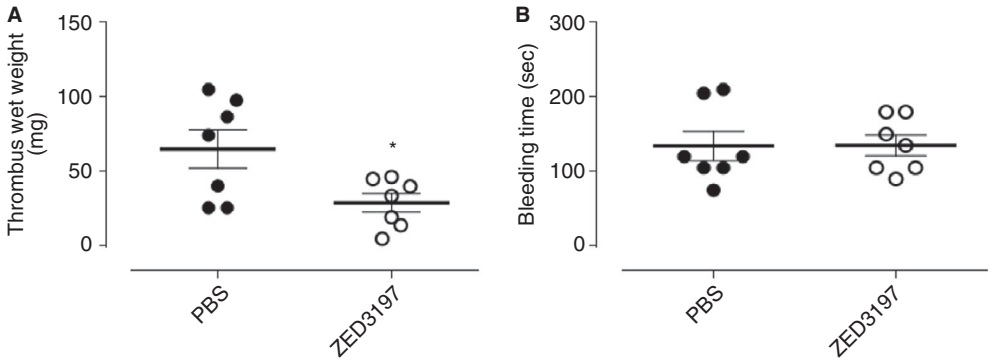

FIGURE 4 A, Thrombus wet weight was determined after 135 min of infusion. Data is depicted as dot plot with mean ± S.E.M. (n = 6-7, $P = 0.0265$). The study protocol is detailed in Figure S2 and table S1 supplemental information. B, Template skin bleeding time was determined after 60 min of infusion. No difference between PBS and ZED3197 was observed. Maximal observation time was pre-defined at 300 seconds. Data is depicted as dot plot with mean ± S.E.M. (n = 7). The study protocol is detailed in Figure S2 and table S1 supplemental information.

approach is supported by studies performed in a rabbit sepsis model showing that depletion of FXIII prevents disseminated intravascular coagulation-induced organ damage.[32] The present study proposes ZED3197 as a viable drug candidate. Considering the molecular weight of the compound (838 g/mol) and the high number of peptide bonds oral bioavailability is very unlikely. The physicochemical properties presuppose parenteral administration. The selectivity of the ZED3197 compound against TG2 is acceptable, at least for short-term usage. TG2 is considered to be the major off-target because it is expressed widely throughout the human body. Most notably, TG2 is the only transglutaminase found in blood, not in plasma but in erythrocytes. However, if TG2 is released from trapped erythrocytes during aging of the thrombi,[8] a certain side-activity of ZED3197 against TG2 might be even favorable. Lack of selectivity toward neuronal transglutaminase (TG6) is considered a minor issue. It seems very unlikely that the compound would reach the nervous system, for example, crossing the blood-brain barrier based on the physicochemical features. Another important point is the reversal of anticoagulants in life-threatening or uncontrolled bleeding events. The IV route of administration and the pharmacokinetic profile obtained in rabbits supports the fast-onset and fast-offset concept. However, accumulation of the compound in certain patients may arise due to insufficient metabolization and/or excretion. Accordingly, the reversal in critical conditions could be indicated. It should be noted that two FXIII preparations are approved by the authorities, containing plasma FXIII-A_2B_2 (Fibrogammin®) or recombinant FXIII-A_2 (NovoThirteen®) representing potential future antidotes.

There are some apparent shortcomings to be considered. One is the short half-life of ZED3197, at least in rabbits. This could be overcome by a loading dose and subsequent continuous infusion to obtain sufficient plasma levels in the study presented here. Accordingly, the IV route of administration including a similar dosing regimen in a potential clinical setting seems likely. Further, the pharmacokinetics of the compound in animals must certainly not translate to the human profile. This assumption is supported by yet unpublished pharmacokinetic data of the more advanced clinical stage "sister compound" ZED1227. ZED1227 is a similar peptidomimetic, first-in-class TG2 inhibitor carrying the identical warhead.[33,34] The inhibitor proved to be safe and well tolerated in humans.

With respect to FXIII inhibition, two issues appear most obvious. First, congenital FXIII deficiency may (but must not) be associated with bleeding diathesis including intracranial bleeding. It has to be underlined that pharmacologic intervention as such is not comparable to severe FXIII deficiency, where both plasma and cellular FXIII is reduced or absent permanently and throughout the whole organism. The optimal benefit-to-risk ratio has to be determined in dose-finding studies. Second, the covalent attachment of clots by FXIIIa to the matrix potentially reduces the risk for thromboembolic events linked to travelling clots within the bloodstream. However, this study and data by others[35] clearly showed that non-crosslinked fibrin clots are prone to fibrinolysis. Therefore, clots may readily be lysed by the endogenous fibrinolytic system.

The study presented here is certainly more mechanistic than disease related. However, data showed that ZED3197 not only modulates the clot structure and composition in vitro. The approach yielded significant flow restoration of an otherwise occluded vein in the rabbit model used, even without the addition of t-PA as suggested in the very few former studies. It remains to be shown if the promising findings translate into therapeutic benefit in certain human disease states. For example, FXIIIa inhibitors could be potentially used clinically to reduce the incidence of acute kidney injury in critically ill patients. Patients that were subjected to continuous renal replacement therapy could also benefit, either in regular hemodialysis or in the intensive care unit. The treatment might even be considered on top of standard of care (still heparins) due to the low bleeding risk with an additional efficacy benefit and ultimately better outcome. However, comprehensive preclinical safety studies are mandatory before the pharmacokinetics and pharmacodynamics of the new chemical entity can be explored in clinical trials. In conclusion, we consider ZED3197 to be a viable drug candidate at least for short-term anticoagulation to modulate clot structure and enhance fibrinolysis. The approach is predicted to dissociate an antithrombotic effect from increased bleeding tendency.

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CONFLICT OF INTEREST

C.B., C.P., R.J., M.S., A.H. are employees; R.P. and M.H. are employees and shareholders of Zedira. C.B., M.H., and R.P. are co-inventors of a patent application claiming the ZED3197 compound. P.F., G.N., U.G. received payments for consultancy.

AUTHOR CONTRIBUTIONS

R. Pasternack and M. Hils created the project, planned experiments, and wrote the article; C. Büchold planned the organic synthesis, analyzed data, and provided intellectual input; R. Jähnig and M. Sommer synthesized key reagents; C. Pelzer and A. Heil planned, analyzed, and performed experiments; P. Florian, G. Nowak, and U. Gerlach planned the experiments and provided intellectual input.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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