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ORIGINAL ARTICLE

A small-molecule hemostatic agent for the reversal of direct oral anticoagulant–induced bleeding

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Abstract

Background: The bleeding risk associated with direct oral anticoagulants (DOACs) remains a major concern, and rapid reversal of anticoagulant activity may be required. Although specific and nonspecific hemostatic biotherapies are available, there is a need for small-molecule DOAC reversal agents that are simple and cost-effective to produce, store, and administer.

Objectives: To identify and characterize a small molecule with procoagulant activity as a DOAC reversal agent.

Methods: We sought to identify a small procoagulant molecule by screening a chemical library with a plasma clotting assay. The selected molecule was assessed for its procoagulant properties and its ability to reverse the effects of the DOACs in a thrombin generation assay. Its activity as a DOAC reversal agent was also evaluated in a tail-clip bleeding assay in mice.

Results: The hemostatic molecule (HeMo) dose-dependently promoted thrombin generation in plasma, with dose values effective in producing half-maximum response ranging between 3 and 5 μM, depending on the thrombin generation assay parameter considered. HeMo also restored impaired thrombin generation in DOAC-spiked plasma and reversed DOAC activity in the mouse bleeding model. HeMo significantly reduced apixaban-induced bleeding from 709 to 65 μL (vs 43 μL in controls; $P < .01$) and dabigatran-induced bleeding from 989 to 155 μL (vs 126 μL in controls; $P < .01$).

Conclusion: HeMo is a small-molecule procoagulant that can counterbalance hemostatic disruption by a thrombin inhibitor (dabigatran) or factor Xa inhibitors (apixaban and rivaroxaban). The compound's effective clot formation and versatility make it a possible option for managing the inherent hemorrhagic risk during DOAC therapy.

KEYWORDS
compound screening, DOAC reversal, hemostatic agent, thrombin generation assay

Essentials

- Despite available biotherapies, the need for direct oral anticoagulant (DOAC) activity reversal remains.
- This study aims at searching for a small hemostatic molecule (HeMo) as an antidote to DOACs.
- HeMo restores impaired thrombin generation in a dose-dependent manner in DOAC-containing plasma.
- HeMo also effectively reverses DOAC activity in a mouse model of bleeding.

1 | INTRODUCTION

Direct oral anticoagulants (DOACs) are widely and increasingly used to prevent and treat venous thromboembolism and prevent stroke in patients with nonvalvular atrial fibrillation. DOACs are direct, reversible, competitive inhibitors; they include dabigatran (which targets thrombin) and various "xabans" (ie, rivaroxaban, apixaban, edoxaban, and betrixaban, which target factor [F]Xa). DOACs have many advantages over vitamin K antagonists (including a more predictable anticoagulant effect, fewer dietary and drug interactions, and shorter plasma half-lives) and so do not require routine laboratory monitoring. Although DOACs are generally recommended as the first-line anticoagulant therapy, they are associated with an inherent risk of bleeding complications; hence, neutralization of their anticoagulant activity may be required in patients with life-threatening bleeding or in those requiring emergency surgery or invasive procedures [1,2].

The results of several clinical trials have shown that DOACs are slightly safer than vitamin K antagonists, with a reduced risk of intracranial and fatal bleeding. However, some DOACs increase the risk of gastrointestinal hemorrhage [3–6]. As with all anticoagulants, the main fear during DOAC therapy is the risk of bleeding. The annual incidence of major bleeding in DOAC-treated patients is around 2% to 4%, and the incidence of minor bleeding is much higher [7]. Although a "wait and support" strategy can be considered for minor bleeding, major bleeding or bleeding in a critical organ requires the physician to consider neutralization of the anticoagulant effect with either a DOAC-specific reversal agent or nonspecific hemostatic therapy. Specific reversal of dabigatran can be achieved with idarucizumab, a monoclonal antibody fragment that binds tightly to the inhibitor and thus diverts it from its target. Idarucizumab was approved in 2015 for dabigatran reversal in the context of major bleeding or emergency surgery [8]. More recently, andexanet alfa was also approved for the specific reversion of "xabans." Andexanet alfa is a catalytically inactive recombinant FXa that retains affinity for FXa inhibitors and acts as a decoy by binding and sequestering FXa inhibitors [9]. Despite overt efficiency, the indication of andexanet alfa remains limited to life-threatening or uncontrolled bleeding in patients on apixaban or rivaroxaban. Andexanet alfa is not yet approved for the reversal of edoxaban and betrixaban or for the prevention of bleeding before surgery [10]. Moreover, andexanet alfa may be associated with a prothrombotic risk (due to its interaction with the tissue factor pathway inhibitor) and is not widely available [11–14]. Furthermore, the choice of a specific antidote requires knowledge of the type of anticoagulant used; in certain emergency situations (eg, intracranial hemorrhage), this knowledge is not readily available.

Thus, when a specific reversal agent is not available or cannot be used, prothrombin complex concentrates (PCCs) are authorized in the treatment guidelines as a nonspecific hemostatic treatment for DOAC-associated bleeding [15,16]. PCCs are widely available because they are often used to reverse the effects of vitamin K antagonists. Like specific reversal agents, PCCs remain expensive and require intravenous administration. The objective of the present study was therefore to develop a hemostatic small molecule with the following characteristics: ease of production and storage, cost-effectiveness, and a low risk of humoral immunization. Ideally, to prevent or stop DOAC-induced bleeding, this small-molecule reversal agent would be administered orally for prophylaxis or parenterally in an emergency situation.

2 | METHODS

2.1 | Materials

Apixaban, rivaroxaban, and dabigatran were purchased from Alsachim. Human plasma was either a pool of normal human plasma (CRYOcheck from Cryopep) or fresh frozen plasma from a single healthy donor obtained from the French Blood Establishment and collected by plasmapheresis using anticoagulant citrate dextrose solution (Etablissement Français du Sang, reference: C CPSL UNT-N° 18/EFS/031). The compounds screened for their procoagulant properties came from our in-house library (BioCIS chemical library [17]). The syntheses of the initial hits and the optimized candidates are detailed in the Supplementary Method.

2.2 | Fibrin formation assay

The fibrin formation assay was performed with a pool of normal human plasma (Cryopep) or with factor-deficient plasma (HemosIL, Werfen) in 96-well half-area microplates. Each well was prefilled with 60 μL of plasma containing the chemical compounds at a final concentration of 50 μM and prewarmed at 37 °C for 5 minutes. Fibrin formation was initiated by adding 30 μL of trigger mix, composed of either FXIa (105 pM, Haematologic Technologies Inc), phospholipid vesicles (12 μM), and $CaCl_2$ (50 mM) or lipidated tissue factor (3 pM tissue factor and 12 μM phospholipids, PPP Reagent Low,

Stago) and $CaCl_2$ (50 mM) in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1 M $CaCl_2$, 6% (weight per volume) bovine serum albumin, pH 7.4. The samples were shaken for 10 seconds, and the absorbance at 405 nm was read every 8 seconds for 30 minutes at 37 °C using a microplate reader (MP96, Safas). The clotting time was defined as the time needed to reach the midpoint between the minimum and maximum absorbance at 405 nm.

2.3 | Thrombin generation assay

Thrombin generation assays (TGAs) were performed with fresh frozen plasma from a healthy donor using the calibrated automated thrombogram method (Stago). All TGAs were run in duplicate in 96-well plates, as described previously [18]. Briefly, 72 μL of normal plasma or DOAC-containing plasma was supplemented with 8 μL of the test compound (extemporaneously prepared as a 10X solution in phosphate-buffered saline) and mixed with 20 μL PPP Reagent Low in test wells or thrombin calibrator in calibration wells. Thrombin generation was triggered by dispensing 20 μL of fluorescent substrate in calcium-containing buffer (FluCa Kit, Stago). The thrombin generation curves were monitored and analyzed with Thrombinoscope software (Stago). For experiments with DOAC-containing plasma, DOACs previously solubilized and stored in dimethyl sulfoxide were diluted at least 200-fold in plasma so that the dimethyl sulfoxide never represented more than 0.5% of the total plasma volume. The TGA parameters (ie, the lag time [LT], time to peak [TTP], thrombin peak [TP], and endogenous thrombin potential [ETP]) were calculated using Thrombinoscope software. The dose-response experiments were analyzed using an exponential equation (1-phase decay or 1-phase association) in GraphPad Prism 7 (GraphPad Software) in order to calculate the concentration of compound that gave the half-maximum response (EC_{50}) or that restored the DOAC-impaired response to its initial value.

2.4 | Tail bleed assays

C57BL/6JOlaHsd female wild-type mice (Envigo) were used at 10 to 12 weeks of age. The animals were housed and handled in compliance with French regulations and the European Union's guidelines. This project was approved by the ethical committee CEEA26 (number APAFIS#21597-2019072415413447 v2).

Before administration, dabigatran was dissolved in acidified water (1 mM HCl) and diluted in a 5% glucose solution. The apixaban suspension was diluted in a vehicle solution of 5% glucose containing 10% (volume per volume [v/v]) glycerol, 10% (v/v) ethanol, and 10% (v/v) polyethylene glycol 400 [19]. Anesthetized mice were intravenously injected with dabigatran (0.4 mg/kg), apixaban (8 mg/kg), or their respective vehicle. Thirty minutes later, the anesthetized mice received a bolus (50 mg/kg; intravenously) of the test compound dissolved in 5% glucose solution or vehicle. The tail was immersed in a saline solution (0.9% NaCl) at 37 °C for 1 minute and then clipped (diameter of the cut section: 2 mm).

Blood was collected in saline solution for 20 minutes, and the volume of blood loss was evaluated after centrifugation (750 × *g* for 15 minutes at room temperature). Red blood cells were lysed by adding distilled water. Hemoglobin was quantified by measuring the absorbance at 420 nm using a standard curve obtained from serial dilutions of a known volume of blood.

Data were evaluated in a 1-way analysis of variance with Tukey's post hoc test for multiple comparisons, using GraphPad Prism 7.

3 | RESULTS

3.1 | Identification and optimization of a small molecule with hemostatic properties

With a view to finding an orally bioavailable small molecule with hemostatic properties, a chemical library of approximately 2000 molecules was screened in a tissue factor-triggered clotting assay with normal human plasma. A small group of polyhydroxylated styrylquinolines (formerly synthesized as HIV-integrase inhibitors [20]) was identified for its ability to shorten the clotting time. The best of these was found to be the 5-bromo-8-hydroxystyrylquinoline 2a (Figure 1). However, this structure had 2 major shortcomings. Firstly, like most quinolines, the compound was totally insoluble in water. Luckily, 2a had a carboxylic acid group that could easily be converted into the corresponding sodium salt, resulting in a water-soluble derivative 3a. The second potential shortcoming was the presence of a catechol group, which is generally considered to be unsuitable for drug development [21]. Thus, to reduce the number of phenol groups and mask the potentially toxic catechol, we modified the ancillary aromatic part of 2a. To rapidly ascertain the pharmacophoric contributions of the substituents on the aromatic ring, we designed a small set of derivatives. Accordingly, the 3′-OH group was replaced with an electron-donating group (O-CH_3 in 2b) or an electron-withdrawing group (Cl, F, or NO_2 in 2c, 2d, or 2e, respectively). The carboxylic acid was then prepared by sodium hydroxide treatment and freeze-drying to give the water-soluble candidates 3a to 3e.

3.2 | *In vitro* characterization of the optimized compounds

The hemostatic properties of compounds 3a to 3e were confirmed: they shortened the fibrin formation time in a clotting assay with normal human plasma (Figure 2A). The most potent molecule was 3e, which reduced the clotting time by 53%. However, this molecule was discarded because it considerably reduced the difference between the maximum absorbance and minimum absorbance (Figure 2B). The analogs 3b and 3d shortened the clotting time by 42% and 41%, respectively, whereas 3a and 3c both shortened the clotting time by 24% only. We thus decided to characterize the hemostatic molecule (HeMo) 3d because it was the most effective in

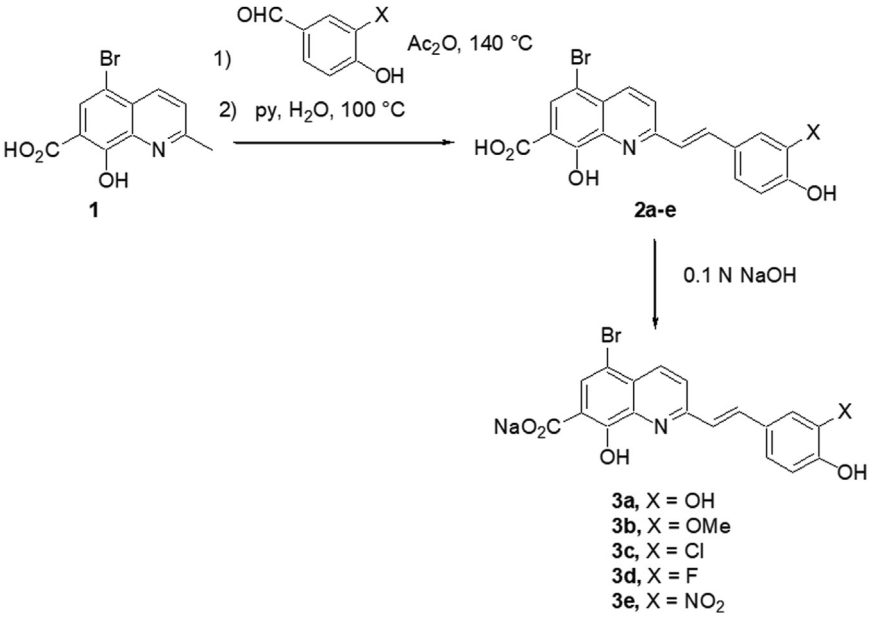

FIGURE 1 Chemical structures of the initial hits and optimized candidates. OMe, O-CH_3.

accelerating the fibrin formation without paradoxical effect on the absorbance amplitude.

The effect of HeMo on fibrine formation was also evaluated in factor-deficient plasma upon activation with tissue factor to explore the extrinsic pathway or with FXIa to explore the extrinsic pathway (Supplementary Figure). HeMo consistently conserved its ability to shorten the clotting time, regardless of the activation pathway and the missing factor. These results suggested that procoagulant activity of HeMo did not require the presence of any of the tested factors (ie, FVII, FX, FII, FV, FIX, FVIII, FXI, or FXII). Unfortunately, these results also failed to identify the biological target of HeMo.

HeMo's hemostatic activity was further investigated in a TGA performed with normal human plasma (Figure 3A). All the TGA parameters (including the ETP, TP, LT, and TTP) were affected in a dose-dependent manner by the addition of HeMo (0-50 μM). The EC_{50} was in the micromolar range but varied with the TGA parameter considered (Figure 3B). The 2 time-dependent parameters followed a 1-phase exponential decay as the HeMo concentration increased: the LT started at 6.2 ± 0.4 minutes and leveled off at 3.4 ± 0.4 minutes, with an EC_{50} of 5.3 ± 1.9 μM. The TTP started at 10.0 ± 0.9 minutes and leveled off at 5.1 ± 0.5 minutes, with an EC_{50} of 3.3 ± 0.8 μM. Conversely, HeMo enhanced the 2 quantitative parameters in an

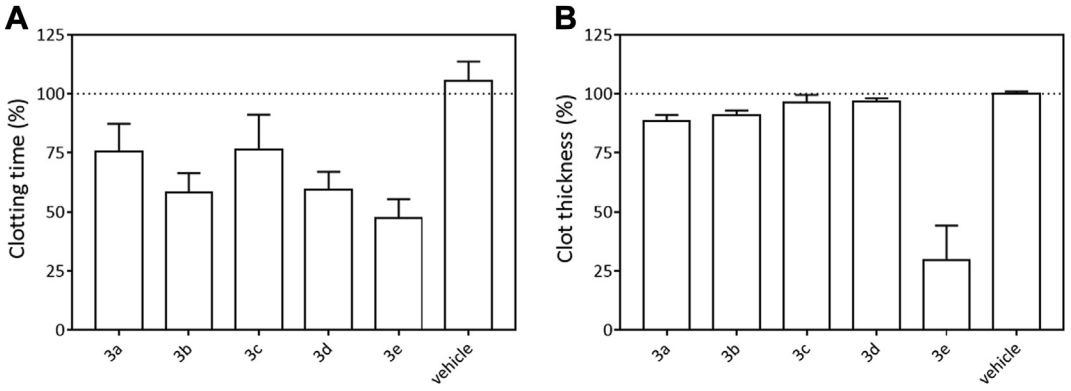

FIGURE 2 Procoagulant activity of the optimized candidates in a fibrin formation assay. Each of the candidates (final concentration: 33.3 μM) was added to a pool of normal human plasma. Fibrin formation was initiated by the addition of tissue factor (final concentration, 1 pM), phospholipid vesicles (final concentration, 4 μM), and $CaCl_2$ (final concentration, 16.7 μM), and absorbance at 405 nm was recorded over a period of 30 minutes. (A) The clotting time was calculated as the time needed to reach the midpoint between the minimum and maximum absorbance, normalized against the value measured in the absence of chemicals. (B) The clot thickness was estimated as the difference between the maximum and maximum absorbance, normalized against the value measured in the absence of chemicals. Three individual experiments were performed, and the results were expressed as the mean ± SD.

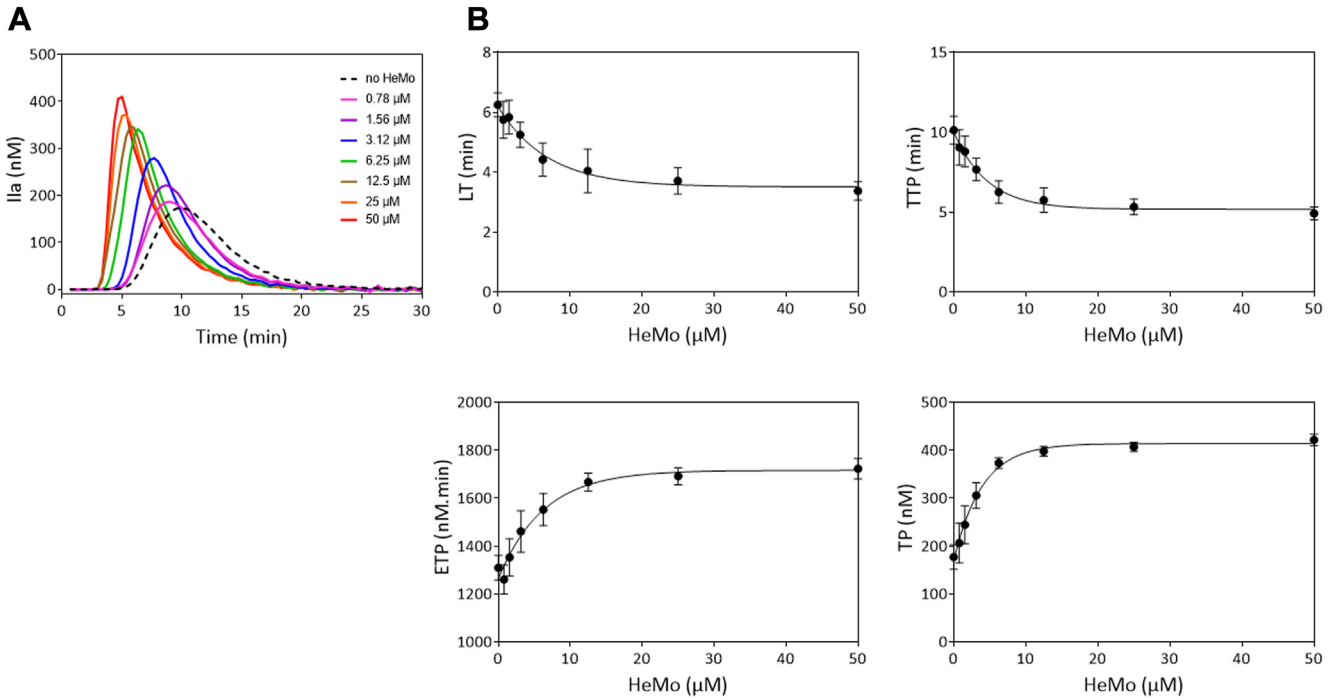

FIGURE 3 Procoagulant activity of the hemostatic molecule (HeMo) in a thrombin generation assay. Tissue factor–induced thrombin generation was measured by calibrated automated thrombography in fresh frozen plasma from a healthy donor. (A) Thrombin generation time curves measured in the absence (dotted line) or presence of increasing concentrations of HeMo (plain lines). The figure shows the results of a representative experiment (1 of 4 performed). (B) The thrombin generation assay parameters were calculated from the thrombin generation curves and plotted as a function of the HeMo concentration. The results were expressed as the mean ± SD of 4 experiments. For each experiment, an exponential equation (1-phase decay or 1-phase association) was used to analyze the dose-response curves and calculate the dose value effective in producing half-maximum response. ETP, endogenous thrombin potential; LT, lag time; TP, thrombin peak; TTP, time to peak.

exponential manner. The ETP ranged from 1254 ± 38 nM·min in the absence of HeMo to 1719 ± 45 nM·min at the highest concentration tested, with an EC_{50} of 4.4 ± 2.6 µM. The TP ranged from 168 ± 31 nM to 414 ± 12 nM, with an EC_{50} of 2.7 ± 0.8 µM.

3.3 | Reversal of DOAC activity *in vitro*

HeMo's ability to reverse the DOACs' anticoagulant activity was initially investigated *in vitro* in a TGA with DOAC-spiked normal human plasma. Firstly, HeMo was evaluated for the reversion of apixaban anticoagulant activity (Figure 4A). Apixaban was added to the plasma at a concentration of 200 ng/mL, which corresponds to the typical plasma concentration found in treated patients [22,23]. At this concentration, the anticoagulant effect of apixaban enhanced the LT by 203% ± 27% and decreased the ETP to 38% ± 18% of its initial value. The addition of increasing concentrations of HeMo to the apixaban-spiked plasma restored thrombin generation in a dose-dependent manner (Figure 4A). Based on an exponential equation, the concentrations of HeMo required to obtain the initial LT and ETP values were 4.9 ± 1.7 µM and 3.2 ± 2.5 µM, respectively.

Similar results were observed in experiments in normal human plasma spiked with 200 ng/mL rivaroxaban (Figure 4B) or 400 ng/mL dabigatran (Figure 4C). HeMo also reversed rivaroxaban's and dabigatran's anticoagulant effect. Indeed, HeMo dose-dependently enhanced the amount and rate of thrombin generation, both of which were reduced by the addition of an anticoagulant. In the presence of rivaroxaban, the concentrations of HeMo that restored the LT and ETP to their normal values were 3.1 ± 1.2 µM and 0.6 ± 0.2 µM, respectively (Figure 4B). In the presence of dabigatran (which had a more pronounced anticoagulant effect, lengthening the LT by 637% ± 111% and reducing the ETP to 46% ± 6%), the addition of up to 50 µM HeMo failed to completely normalize all the TGA parameters. Nevertheless, the LT and the ETP were restored to 130% ± 9% and 79% ± 3% of their initial values, respectively (Figure 4C).

3.4 | Reversal of DOAC activity *in vivo*

We next evaluated HeMo's hemostatic effect in a mouse model in which hemorrhage was induced by overdosing with apixaban (8 mg/kg) or dabigatran (0.4 mg/kg) [19]. Mice treated with apixaban (Figure 5A) or dabigatran (Figure 5B) bled for significantly longer and more than control mice. Indeed, the bleeding time and blood loss volume were 974 ± 290 seconds and 709 ± 587 µL in apixaban-treated mice and 240 ± 342 seconds and 43 ± 80 µL in controls,

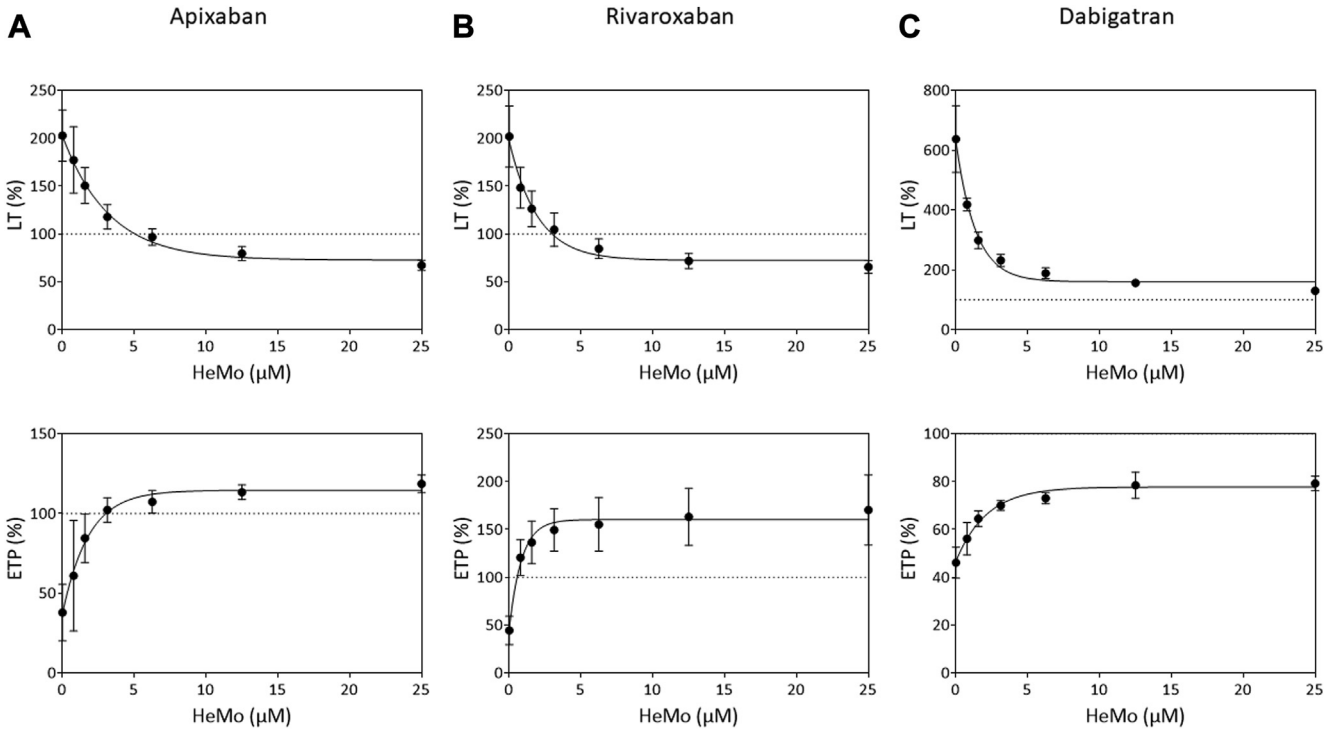

FIGURE 4 Hemostatic molecule (HeMo) as a direct oral anticoagulant (DOAC) activity reversal agent in a thrombin generation assay. Thrombin generation was measured in fresh frozen plasma from a healthy donor spiked with apixaban (A; final concentration, 200 ng/mL), rivaroxaban (B; final concentration, 200 ng/mL), or dabigatran (C; final concentration, 400 ng/mL), with increasing concentrations of HeMo. The thrombin generation assay parameters were calculated from the progress curves, normalized against the initial values obtained in the absence of DOAC and HeMo, and plotted as a function of HeMo concentrations. The results were expressed as the mean ± SD of 4 experiments. For each experiment, an exponential equation (1-phase decay or 1-phase association) was used to analyze the dose-response curves and calculate the concentration of HeMo that rescued the DOAC-altered response (ie, a return to the initial value). ETP, endogenous thrombin potential; LT, lag time.

respectively. When the mice received HeMo in addition to apixaban, both the bleeding time and blood loss volume were partially but significantly restored (to 507 ± 371 seconds and 65 ± 104 μL, respectively; $P < .01$). Similarly, treatment with dabigatran resulted in a greater bleeding time (915 ± 344 vs 408 ± 371 seconds) and blood loss volume (1046 ± 432 μL vs 65 ± 104 μL) in control mice. When the mice received HeMo in addition to dabigatran, the bleeding time and the blood loss volume were significantly lower (155 ± 111 seconds and 21 ± 18 μL, respectively; $P < .01$). Taken as a whole, these results demonstrated HeMo's hemostatic properties and its ability to reverse DOAC activity *in vivo*.

4 | DISCUSSION

Chemical library screening is an efficient and cost-effective way of identifying pharmacologically active compounds. In the present study, an initial procoagulant hit derived from 8-hydroxystyrylquinoline was identified in a general clotting assay. Unfortunately, the hit's chemical structure was unfavorable for the development of an orally administered drug. Like most quinolines, the compound was totally insoluble in water. Given the presence of 3 hydroxyl groups, the compound had a polyphenolic structure. Polyphenol derivatives are typical "frequent hit compounds" that interact weakly with many target proteins through hydrogen bonds but are unable to yield high-affinity inhibitors [24]. Furthermore, this problem was worsened by the presence of a catechol group (1,2-dihydroxybenzene). Catechols are problematic because they are easily oxidized to o-quinones that, in turn, may react with biomolecules. Although some very important drugs harbor a catechol group (eg, L-dopamine and norepinephrine), the latter group is generally considered to be unsuitable for drug development [21]. Examination of the structure-activity relationships in the initial screening step showed clearly that few modifications of quinoline moiety were possible. Indeed, analogs lacking the 5-bromine or the 8-hydroxyl group were poorly active or inactive. Furthermore, the carboxylic acid group was required for water solubility. We therefore decided to keep the general styrylquinoline scaffold and to modify the ancillary aromatic part. This strategy was straightforward because the synthetic route used to prepare styrylquinolines was based on a Perkin-type reaction between a quinaldine derivative and an aromatic aldehyde (Figure 1) [20]. Thus, a large array of analogs could be obtained quite easily by fine-tuning the aromatic aldehyde—a large number of which are commercially available. Of the few water-soluble candidates, the compound 3d ("HeMo") was found to have procoagulant activity (shortening the plasma clotting time) and did not greatly influence the clot thickness (ie, clot absorbance). We

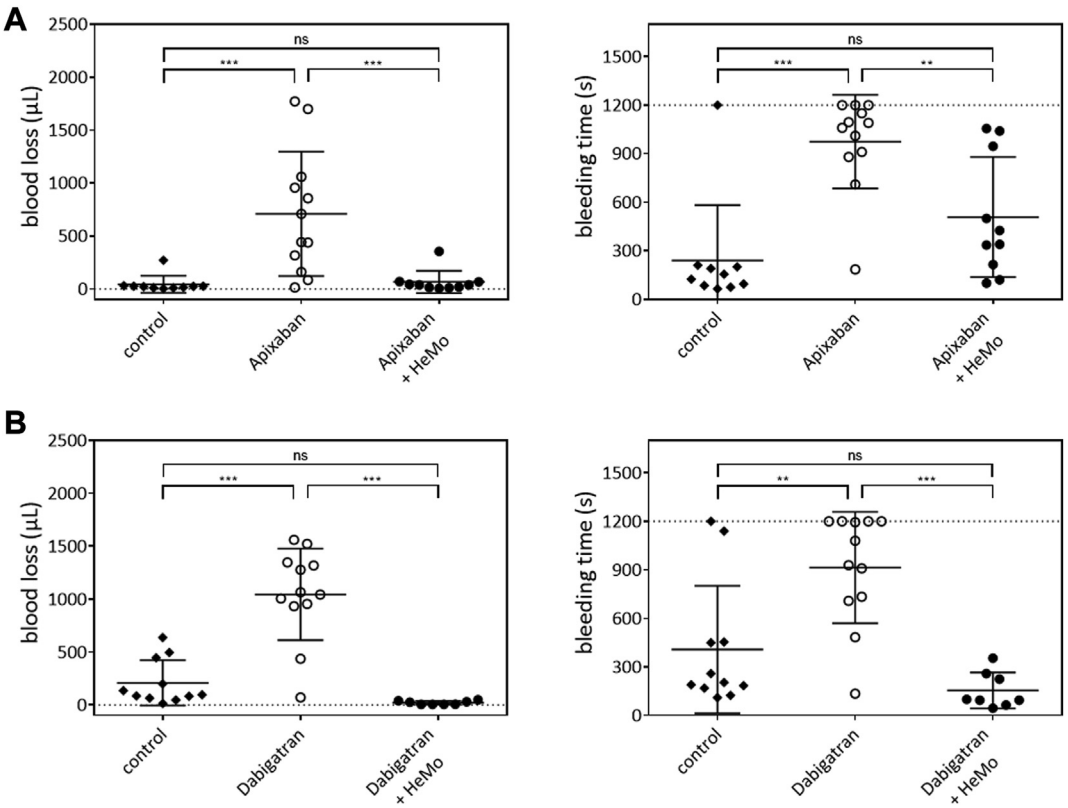

FIGURE 5 Hemostatic molecule (HeMo) as a direct oral anticoagulant activity reversal agent in a mouse tail-clip bleeding model. Anesthetized wild-type mice were intravenously injected with (A) apixaban (8 mg/kg) or (B) dabigatran (0.4 mg/kg) in the presence (closed circles) or absence (open circles) of intravenously administered HeMo (50 mg/L). In the control group (diamonds), mice were treated with the corresponding vehicle solutions instead of anticoagulant and HeMo. Tails were immersed in a saline solution at 37 °C before the tip of the tail was cut off (diameter of the cut section: 2 mm). The bleeding time (right panels) was defined as the time until the bleeding first stopped for at least 1 minute. Blood was collected for 20 minutes, and the total blood loss volume (left panels) was quantified by measuring the amount of hemoglobin. Graphs represent the mean ± SD, and each dot represents 1 measure ($8 \leq n \leq 12$). Statistical difference was evaluated by 1-way analysis of variance with Tukey's post hoc test for multiple comparisons. ns, nonsignificant. $^{**}P < .01$; $^{***}P < .001$.

therefore decided to further characterize HeMo's hemostatic properties and reversal of DOAC activity in a model of DOAC-induced bleeding.

Our present results showed that *in vitro*, HeMo had procoagulant activity in a fibrin formation assay (shortening the clotting time) and a TGA (promoting thrombin generation). Although HeMo's biological target and mechanism of hemostatic action are not known, this compound was effective enough to counterbalance DOAC activity, as demonstrated *in vitro* by its ability to rescue thrombin generation in DOAC-containing plasma and *in vivo* by its ability to attenuate bleeding in DOAC-treated mice. Further studies will be required to elucidate HeMo's biological target and mechanism of action. Like all hemostatic agents, HeMo exhibited intrinsic procoagulant activity *in vitro*; this obviously raises concerns about a potential thrombotic risk, especially in patients taking anticoagulants. Although PCC also has procoagulant activity *in vitro* (in thrombin or fibrin formation assays [25–27]), its use as a DOAC reversal agent was nevertheless supported by clinical data recorded in the context of therapeutic failure [8,28,29]. Here, we sought to demonstrate that HeMo is an effective DOAC reversal agent in anticoagulated or normal plasma at concentrations of up to 25 µM and 50 µM, respectively. Interestingly, HeMo's procoagulant activity tended to level off, with an approximately 2-fold increase in thrombin generation in the absence of an anticoagulant (Figure 3). It is noteworthy that in the presence of therapeutic DOAC concentrations, HeMo barely overcorrected xaban-associated anticoagulant activity and almost completely rescued dabigatran's anticoagulant effect (Figure 4).

Besides its potential use as a DOAC reversal agent, HeMo might also be an asset in the management of other kinds of hemorrhage, such as those resulting either from rare bleeding disorders (eg, hemophilia) or from trauma. Despite significant recent progress, the treatment of hemophilia is still challenging. Replacement therapies and other treatments all have several drawbacks, such as the risk of alloimmunization, the parenteral administration route, and the high cost [30–32]. HeMo might be hemostatically efficient enough to counterbalance a procoagulant factor deficiency and might therefore be a treatment option in hemophilia and other rare bleeding disorders lacking a specific treatment (eg, severe FV deficiency). HeMo is easy to synthesize, and its chemical structure is compatible with oral administration; it might considerably improve the quality of life and lower the cost of treatment for patients with hemophilia. Furthermore, HeMo could also be considered for use on the battlefield, where

uncontrolled hemorrhage is the leading cause of preventable death. Given that some anatomic areas are not amenable to tourniquet placement, the topical application hemostatic dressings, impregnated either with clotting factors activator or adhesive agents, can limit the extent of bleeding and are extensively used by military forces [33,34]. HeMo might have the characteristics needed for the preparation of hemostatic dressings on the battlefield: quick cessation of severe bleeding, ease of use under difficult conditions, a long shelf-life, and low cost.

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ETHICS STATEMENT

The animals were housed and handled in compliance with French regulations and the European Union's guidelines. This project was approved by the ethical committee CEEA26 (number APAFIS#21597-2019072415413447 v2).

AUTHOR CONTRIBUTIONS

M.D. performed research, analyzed data, and cowrote the manuscript. F.A., G.T., and S.J. performed research and analyzed data. C.R. performed research. C.V.D. conceptualized the study and edited the manuscript. D.D. and D.B. conceptualized and designed the study, analyzed data, and edited the manuscript. E.P.B. conceptualized and designed the study, performed research, analyzed data, and wrote the manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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