


Pharmacological profile of asundexian, a novel, orally bioavailable inhibitor of factor XIa

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Abstract

Background: Activated coagulation factor XI (FXIa) contributes to the development and propagation of thrombosis but plays only a minor role in hemostasis; therefore, it is an attractive antithrombotic target.

Objectives: To evaluate the pharmacology of asundexian (BAY 2433334), a small molecule inhibitor targeting FXIa, *in vitro* and in various rabbit models.

Methods: The effects of asundexian on FXIa activity, selectivity versus other proteases, plasma thrombin generation, and clotting assays were evaluated. Antithrombotic effects were determined in FeCl₂- and arterio-venous (AV) shunt models. Asundexian was administered intravenously or orally, before or during thrombus formation, and with or without antiplatelet drugs (aspirin and ticagrelor). Potential effects of asundexian on bleeding were evaluated in ear-, gum-, and liver injury models.

Results: Asundexian inhibited human FXIa with high potency and selectivity. It reduced FXIa activity, thrombin generation triggered by contact activation or low concentrations of tissue factor, and prolonged activated partial thromboplastin time in human, rabbit, and various other species, but not in rodents. In the FeCl₂-injury models, asundexian reduced thrombus weight versus control, and in the arterial model when added to aspirin and ticagrelor. In the AV shunt model, asundexian reduced thrombus weight when administered before or during thrombus formation. Asundexian alone or in combination with antiplatelet drugs did not increase bleeding times or blood loss in any of the models studied.

Conclusions: Asundexian is a potent oral FXIa inhibitor with antithrombotic efficacy in arterial and venous thrombosis models in prevention and intervention settings, without increasing bleeding.

KEYWORDS

anticoagulant, asundexian, BAY 2433334, factor XIa inhibitor, thrombosis

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1 | INTRODUCTION

Direct oral anticoagulants (DOACs) have markedly improved the management of thrombotic disorders compared to vitamin K antagonists or heparins.¹ However, bleeding remains a concern and dosing requires a careful balance between efficacy and bleeding risk, particularly in indications with a need for higher therapeutic doses (e.g., under-treatment conditions) or in which antiplatelets are standard of care and the addition of anticoagulants may be beneficial. Establishing a “sweet spot” in the latter indications remains difficult. So far only dual antiplatelet therapy (DAPT) with rivaroxaban at a very low dose has been successful in the secondary prevention of acute coronary syndrome, coronary artery disease, or peripheral artery disease, albeit with an increased bleeding risk compared to antiplatelets alone.²⁻⁵ Thus, a need remains for new therapies that offer a reduced risk of bleeding and equivalent or superior antithrombotic efficacy.¹

Factor XI (FXI) is the zymogen of the serine protease activated FXI (FXIa). It is activated by factor XIIa (FXIIa) after contact activation, as part of an amplification loop by thrombin or through autoactivation by FXIa.⁶ As such, FXIa or FXI represent interesting therapeutic targets because inhibition of FXIa may block contact pathway-initiated coagulation as well as amplification-associated clot propagation while leaving the tissue factor pathway intact, a pathway that plays a major role in stopping bleeding after vessel wall injury. Consequently, FXIa inhibitors have the potential to be antithrombotic without compromising hemostasis.⁷ This has been confirmed by experimental data with inhibitors of FXIa or with FXI-depleting strategies,⁷⁻¹⁵ as well as by clinical observations from individuals with congenital FXI deficiency,^{16,17} and from randomized controlled trials of FXI or FXIa inhibitors.¹⁸⁻²¹

It has long been a challenge to identify potent FXIa inhibitors with a pharmacokinetic profile suitable for oral administration. However, two orally available small-molecule FXIa inhibitors—milvexian and asundexian—are currently in clinical development. This study investigates the preclinical pharmacology of asundexian (BAY 2433334), which binds to the active center of FXIa.

2 | MATERIALS AND METHODS

2.1 | *In vitro* studies

Asundexian, rivaroxaban, dabigatran, and apixaban were synthesized at the laboratories of Bayer AG.

Whole blood of anesthetized mice, rats, and guinea pigs was drawn from the vena cava. Dog blood was taken from the antecubital vein, while rabbit and pig whole blood were drawn from the carotid artery. All animal blood samples were collected from untreated donor animals into citrate-containing Monovette tubes (Sarstedt), and centrifuged at 2500 ×g for 20 min at 4°C. Citrated plasma samples were stored at -20°C prior to use.

Human blood was obtained by venipuncture from healthy volunteers who had received no medication during the previous 10 days. All

Essentials

- Asundexian is a small molecule inhibitor of human activated coagulation factor XI (FXIa).
- Asundexian dose-dependently reduced arterial and venous thrombosis in animal models.
- Mono- or combination therapy with asundexian did not increase bleeding.
- Further evaluation of asundexian in patients with, or at risk of, thrombosis is ongoing.

blood samples were collected into plastic tubes and diluted 1:10 with 3.8% trisodium citrate. Platelet-poor plasma was obtained immediately after collection by centrifugation at 2500 ×g for 10 min at 4°C and stored at -20°C. Human plasma was sourced from 10 volunteers and pooled. Human citrated plasma was purchased from Octapharma.

2.1.1 | Inhibition of human FXIa and selectivity versus other serine proteases

The potency of asundexian against human FXIa activity in buffer was analyzed in biochemical assays based on the fluorimetric detection of aminomethylcoumarine (AMC) cleaved from a synthetic peptidic substrate by FXIa.

Asundexian was diluted in dimethylsulfoxide (DMSO) to final assay concentrations of 0–50 μM. Human FXIa (Kordia) and the FXIa substrate Boc-Glu(OBzl)-Ala-Arg-AMC (Bachem I-1575) were diluted in buffer solutions (pH 7.4) consisting of 50 mM Tris/HCl, 100 mM NaCl, 5 mM CaCl₂, and 0.1% bovine serum albumin to final concentrations of 0.15 nM and 5 μM, respectively. Diluted asundexian or DMSO (1 μl) in assay buffer (20 μl), diluted FXIa solution (20 μl), and substrate solution (20 μl) were added to 384-well microtiter plates (Greiner Bio-One). Fluorescence (excitation 360 nm, emission 460 nm) was measured using a microtiter plate fluorescence reader (Safire II, Tecan) for 30 min at room temperature. The concentration of asundexian producing 50% inhibition of FXIa activity (IC₅₀) was determined via a nonlinear logistic regression model.

The selectivity of asundexian versus other serine proteases of the hemostatic system was evaluated in buffer in biochemical assays based on the fluorimetric detection of AMC released from specific substrates for respective proteases as described above. The details for each assay are described in Appendix S1 in supporting information. IC₅₀ values were determined via a nonlinear logistic regression model.

2.1.2 | Inhibition of FXIa and plasma kallikrein in plasma

The FXIa inhibitory effects of asundexian in plasma following contact activation were investigated using similar methodologies to those

described above. Asundexian in 3 μ l DMSO was added to 50 μ l of pooled human plasma or rabbit plasma to yield final concentrations of 0–50 μ M. To 20 μ l of these mixtures, 20 μ l of a kaolin/cephalin suspension (C.K. Prest, Diagnostica Stago) were added. After incubation for 3 min at 37°C, 20 μ l of an aqueous FXIa substrate solution (Bachem I-1575; final assay concentration 400 μ M) with a plasma kallikrein inhibitor (BAY 2292992; final assay concentration 5.6 μ M) were added. Fluorescence (excitation 360 nm, emission 460 nm) was measured for 40 min at 37°C, and the slope of fluorescence generation during the first 5 min was calculated. The rate of inhibition was determined relative to the slope of samples without inhibitor; IC₅₀ values were calculated as described above.

To investigate the inhibitory effects on plasma kallikrein after contact activation in plasma, 20 μ l of a mixture of human plasma with asundexian in DMSO were added to 20 μ l of the kaolin/cephalin suspension. After incubation for 3 min at 37°C, 20 μ l of an aqueous plasma kallikrein substrate solution (H-Pro-Phe-Arg-AMC; Bachem I-1295; final concentration 50 μ M) together with 3.3 μ g/ml corn trypsin inhibitor (CTI; Haematologic Technologies) were added and fluorescence was measured for 40 min at 37°C. IC₅₀ values were determined as described above.

2.1.3 | Effect of asundexian on thrombin generation in plasma

For this assay, asundexian was diluted in DMSO to final concentrations of 0–30 μ M. Samples of pooled human plasma (78 μ l) were transferred to 96-well microtiter plates (Greiner Bio-One) and mixed with 2 μ l/well of the serial dilutions of asundexian or DMSO alone. Samples (20 μ l) of a solution containing phospholipids (final concentration 4 μ M) and either tissue factor (final concentrations 5 or 0.1 pM, respectively) or 2.5% ellagic acid (Actin® FS, Siemens) were added, and the mixture incubated for 10 min at 37°C. Thrombin generation was initiated by addition of 20 μ l of a FluCa buffer solution containing the substrate Z-Gly-Gly-Arg-AMC and CaCl₂. Fluorescence measurements (excitation 390 nm, emission 460 nm) commenced immediately using a Fluoroskan Ascent fluorometer (Thermo Fisher). A plot of thrombin concentration versus time was calculated from the slope of the fluorescence curves using the Thrombin Calibrator and thrombinoscope software (Thrombinoscope BV). Lag time and peak thrombin concentration were calculated from the thrombogram.

2.1.4 | Effect of asundexian on clotting times in plasma

The effects of asundexian on clotting times were assessed by measurement of activated partial thromboplastin time (APTT) and prothrombin time (PT) in plasma from various species, including rabbit, dog, pig, guinea pig, mouse, and rat, as well as in human citrated plasma.

For APTT measurement, plasma was incubated with increasing concentrations of asundexian (0–37.5 μ M) for 3 min at 37°C, after

which 50 μ l aliquots were incubated for 3 min with 50 μ l of an APTT reagent (C.K. Prest, Diagnostica Stago). Coagulation was initiated by addition of 50 μ l of 0.025 M CaCl₂, and the clotting time measured by an automated coagulometer (AMAX 200, Trinity Biotech). The concentration of asundexian producing a 1.5-fold increase in APTT was reported as the EC₁₅₀.

Prothrombin time was measured in plasma samples incubated with increasing concentrations of asundexian (0–37.5 μ M) for 3 min at 37°C. Samples (50 μ l) were mixed with 100 μ l of thromboplastin (RecombiPlasTin, Instrumentation Laboratory), and the clotting time measured at 37°C using an automated coagulometer.

2.2 | *In vivo* studies

Further methodological details can be found in Appendix S1, method 2 and Figure S5.

2.2.1 | Combined arterial thrombosis (FeCl₂-injury) and bleeding time model in rabbits

The antithrombotic effects of asundexian in a prevention setting were assessed in a rabbit arterial thrombosis model simultaneously with determination of effects on ear bleeding time. Two series of experiments were performed. In the first series, various intravenous and oral regimens of asundexian were compared to intravenous rivaroxaban and/or control (vehicle). The second series investigated the combination of intravenous asundexian plus DAPT (aspirin [acetylsalicylic acid] and ticagrelor), versus DAPT alone or apixaban plus DAPT. Details of used regimens in these experiments are shown in Table S1 in supporting information.

Anesthesia was induced and maintained in male New Zealand White rabbits (2.7–3.0 kg) with ketamine and xylazine (intramuscular bolus of 40 mg/kg +5 mg/kg, and 800 mg +80 mg in 12 ml with an infusion rate of 5 ml/hour via an ear vein, respectively). Femoral artery and vein were exposed and cannulated for arterial blood sampling and intravenous drug administration, respectively. Thrombosis was induced 30 min after treatment by exposing the carotid artery to FeCl₂ (13% in 100 μ l water, Merck KGaA) on a filter paper for 5 min. Thirty minutes after application of FeCl₂, the injured artery was excised, and the thrombus removed for weighing.

Shortly after FeCl₂ injury and simultaneously to thrombus generation, ear bleeding was initiated by making a standardized 5 mm skin cut using a scalpel blade parallel to the external vein of the ear, avoiding direct injury of a visible vessel. Blood was removed gently by swabbing with a filter paper at 10-s intervals. Bleeding time was recorded as the time at which bleeding stopped. Equivalent techniques were applied to evaluate bleeding times from the gum mucosa.

At the start of the thrombosis experiment, blood was drawn and collected in 2.9 ml citrated tubes (Sarstedt). Plasma was obtained by centrifugation at 2500 \times g for 10 min at 4°C and was stored at –20°C. Rabbit plasma concentrations of asundexian were determined by

liquid chromatography–tandem mass spectrometry using an internal standard. The applied sample preparation procedure involved protein precipitation followed by high-performance liquid chromatography (HPLC) separation and tandem mass spectrometric detection. The HPLC system was coupled in-line to a tandem mass spectrometer (Sciex API 5500) via a Turbo Spray interface. Plasma concentrations were logarithmically transformed and plotted against thrombus weight to determine the half-maximal effective concentration (EC_{50}).

Rate of FXIa inhibition by asundexian was assessed fluorimetrically and the APTT was measured mechanically as described above.

2.2.2 | Combined venous thrombosis (FeCl₂-injury) and bleeding time model in rabbit

Anesthesia was induced and maintained in male New Zealand White rabbits (2.7–3.0 kg) using the same regimen described above. A femoral artery and vein were exposed and cannulated for arterial blood sampling and intravenous drug administration, respectively. Thrombosis was induced 30 min after intravenous treatment with asundexian by exposing the jugular vein to FeCl₂ (13% in 100 μ l water, Merck KGaA) on a filter paper for 5 min. After an additional 30 min, the injured part of the vein was excised, and the thrombus removed for weighing. The effect of drug treatment on ear bleeding was assessed as described above.

2.2.3 | Liver injury in a rabbit model

The effect of asundexian was evaluated in a rabbit liver-injury model compared with vehicle and dabigatran.

Anesthesia was induced and maintained in male New Zealand White rabbits (2.5–3.3 kg) as described above. The rabbits were maintained at 37°C throughout the experiment. Forty-five minutes before the liver-injury procedure was initiated, rabbits received intravenous injections of 6 or 12 mg/kg asundexian, 0.3 or 1 mg/kg dabigatran, or vehicle (10% ethanol/40% polyethylene glycol/50% sodium chloride [0.9%]). The abdominal cavity was opened directly beneath the costal arch, and the liver was exposed. A triangular wedge (1 cm length and 1 cm width) was removed from the outer edge of the liver, and non-adhesive absorbent swabs (Medtronic Inc.) were placed in the wound in 30-s intervals for 30 min. The pre-weighed swabs were collected and the magnitude of blood loss was determined.

Intravenous blood samples were obtained at the start of the bleeding procedure. APTT and PT were measured as described above.

2.2.4 | Arterio-venous shunt model in preventive and intervention settings

The *in vivo* antithrombotic effects of asundexian in an arterio-venous (AV) shunt model were assessed using male New Zealand White rabbits (2.7–3.0 kg). Anesthesia was induced and maintained as described previously.

A femoral artery and vein were used for blood sampling and intravenous drug administration, respectively. Pieces of Braun venous catheter (1.4/2.1 mm inner/outer diameter), filled with physiological saline, were tightly implanted into the left jugular vein and right carotid artery and connected with a 4 cm polyethylene 240 tube containing a 6 cm loop of nylon wire (Mitchell fishing line, 0.14 mm diameter). Thrombosis experiments were started by opening the shunt.

In the prevention protocol, asundexian was administered as an intravenous bolus (0.3, 1, or 3 mg/kg) 10 min prior to opening the shunt, while asundexian was administered as intravenous bolus (1 or 5 mg/kg) 10 min after start of the experiment in the intervention protocol. After shunt opening for 15 min in the prevention setting and 30 min in the intervention protocol, the catheter was excised and the nylon wire, including the attached thrombus, was removed for weighing.

After blood collection in 2.9 ml citrated tubes and plasma preparation, plasma concentrations of asundexian, rates of inhibition of FXIa, and APTT prolongation were determined as described above.

2.3 | Statistical methods

Descriptive statistics were prepared for all variables after confirmation of normality (*in vitro*: mean \pm standard error of the mean [SEM] or standard deviation [SD]; *in vivo*: median with 25th and 75th percentiles). Differences in thrombus weights and in bleeding times between asundexian and comparators were analyzed by Kruskal–Wallis analysis followed by Dunn's multiple comparison tests.

Graphs were prepared and all statistical analyses were performed using GraphPad Prism software and adjusted alpha errors of $P < .05$ were considered statistically significant.

2.4 | Ethical approval

Collection of human blood by venipuncture was approved by the Ethics Committee of the North Rhine Medical Association (Ärzttekammer Nordrhein). All animal experiments were approved by the State Office for Environment, Nature and Consumer Protection North Rhine-Westphalia (Landesamt fuer Umwelt, Natur und Verbraucherschutz NRW), Recklinghausen, Germany.

3 | RESULTS

3.1 | *In vitro* properties of asundexian

3.1.1 | Inhibition of human FXIa and selectivity versus a hemostasis protease panel

In vitro fluorometric assays showed that asundexian produced a concentration-dependent, reversible reduction in human FXIa activity, with a mean (\pm SD) IC_{50} value of 1.0 (\pm 0.17) nM in buffer (Figure 1A; Figure S4 in supporting information), as IC_{50} values were

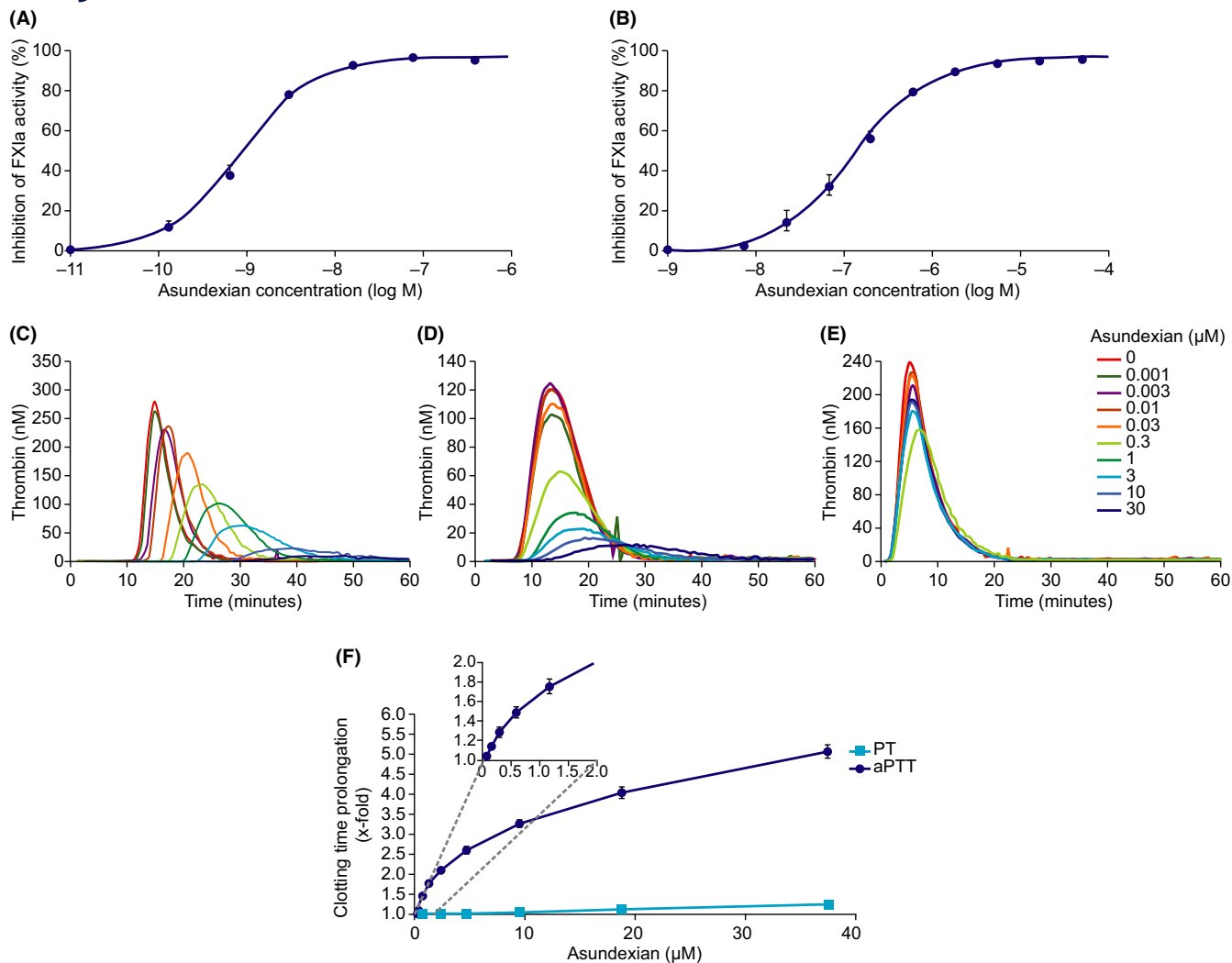


FIGURE 1 Effect of asundexian on human FXIa activity in buffer (A; $n = 6$) and human plasma (B; $n = 4$). Results are presented as mean \pm SEM. Representative thrombograms showing the effect of asundexian on thrombin generation initiated by the FXII activating agent ellagic acid (Actin® FS) 2.5% (C), or 0.1 pM or 5 pM tissue factor (D and E). Effect of asundexian on clotting time prolongation in human plasma, with the inset expanding the curve for APTT over the asundexian 0–2 μ M range (F). Results are presented as mean \pm SEM ($n = 2$ –9). APTT, activated partial thromboplastin time; FXIa, activated coagulation factor XI; PT, prothrombin time; SEM, standard error of the mean

determined at substrate concentrations much lower than the substrate, $K_i \approx IC_{50}$. Asundexian did not inhibit other proteases, including thrombin, FXa, FIXa, FXIIa, FVIIa, urokinase, tissue plasminogen activator, plasmin, activated protein C, trypsin, and chymotrypsin at concentrations more than 1000-fold higher, except for plasma kallikrein, which had a mean (\pm SD) IC_{50} of 6.7 (\pm 1.5) nM (Table S2 in supporting information).

3.1.2 | Inhibition of FXIa or plasma kallikrein in plasma

When triggering FXI activation via the contact activation pathway, asundexian reduced FXIa activity in human plasma with a mean (\pm SD) IC_{50} of 0.14 (\pm 0.04) μ M (Figure 1B). In rabbit plasma,

asundexian was 3.8-fold less potent (IC_{50} 0.54 [\pm 0.11] μ M). Under similar conditions, but with an adequate substrate, human plasma kallikrein was inhibited with a mean (\pm SD) IC_{50} of 1.23 (\pm 0.07) μ M.

3.1.3 | Impact on thrombin generation in plasma

Following initiation of the coagulation system by either a low concentration (0.1 pM) of tissue factor or a FXII activating agent (Actin® FS), asundexian markedly inhibited thrombin formation, with an IC_{50} for peak height of 0.3 μ M (representative examples in Figure 1C,D). While asundexian resulted in a concentration-dependent effect on the lag time following contact activation by Actin® FS, this effect was small when triggered using a low tissue factor (TF) concentration.

TABLE 1 Prolongation of APTT by asundexian in plasma (mean, $n = 2-9$)

	Human	Rabbit	Dog	Pig	Guinea pig	Mouse	Rat
APTT prolongation, EC_{150} , μM	0.61	1.50	1.59	0.51	2.13	>12.5	>12.5

Abbreviations: APTT, activated partial thromboplastin time; EC_{150} , effective concentration to achieve 50% prolongation.

By contrast, asundexian had no effect on thrombin generation in the presence of a high concentration (5 μM) of tissue factor (representative example in [Figure 1E](#)).

3.1.4 | Effect of asundexian on clotting times in plasma

Asundexian produced a concentration-dependent prolongation of APTT in human plasma, with a mean (\pm SD) EC_{150} of 0.61 (\pm 0.10) μM ([Figure 1F](#)). By contrast, asundexian had little to no effect on PT. Experiments using plasma from other species showed that asundexian prolonged APTT to a similar extent in pig plasma (EC_{150} 0.51 μM), and to a lesser extent in rabbit (EC_{150} 1.50 μM), dog (EC_{150} 1.59 μM), and guinea-pig plasma (EC_{150} 2.13 μM), but had virtually no effect in mouse or rat plasma (EC_{150} >12.5 μM ; [Table 1](#)).

3.2 | In vivo profile of asundexian

3.2.1 | Evaluation in an arterial FeCl_2 -injury rabbit model after intravenous or oral administration and in combination with DAPT

Prophylactic intravenous administration of asundexian produced a dose-dependent reduction in thrombus weight compared to control in the FeCl_2 -injury model ([Figure 2A](#)) with no increase in bleeding time ([Figure 2B](#)). Thrombus weight decreased from a mean (\pm SEM) of 18.7 (\pm 1.1) mg in the control group to 14.0 (\pm 1.8) mg (25% reduction) in animals receiving asundexian 0.6 mg/kg, and 2.2 (\pm 0.6) mg (88% reduction) in animals receiving 20 mg/kg ([Figure 2A](#)). The EC_{50} of asundexian was 380 $\mu\text{g/L}$. Following oral administration of asundexian 10 mg/kg and 30 mg/kg, thrombus weight was significantly reduced by 30% ($P < .05$) and 91% ($P < .001$) compared to the control group, respectively, with no prolongation of ear bleeding times at either dose ([Figure S1](#) in supporting information).

Intravenous administration of rivaroxaban had a similar dose-dependent effect on thrombus weight as intravenous asundexian, with mean reductions of 51% and 77% for the 0.08 mg/kg +0.09 mg/kg/hour and 0.24 mg/kg +0.27 mg/kg/hour doses, respectively ([Figure 2A](#)). In contrast to asundexian, rivaroxaban prolonged ear bleeding time compared to controls (significant for the higher dose; [Figure 2B](#)). Similar results were obtained for gum bleeding ([Figure 2C](#)).

Ex vivo measurements revealed a dose-dependent increase in asundexian plasma concentrations, FXIa inhibition, and APTT prolongation following intravenous administration of asundexian ([Figure 2D-F](#)). At the EC_{50} of 380 $\mu\text{g/L}$, APTT was prolonged by a factor of approximately 1.4. There was a strong negative correlation between thrombus weight and APTT prolongation ($R^2 = .76$; [Figure 2I](#)), consistent with the strong negative correlation seen between thrombus weight and asundexian plasma level and FXIa activity inhibition, respectively ([Figure 2G,H](#)). Following oral administration, asundexian 10 mg/kg and 30 mg/kg prolonged *ex vivo* APTT by 1.15-fold and 1.45-fold, respectively, and reduced FXIa activity by a mean (\pm SD) of 17% (\pm 22%) and 44% (\pm 29%), respectively, compared to the control group (data not shown).

Coadministration of intravenous asundexian with aspirin and ticagrelor produced additional dose-dependent reductions in thrombus weight: an additional 32% with an asundexian dose of 2 mg/kg, 70% with a 6 mg/kg dose, and 98% with a 12 mg/kg dose ([Figure 3A](#)). However, it did not further increase bleeding time beyond that observed following coadministration of aspirin and ticagrelor alone ([Figure 3B](#)). Coadministration of intravenous apixaban 1 and 3 mg/kg with aspirin and ticagrelor was also associated with a significant additional reduction of 46% and 83% in thrombus weight, respectively ([Figure 3A](#)). However, apixaban significantly increased bleeding time compared to aspirin and ticagrelor alone by 2.8-fold and 6.6-fold, respectively ([Figure 3B](#)).

3.2.2 | Evaluation of asundexian in a combined FeCl_2 -injury venous thrombosis and ear bleeding rabbit model

Intravenous asundexian produced a dose-dependent reduction in thrombus weight ([Figure 4A](#)) in the venous FeCl_2 -injury model, from a mean (\pm SEM) of 6.1 (\pm 1.6) mg in the control group to 4.5 (\pm 1.2) mg (25% reduction), 1.6 (\pm 0.5) mg (74% reduction), or 0.6 (\pm 0.6) mg (90% reduction) in animals receiving asundexian 0.6, 2, or 6 mg/kg, respectively. No ear bleeding time prolongation was observed in any of the dose groups ([Figure 4B](#)).

3.2.3 | Impact of asundexian on bleeding in the rabbit liver-injury model

In this model, neither 6 nor 12 mg/kg asundexian led to any significant effect on bleeding time or blood loss compared to

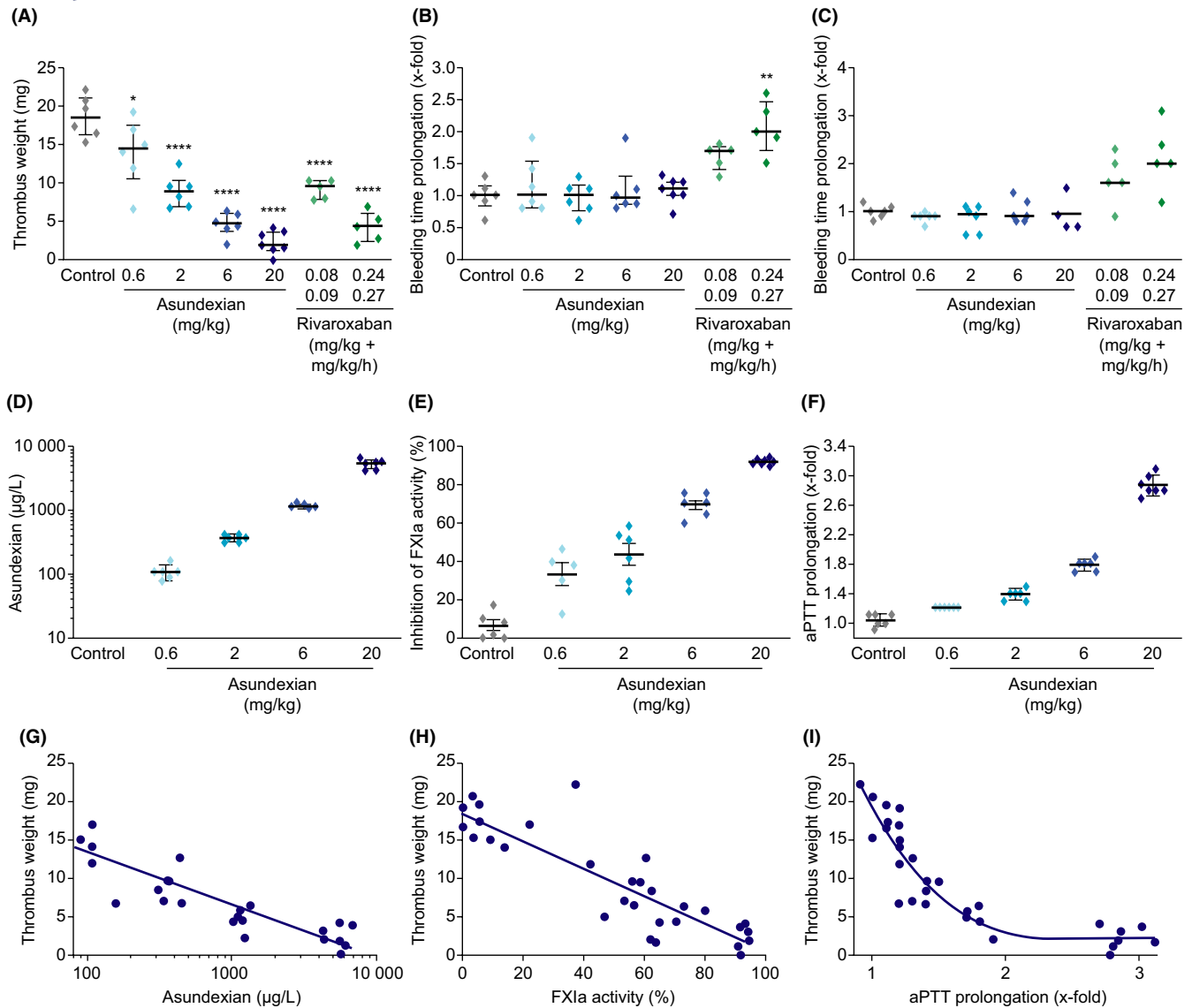


FIGURE 2 Effects of asundexian versus rivaroxaban on thrombus weight following FeCl_2 -induced damage to the carotid artery (A), ear bleeding time (B), and gum bleeding time (C) in rabbits. Plasma concentrations of asundexian (D), inhibition of FXIa activity (E), and APTT prolongation (F) according to asundexian dose, and correlation of plasma concentrations (G), inhibition of FXIa (H), and APTT prolongation (I) with thrombus weight. Asundexian was administered as an intravenous bolus, whereas rivaroxaban was administered as an intravenous bolus followed by continuous infusion. Results are presented as individual values and median with 25th and 75th percentiles (Parts A–F). * $P < .05$, **** $P < .001$. APTT, activated partial thromboplastin time; FXIa, activated coagulation factor XI

controls (Figure 5). By contrast, dabigatran 0.3 mg/kg increased bleeding time approximately 5-fold compared to the mean value of 5.7 min (340 s) in controls, and with a dose of 1 mg/kg dabigatran bleeding continued beyond the maximum observation period of 30 min (Figure 5).

Both doses of asundexian significantly prolonged APTT (low dose: 1.7-fold increase; high dose: 2.3-fold increase), but had no effect on PT, whereas the higher dose, but not the lower dose, of dabigatran significantly prolonged both APTT (low dose: 1.4-fold increase; high dose: 2.2-fold increase) and PT (low dose: 1.1-fold increase; high dose: 1.7-fold increase).

3.2.4 | Impact of asundexian on already initiated thrombus formations in a rabbit AV shunt model

Preventative intravenous administration of asundexian produced a dose-dependent reduction in thrombus weight (Figure 6A), without increasing bleeding (Figure S2 in supporting information). Thrombus weight was decreased from a mean (\pm SEM) of 14.8 (\pm 2) mg in the control group to 12.7 (\pm 2.2) mg (14% reduction), 6.1 (\pm 0.9) mg (59% reduction; $P < .01$), and 4.3 (\pm 0.5) mg (71% reduction; $P < .001$) with the intravenous doses of 0.3, 1, and 3 mg/kg, respectively (Figure 6A). Bleeding times following ear incision were

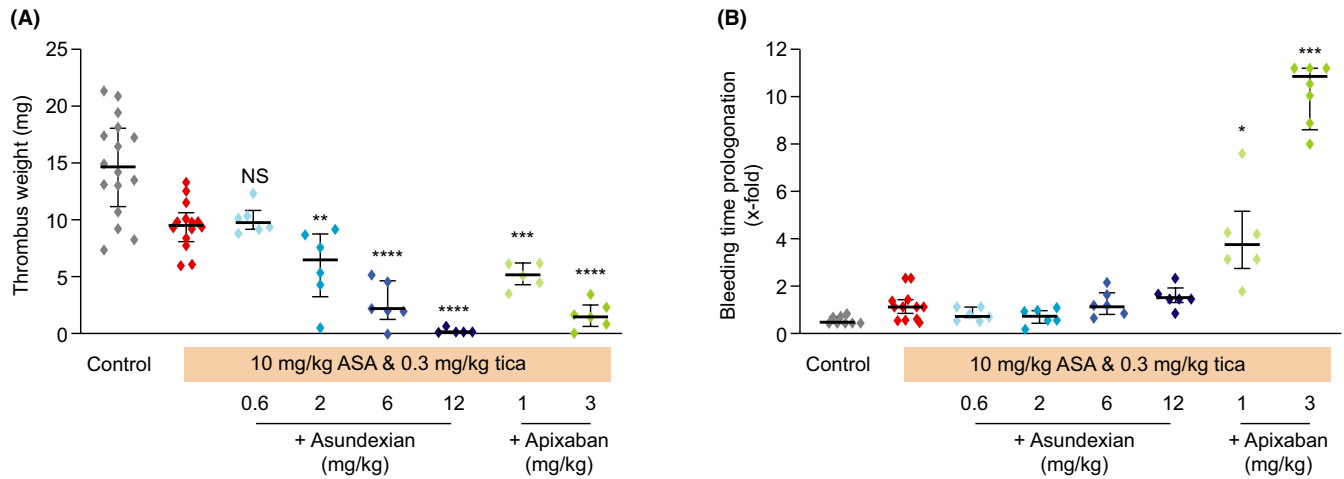


FIGURE 3 Effects of asundexian in combination with aspirin/ticagrelor versus apixaban in combination with aspirin/ticagrelor on thrombus weight following FeCl_2 -induced damage to the carotid artery (A) and ear bleeding time (B) in rabbits. Results are presented as individual values and medians (\pm interquartile range). Adjusted *P*-values versus control: **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. ASA, acetylsalicylic acid (aspirin); Tica, ticagrelor

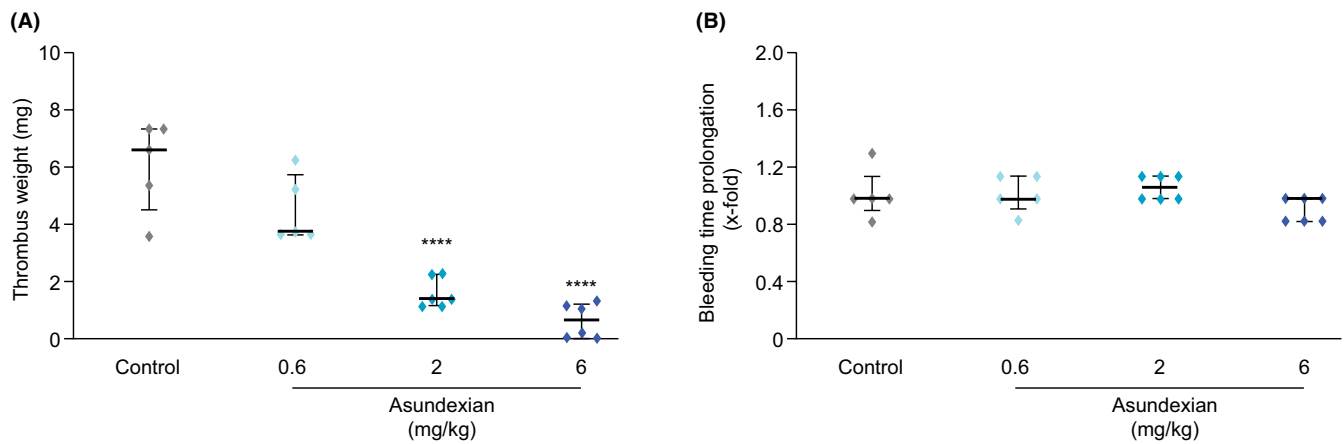


FIGURE 4 Effects of asundexian on thrombus weight (A) and ear bleeding time prolongation (B) following FeCl_2 -induced damage to the jugular vein in rabbits. Asundexian was administered as an intravenous bolus. Results are presented as individual values and medians (\pm interquartile range). *****P* < .001

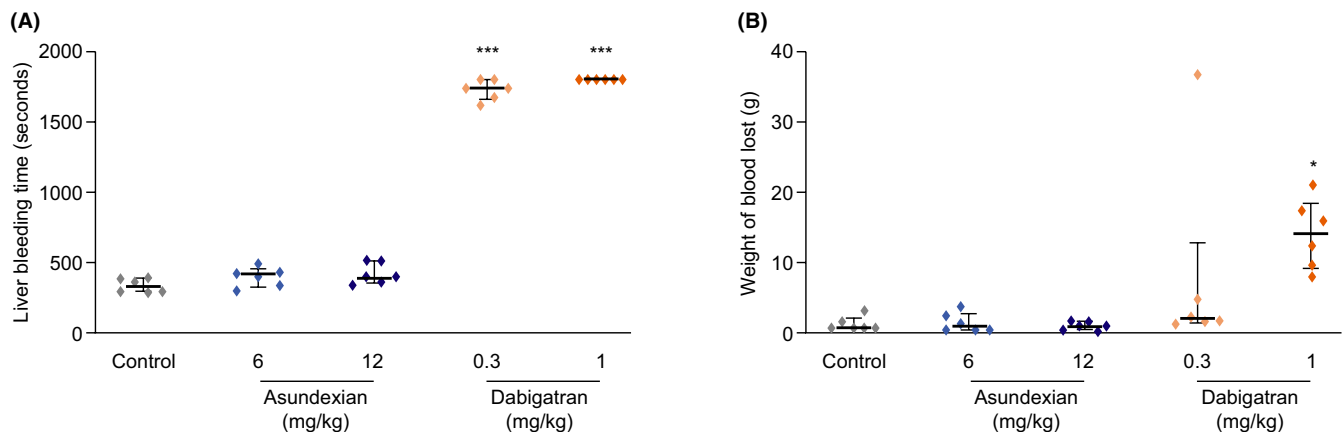


FIGURE 5 Effect of intravenously administered asundexian 6 mg/kg and 12 mg/kg on bleeding time (A) and blood loss (B) in the rabbit liver-injury model. Results are presented as medians with interquartile ranges (*n* = 6). **P* < .05, ***P* < .001, ****P* < .0001 versus controls

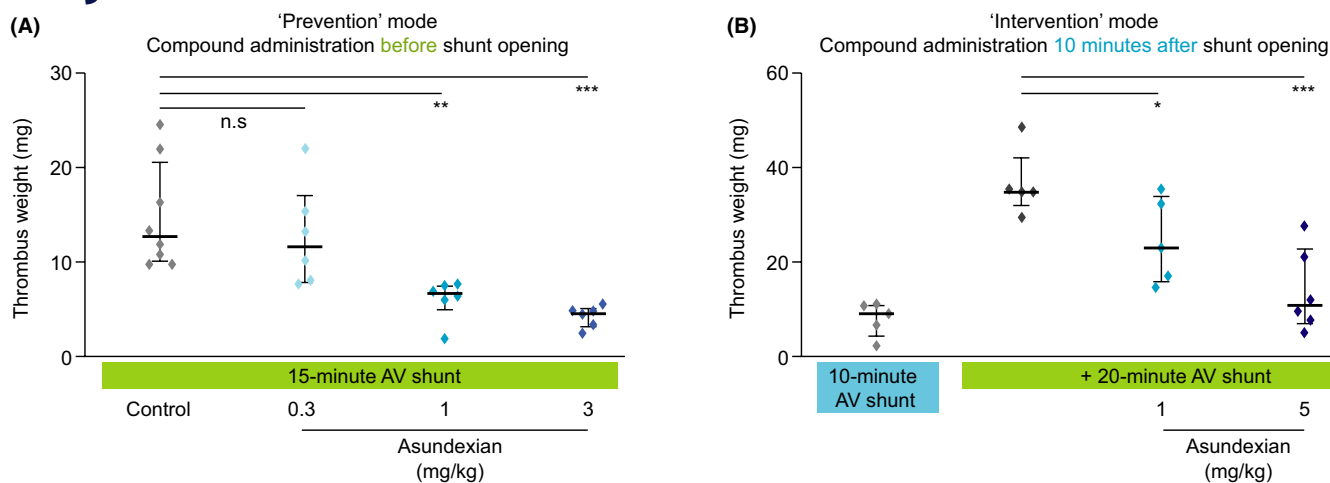


FIGURE 6 Effects of asundexian in the AV-shunt model in rabbits on thrombus weight in the prevention protocol (A) and intervention protocol (B). Asundexian was administered as an intravenous bolus 10 min before or 10 min after the shunt was opened, respectively. Results are presented as individual values (diamonds) and mean values (bars) (\pm interquartile range). * $P < .05$, ** $P < .01$, *** $P < .001$. AV, arterio-venous

not prolonged by asundexian at any dose or time point after administration (Figure S2).

When given during ongoing thrombus formation (interventional protocol), intravenous asundexian significantly reduced thrombus weight from a mean (\pm SEM) of 36.7 (\pm 3.2) mg in controls to 24.6 (\pm 4.1) mg (33% reduction; $P < .05$), and 14.0 (\pm 3.5) mg (62% reduction; $P < .001$) in the 1 mg/kg and 5 mg/kg groups, respectively (Figure 6B).

In the AV shunt model, preventative and interventional asundexian treatment were associated with a dose-dependent prolongation of APTT (Table S3A,B in supporting information) and reduced FXIa activity (Figure S3 in supporting information).

4 | DISCUSSION

The studies reported here show that asundexian is a potent small molecule inhibitor of FXIa activity and coagulation in buffer and plasma of various species and demonstrated antithrombotic effects in rabbit models after intravenous or oral administration without affecting bleeding time or blood loss.

Asundexian produced a direct, potent, and reversible inhibition of FXIa activity in buffer and human plasma with the 94% plasma protein binding of asundexian in human plasma accounting for the difference in potency observed in these two assays. X-ray cocrystal structures show the binding mode of asundexian in the active site of the activated form FXIa (manuscript in preparation).

Asundexian also demonstrated high selectivity versus other proteases of the coagulation and fibrinolytic system and proteases of the gastrointestinal tract (inhibited ≥ 1000 -fold less potently). The "close relative" plasma kallikrein was inhibited by asundexian 10-fold less potently in human plasma. It is therefore possible that at very high doses, where FXIa is fully inhibited, a concomitant plasma

kallikrein inhibitory effect may contribute to the biological effect of asundexian.

The results from the thrombin generation assays may reflect different processes for FXI activation: When the coagulation system was activated with the standard concentration of 5 pM TF in human plasma, the high TF concentration resulted in strong thrombin generation via the extrinsic pathway without FXI(a) involvement and consequently no impact of asundexian. When coagulation was triggered via contact activation, asundexian delayed and reduced thrombin generation. Reduced thrombin generation was predominantly observed when low amounts of TF were used as trigger, which can be explained by an initial activation of a small amount of thrombin via the extrinsic pathway followed by initiation of the thrombin amplification loop, which includes FXI activation by thrombin and can thereby be reduced by asundexian.

These findings are in line with the clotting assay results showing a concentration-dependent prolongation of APTT without affecting PT. Overall, the *in vitro* results correspond well to the proposed mode of action: fibrin generation initiated by high tissue factor concentrations, found for example in the subendothelial "tissue factor envelope," is thought to be critical for hemostasis after vessel wall injury and may not be affected by asundexian. In contrast to vitamin K antagonists or DOACs, which block the common pathway of the coagulation system irrespective of the trigger origin, FXIa inhibitors act as selective coagulation modulators via the two specific processes mentioned earlier: reducing coagulation after contact activation by negatively charged macromolecules or surfaces and by attenuating the effect of the amplification loop on clot progression, while leaving the TF pathway as a cornerstone of hemostasis intact. This is consistent with findings in *in vitro* studies of other inhibitors of FXI and FXIa.^{7,11}

Of note, asundexian prolonged the APTT in the plasma of guinea pigs, rabbits, dogs, pigs, and monkeys, but had little effect

in rodent plasma, potentially due to amino acids preventing appropriate binding in the active site. Exchanging two amino acids in the active center of murine FXIa with the equivalent ones from the human enzyme increased the potency of asundexian in such humanized FXI transgenic mouse experiments.²² However, the *in vivo* thrombosis studies were carried out in rabbits instead of transgenic mice to allow comparative studies with other anticoagulant approaches.

In arterial or venous thrombosis models in rabbits (FeCl₂-injury or AV shunt), intravenous or oral asundexian administration alone or on top of dual platelet inhibition reduced the thrombus weight versus control in a dose-dependent manner, without affecting the bleeding times in simultaneously conducted ear or gum bleeding models compared to control. This decoupling of antithrombotic efficacy and bleeding time prolongation is uncommon, as thrombus weight reductions with higher doses of the clinically established DOACs are accompanied by longer bleeding times, as demonstrated herein for the different members of this class used as references in the described studies. Balancing efficacy with bleeding risk appears to be particularly challenging when current anticoagulants are given in combination with DAPT in arterial thrombosis indications. It is therefore encouraging that under these conditions, no further increase of bleeding risk was observed when asundexian was combined with antiplatelets in these animal models.

In addition to ear bleeding, gum bleeding times were also measured to simulate mucosal bleedings, which have been observed to be more sensitive to reduced FXI concentrations in humans.¹ However, no increase in gum bleeding times was observed in our models. In the liver injury model, conducted as a representative model for potential trauma bleedings, asundexian had no significant effect on bleeding time or blood loss compared to controls, whereas dabigatran (0.3 mg/kg) as reference increased bleeding time approximately 5-fold.

In these studies, close correlations were observed between the reductions of thrombus weight versus control and increasing plasma concentrations of asundexian, increasing FXIa inhibition and increasing APTT prolongation. Considered collectively with the human *in vitro* data described here, these observations support further investigation of asundexian in clinical trials.

Experiments with orally administered asundexian indicate the possible utility of this agent, for example for long-term thromboprophylaxis. In addition to evaluation in a prophylactic setting, asundexian was also tested in the rabbit AVbshunt model under treatment conditions: when given 10 min after shunt opening, asundexian minimized further clot growth. Therefore, asundexian could have potential in interventional settings like venous thromboembolism treatment.

While the described general pharmacological profile of asundexian is consistent with those of other agents targeting FXIa, including milvexian,²³ osocimab,^{7,18,24} abelacimab,²⁵ and the antisense oligonucleotide IONIS FXI-R_x, which blocks the hepatic synthesis of FXI,¹⁹ important differences exist in their route of administration, onset of action, and duration of effect. Asundexian and milvexian

can be administered orally, whereas osocimab, abelacimab, and IONIS FXI-R_x need to be administered subcutaneously or intravenously. The onset of action of asundexian, milvexian,²⁶ osocimab,²⁴ and abelacimab²⁷ is rapid, whereas it takes longer time periods for IONIS FXI-R_x to lower FXI concentrations to therapeutic levels with a correspondingly long duration of the anticoagulant effect.¹⁹ Osocimab (mean elimination half-life, 30–44 days²⁴) and abelacimab (elimination half-life, 25–30 days²⁷) have been shown to have a long duration of effect, whereas milvexian has a half-life that may be suitable for once- or twice-daily dosing,²⁶ and phase I data suggest asundexian is a suitable candidate for once-daily oral anticoagulation^{28,29}; this is being further evaluated in phase II.

It is important to note that anticoagulants currently in clinical use were initially proven to be effective in animal models, which also identified their concomitant bleeding risk at high exposures, indicating that mechanisms of thrombus initiation and propagation in animal models are similar to those in humans.³⁰ However, the limitations of this study are inherent to preclinical studies in that no animal model can fully replicate human pathology. For example, in humans, thrombosis usually develops in diseased vessels, whereas in the animal models used in this study, thrombosis was induced in healthy vessels. While clinically available anticoagulants were used as comparators in our rabbit models, it was not considered ethically feasible to include all DOACs in every model, as these studies have already been published.^{31–35}

In summary, these preclinical findings show that asundexian, an oral, direct, potent, and selective FXIa inhibitor, represents a novel type of selective coagulation modulator by reducing thrombus formation in arterial or venous thrombosis without prolonging bleeding times. Asundexian may therefore have potential as an antithrombotic agent with a minimal bleeding risk. Further, due to the apparent lack of an effect of asundexian on bleeding shown in these studies, higher dosing in humans to further increase efficacy may be possible. The lack of bleeding time prolongation when asundexian is added to DAPT may also support a role for coagulation modulation in the prevention of severe arterial thrombotic events like ischemic stroke or myocardial infarction.

Further investigations are warranted to establish the clinical relevance of these preclinical findings. The efficacy and safety of asundexian are currently being investigated in a number of phase II studies, namely PACIFIC-AF (NCT04218266) in patients with atrial fibrillation, PACIFIC-STROKE (NCT04304508) in patients with non-cardioembolic stroke, and PACIFIC-AMI (NCT04304534) in patients with acute myocardial infarction.

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CONFLICTS OF INTEREST

Stefan Heitmeier, Adrian Tersteegen, Julia Glunz, Jan Stampfuss, Christoph Gerdes, Susanne Roehrig, and Julia Dietze-Torres are

all employees of Bayer AG. At the time this work was conducted, Mayken Visser and Volker Laux were both employees of Bayer AG but have subsequently left. Christoph Gerdes, Julia Glunz, Jan Stampfuss, Susanne Roehrig, and Adrian Tersteegen also own shares in Bayer AG.

AUTHOR CONTRIBUTIONS

All authors contributed to the study concept and design, analysis and/or interpretation of data, and writing or critical revision of the manuscript for important intellectual content. All authors approved the final version of the manuscript for publication.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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