Solulin increases clot stability in whole blood from humans and dogs with hemophilia

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Solulin is a recombinant form of thrombomodu-
lin that is resistant to proteolysis and oxidation. It has been shown to increase the clot lysis time in factor VIII (fVIII)–deficient plasma by an activated thrombin-activatable fibrinolysis inhibitor (TAFIa)–dependent mechanism. In the present study, blood was drawn from humans and dogs with hemophilia, and thromboelas-
tography was used to measure tissue factor–initiated fibrin formation and tissue-plasminogen activator–induced fibrinoly-
is. The kinetics of TAFI and protein C activation by the thrombin-Solulin com-
plex were determined to describe the relative extent of anticoagulation and anti-
fibrinolysis. In severe hemophilia A, clot stability increased by >4-fold in the presence of Solulin while minimally affecting clot lysis time. Patients receiving fVIII/fIX prophylaxis showed a similar trend of increased clot stability in the presence of Solulin. The catalytic efficiencies of TAFI and protein C activation by the thrombin-
Solulin complex were determined to be 1.53 and 0.02/μM/s, respectively, explain-
ing its preference for antifibrinolysis over anticoagulation at low concentrations. Fi-
ally, hemophilic dogs given Solulin had improved clot strength in thromboelas-
tography assays. In conclusion, the antifib-
brinolytic properties of Solulin are exhib-
ted in hemophilic human (in vitro) and dog (in vivo/ex vivo) blood at low concen-
trations. Our findings suggest the therapeu-
tic utility of Solulin at a range of very low doses. (Blood. 2012;119(15): 3622-3628)

Introduction

Patients with hemophilia A have a bleeding diathesis that is usually predicted by their factor VIII (fVIII) activity level (fVIII:C).1-2 The primary form of treatment for severe hemophilia A is replacement therapy, which involves administration of recombinant or plasma-derived fVIII. fVIII can be given either on demand or by prophylaxis,3 and the amount needed can vary drastically depend-
ing on the treatment schedule and the type and severity of the bleed in the case of on-demand treatment.4

The treatment developments to date have greatly improved both mortality and morbidity for individuals with hemophilia5,6; how-
ever, current treatments are not 100% effective, are expensive, and are often considered inconvenient. Because single bleeding events can have devastating consequences, it is important to continue to strive for maximally effective treatments. The recent improvements in mortality and morbidity have only been observed in developed countries with the resources to fund treatment. It is currently estimated that 80% of the world’s hemophilia population has little or no access to therapy7; therefore, the development of cost-effective alternate treatment strategies or effective factor-sparing regimes to treat bleeding is clearly necessary.

Many new and adjunctive therapeutic options have been explored, including platelet infusion,8 tranexamic acid,9 ε-amino caproic acid,10 molecules that block tissue factor pathway inhibitor,11,12 and a combination of phospholipid and FIXa13 and FXIII.14 Solulin is a recombinant soluble analog of human thrombomodu-
lin. Consisting of the extracellular domains of thrombomodulin, it is distinguished by several directed mutations, providing for lack of a chondroitin sulfate attachment site, resistance to exocarboxypepti-
dase/protease activity and to oxidation/irradiation, and, finally, abolishing the N-terminal heterogeneity arising in the wild-type sequence from 2 common signal cleavage sites.15-17

Recently, we demonstrated that soluble thrombomodulin (Solulin) may be used to partially correct the premature lysis defect in fVIII-deficient plasma through an activated thrombin-activatable fibrinolysis inhibitor (TAFIa)–dependent mechanism,18 which supports the hypoth-
thesis that bleeding in hemophilia may be due to unregulated fibrinolysis19 in addition to the well-documented clotting defect.20 This hypothesis is also supported by a preliminary clinical study showing that ε-amino caproic acid, an antifibrinolytic lysine analog, may be used adjunctively with FIXa inhibitor bypass activity or activated prothrombin complex to control bleeding.10 Full-length thrombomodulin (TM) has been shown to bind tightly to thrombin,21 which prevents cleavage of fibrinogen22 and therefore fibrin formation. Furthermore, the cofactor activity of TM for thrombin-mediated protein C activation diminishes thrombin generation by proteolytically inactivating the coagulation cofactors Vα and VIIα.23 It is for these reasons that TM was thought to be an unlikely candidate for the treatment of bleeding in hemophilia. An important difference between Solulin and full-length TM is that Solulin has reduced affinity for thrombin, which greatly reduces its anticoagulant function but still adequately promotes TAFI activation.24 Part of the


There is an Inside Blood commentary on this article in this issue.
**Methods**

**Materials**

The thrombin inhibitors D-Phe-Pro-Arg chloromethyl ketone (PPA-ck) and hirudin were purchased from Calbiochem. The TAFIa substrate anisylazofenyl arginine (AAFR; catalog number 2525) was purchased from Bachem Americas, and the activated protein C substrate S-2366 was purchased from DiaPharma Group. Recombinant human soluble thrombomodulin (Solulin) was provided by PAION Deutschland. Tissue-type plasminogen activator (Activase) was purchased from the pharmacy at King’s College Hospital, London, United Kingdom. Solulin (25nM) and thrombin (0.5nM) were incubated in HEPES-buffered saline (20mM HEPES, 150mM NaCl, pH 7.4). Other reagents were of analytical quality.

**Subjects**

A total of 17 patients with hemophilia (15 with hemophilia A and 2 with hemophilia B) were recruited. All subjects gave informed, written consent before sample collection in accordance with the Declaration of Helsinki (St. Thomas’ Hospital ethics approval number 10/H0805/023). The 2 subjects with hemophilia B had 5.8 and 1.2 IU/dL of fIX at baseline and 12 of 15 hemophilia A subjects had a clinically severe phenotype (with fVIII:C < 1.2 IU/dL at the time of baseline sampling). One hemophilia A subject (patient 11) had a history of inhibitor, which was treated with recombinant human activated protein C (Activase) and apheresis. The thromboelastography parameters were comparable to other patients enrolled. In addition, 2 patients had a history of hepatitis C, which was treated and resolved.

**Results**

**Kinetics of protein C and TAFI activation by thrombin-Solulin**

Solulin (25nM) and thrombin (0.5nM) were incubated in HEPES-buffered saline at room temperature in wells of a transparent 96-well plate. TAFI or protein C (both 25nM), hirudin, and thrombin were incubated in HEPES-buffered saline (20mM HEPES, 150mM NaCl, pH 7.4). All other reagents were of analytical quality.

**Thromboelastography**

Blood was drawn from human subjects with hemophilia A or B into 3.2% sodium citrate (1:10) and 350mM of corn trypsin inhibitor (9 parts blood and 1 part anticoagulant) before and, for select subjects, at various times after fVIII or fIX administration, which was given for clinical purposes. Within 5 minutes, the blood (260 µL) was titrated with Solulin (20 µL; 0-100nM) and induced to clot with hirudin (1:17000) and CaCl2 (5mM excess; 20 µL). Tissue-plasminogen activator (20 µL, 1nM) was included to induce fibrinolysis (final concentrations). Rotational thromboelastometry (ROTEM; Pentapharm) measurements were used to determine the clot lysis time (CLT; in minutes) and maximum clot firmness (MCF; in millimeters). In addition, the area under the elasticity curve (AUEC; in millimeters per minute) was used to characterize and quantify the stability of fibrin.

**Animals**

Dogs with hemophilia A were bred and maintained at Queen’s University, Kingston, ON. All procedures were in compliance with the institutional animal care committee and the Canadian Council for Animal Care. Blood from 4 dogs with severe hemophilia A was collected before and at regular intervals after a 10-minute reaction time. Each experiment was conducted in the presence of 2.5mM CaCl2. After a 10-minute reaction time, 160 µL of a solution containing 150mM AAFR and 1.25µM PPA-ck was added to the TAFI series and 160 µL of a solution containing 375µM S-2366 and 3.125 antithrombin units/mL of hirudin was added to the protein C series. In these experiments, PPA-ck and hirudin were used to quench the respective reactions and the small substrates AAFR and S-2366 were used in conjunction with standards to determine the amount of TAFI or protein C activated over the 10-minute experimental time frame. The results of TAFI and protein C activation were determined using these methods and subsequently analyzed by nonlinear regression to the Michaelis-Menten model of enzyme kinetics.

**Conclusion**

Solulin was effective in the prevention of fibrinolysis in vitro. This in vitro effect was confirmed in vivo in dogs with severe hemophilia A. The implications of these findings for the clinical setting are currently being investigated.

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**Table 1. Hemophilia patient demographics**

<table>
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<tr>
<th>Patient no.</th>
<th>Clinical details</th>
<th>Age, y</th>
<th>fVIII/FIX, IU/dL</th>
<th>APTT ratio</th>
<th>Fibrinogen, mg/mL</th>
<th>Platelets, x 10⁹/mL</th>
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APTT indicates activated partial thromboplastin time.
intervals after IV Solulin administration. Solulin was given by bolus injection IV in a volume of 3 mL (3 dogs: 5 or 10 μg/kg; 1 dog: 500 μg/kg). The dosing of Solulin is described in relative terms with a “low” dose being 5 or 10 μg/kg and a “high” dose being 500 μg/kg. The blood was subsequently analyzed by thromboelastography (see next section). A parallel sample was used for the determination of plasma concentration of Solulin using an ELISA assay validated for dog plasma.

Ex vivo thromboelastography using hemophilic dog blood

Citrated blood (9 parts blood and 1 part 3.8% trisodium citrate) was obtained from hemophilic dogs before and after IV administration of Solulin. The blood was subjected to thromboelastography using methods similar to those described in “Thromboelastography.” Briefly, 340 μL of whole blood was added to channels of a Hemoscope TEG 5000 (Hemonetics) containing a 20 μL solution of Innovin (1/15 000 dilution) and CaCl2 (5 mM excess) to induce coagulation and tissue-type plasminogen activator (1 nM) to induce fibrinolysis (final concentrations). After mixing thoroughly, the pin was seated and coagulation and fibrinolysis were monitored continuously. Because of equipment limitations, the maximum amplitude (MA; maximum clot strength) was used to describe the efficacy of Solulin in stabilizing fibrin.

Figure 1. Solulin improves fibrin viscoelasticity. At low concentrations (1.4-35.7nM), Solulin increases the MCF and CLT in human hemophilic blood. Increases in MCF and CLT are accompanied by an increase in the AUEC, which is used as a measure of clot stability or resistance to fibrinolysis. At high concentrations of Solulin (71.4nM), the MCF, CLT, and AUEC are reduced compared with the no-Solulin control. These data are representative of those obtained when Solulin and hemophilic blood (< 1% VIII:C) are titrated.

Using ROTEM, the effect of Solulin on fibrin viscoelasticity was monitored using thromboelastography. At Solulin concentrations ranging from 1.4 to 35.7 nM, ROTEM parameters generally improved compared with the control containing no Solulin (Figure 1). The MCF and CLT increased dramatically over this range of Solulin concentrations, which resulted in an associated increase in the AUEC. In the absence of Solulin, the MCF of fibrin formed from severe hemophilic blood was 25.3 ± 4.5 mm (Figure 2A). Solulin concentrations ranging from 1.4-17.9 nM significantly increased the MCF (P < .01) by an average of 1.7-fold (43.2 ± 6.6 mm). At 35.7 nM Solulin, a nominal increase in MCF was still observed (P = .12); however, the MCF decreased from its maximal level (31.1 ± 11.6 mm). This reduction was likely because of increased protein C activation, as may be conjectured from the virtually complete suppression of thrombin generation observed when Solulin was studied ex vivo in healthy volunteers.28 Like MCF, the AUEC was also significantly increased in severe hemophilia in the presence of Solulin (1.4-17.9 nM; P < .05), due in part to an increase in MCF, but also to an increased CLT. Figure 2B shows that the AUEC was increased by approximately 4-fold compared with the no-Solulin controls (16.8 ± 4.6 × 10^4 vs 4.1 ± 0.8 × 10^4 mm/min, respectively). Consistent with the concurrent (nominal) increase in MCF, 35.7 nM Solulin still improved the AUEC (by 2.5-fold, P < .05) compared with controls, but the level was lower (10.2 ± 4.7 × 10^4 mm/min) than that achieved with 1.4-17.9 nM Solulin. At 71.4 nM Solulin a decrease in both the MCF (11.6 ± 12.5 mm) and AUEC

Statistics

Each Solulin dosage was grouped and between-group comparisons were conducted using ANOVA and the Tukey HSD test. Data were presented as means ± SD. For all statistical analyses, P < .05 was considered significant.

Results

Effect of Solulin on clot stability and CLT in patients with severe hemophilia A

Figure 2. Solulin increases the MCF and AUEC in human hemophilic blood with minimal effect on CLT. (A) The MCF increased from 25.3 ± 4.5 mm to 43.2 ± 6.6 mm in the presence of 1.4-17.9 nM Solulin (P < .01). At higher concentrations, the MCF decreased from peak values. (B) In severe hemophilia A, the AUEC is increased by approximately 4-fold in the presence of Solulin due in part to an increase in MCF but also to an increased CLT. (C) Solulin marginally increases the CLT in hemophilia A blood. At the lowest concentrations of Solulin (< 7.1 nM), the CLT increased marginally from 11.2 ± 1.9 to 12.9 ± 3.7 minutes. The increase in CLT with Solulin was not statistically significant even at 35.7 nM (P = .07).
(3.4 ± 3.3 × 10⁴ mm/min) was observed, which is best explained by the anticoagulant properties of Solulin prevailing over its antifibrinolytic properties. To demonstrate that Solulin minimally affects the CLT in hemophilic whole blood, CLT data were analyzed for the concentrations of Solulin that improved the MCF and AUEC (ie, 1.4-35.7nM; Figure 2C). In the absence of Solulin, the CLT was 11.2 ± 1.9 minutes and increased slightly (by < 16%; P > .05) in the presence of 1.4-7.1nM Solulin (from 11.8 ± 2.2 to 12.9 ± 3.7 minutes). The increase in CLT did not reach the level of statistical significance at Solulin concentrations less than 40nM.

**Effect of fVIII or fIX on the Solulin-dependent improvement of clot stability**

Three of the patients enrolled (2 with hemophilia A and 1 with hemophilia B) were studied longitudinally after an approximately 5-day washout period. The first sample in this series of experiments was collected just before the patient received fVIII or fIX for clinical purposes. This baseline sample and other samples collected at low concentrations (≤ 1% fVIII), Solulin, at low concentrations (< 17.1nM), improved clot stability (as shown by the AUEC) by up to 6-fold in this particular subject's blood. In absolute values, the increment was similar after administration of fVIII (102% fVIII:C). However, as clot stability increased after fVIII:C administration (3.7 just before and 11.6 × 10⁴ mm/min after fVIII:C, no Solulin), the improvement by Solulin relative to the no-Solulin control (from 11.6 × 10⁴ to 21.3 × 10⁴ mm/min) was only 1.84-fold. As the time from fVIII infusion increased, the plasma concentration of fVIII decreased and the (relative) > 5.5-fold improvement in AUEC at 3.6nM Solulin was restored. Therefore, the absolute AUEC values at Solulin concentrations ranging from 1.4-17.1nM were similar regardless of fVIII:C (range, 13.7 × 10⁴ to 21.3 × 10⁴ mm/min), whereas the relative increases were most pronounced at low fVIII:C.

The hemophilia B subject, who was studied longitudinally, demonstrated a Solulin-dependent increase in the AUEC before fIX infusion (Figure 3B), which was similar to that observed in the hemophilia A subject (Figure 3A). Two hours after fIX infusion, the fIX:C was 38.2% and the AUEC in the absence of Solulin had increased from 2.3 × 10⁴ to 7.3 × 10⁴ mm/min. As with the hemophilia A subject, the absolute AUEC values at a given Solulin concentration were comparable throughout, whereas the increases relative to the values without Solulin were almost exclusively a function of the latter. For example, before fIX infusion (ie, 1.6% fIX), 3.6nM Solulin caused a 7.3-fold increase in AUEC, from 2.3 × 10⁴ to 16.7 × 10⁴ mm/min. This AUEC value remained virtually unchanged at 16.3 × 10⁴ mm/min 2 hours later, when fIX:C had reached 38.2%, whereas the relative increase was only 2.23-fold (16.3/7.3). As the fIX:C level dropped further to 14.4%, the relative improvement in the AUEC approached the pre-infusion level.

**Kinetics of protein C and TAFI activation by the thrombin-Solulin complex**

To rationalize the preference for antifibrinolysis over anticoagulation at low concentrations of Solulin, the kinetics of TAFI and protein C activation by the thrombin-Solulin complex were determined and compared. Figure 4 shows that the thrombin-Solulin complex activated TAFI much more efficiently than it did protein C. As described previously,²⁹ TAFI activation by the thrombin-Solulin complex can be described using the Michaelis-Menten model. Using the Michaelis-Menten model to fit the data, a $K_m$ of 0.71µM and a $k_{cat}$ of 1.09/s were determined, which implies a catalytic efficiency of 1.53/µM/s. The slope derived from the protein C activation data provides a reasonable estimate of the catalytic efficiency of the reaction. The catalytic efficiency of protein C activation by the thrombin-Solulin complex was 0.02/µM/s, which is 76-fold less than that observed for TAFI activation by the same complex.

**Ex vivo effect of Solulin on clot stability in blood from hemophilic dogs**

Dogs given a low dose of Solulin (5 or 10 µg/kg) generally showed improvement in their maximal clot strength after
Solulin administration (Figure 5A-C). Because of time and equipment constraints, clot strength (MA) was used to describe the effect of Solulin on fibrin stabilization. Figure 2A and B show that clot firmness (MA) and clot stability (AUEC) follow very similar trends. Dog 1 (Figure 2A) had a 40-mm improvement in maximal clot strength (amplitude) 2 hours after Solulin administration, at an actual plasma concentration of Solulin of 0.8nM Solulin. Dog 2 (Figure 2B) showed the best improvement in clot strength. A marginal increase in clot strength was observed after 0.5 and 2 hours (1.1 and 0.87nM Solulin, respectively). During the period from 8-72 hours (0.9-0.1nM Solulin), there was a sustained improvement in clot strength compared with the baseline value (20-35 mm improvement). Dog 3 (Figure 2C) also exhibited an improvement in clot strength during the 72-hour period after Solulin administration (up to a 25-mm improvement at 72 hours; 0.16nM Solulin). The dog given a high dose of Solulin (500 µg/kg; Figure 5D) was actually the first experiment in the series, the dose being selected from the range of doses studied in human volunteers.28 The dog showed a decrease in clot strength during the first 24 hours after receiving Solulin. As the concentration of Solulin decreased to approximately 40nM at the 48-hour time point, a marginal increase in clot strength was observed. The clot strength improved further at the 72-hour time point, when the Solulin concentration had decreased to approximately 22nM, indicating that lower doses were appropriate for further exploration.

Discussion

In the present study, we report proof of principle that Solulin may be used to improve clot strength and stability in hemophiliic whole blood. We demonstrate that in vitro Solulin improves clot stability in severe hemophilia A and B, and that the Solulin-dependent improvement in clot strength is maintained throughout the peaks and troughs of factor replacement therapy. As could be predicted, such improvement is less marked when high factor concentrations already provide for an acceptable clot firmness, and more marked when such factor effects are lower because of lower factor concentrations. We also show herein that at low concentrations (30 pM to 35nM), the antifibrinolytic properties of Solulin are more pronounced than anticoagulant properties, which are rationalized by the relative efficiencies of TAFI and protein C activation by the thrombin-Solulin complex. Finally, our preliminary data suggest that Solulin administered to dogs with severe hemophilia improves ex vivo thromboelastography parameters in a manner similar to that observed in vitro with blood from severe hemophilia human subjects.

The relative kinetics of TAFI and protein C activation by the thrombin-Solulin complex provides a biochemical rationale for the preference for antifibrinolysis at low Solulin concentrations with minimal anticoagulation. The $k_{cat}$ (1.1/s) and $K_m$ (0.71µM) of TAFI activation by the thrombin-Solulin complex suggest that TAFI is a much better substrate for the complex than is protein C, which has a linear Michaelis plot (ie, a high $K_m$). Given the potency of TAFIa in down-regulating fibrinolysis,18,24 only a very small fraction is needed to maximally attenuate fibrinolysis. Once this level of TAFIa is achieved, increasing the Solulin concentration would have no additional effect on fibrinolysis, but would result in increased protein C activation. The impact of this shift from a state of antifibrinolysis to that of antifibrinolysis combined with anticoagulation is evident when examining the effect of Solulin on clot stability. In the present study, at 3.6nM Solulin, the AUEC was increased by 4.5-fold, whereas at 71.4nM Solulin, the AUEC was decreased compared with the no-Solulin control (Figure 2B). Our data demonstrate that a higher concentration of Solulin compared with full-length thrombomodulin (rabbit lung) is required28 to shift the hemostatic balance from antifibrinolytic to anticoagulant (> 35nM compared with approximately 15nM).

The advantages of using an agent such as Solulin to stabilize fibrin are intriguing, but do not replace the need for factor replacement or bypassing products, and therefore one envisages that Solulin would be most efficacious as an adjunctive pro-hemostatic agent. The key features of Solulin that make it an interesting option for the treatment for
hemophilia is its long half-life and wide effective dose range (Figure 2). With a 15- to 30-hour half-life and effective dose range estimated to range from the sub-nanomolar to approximately 40nM, Solulin could potentially be administered on a weekly basis and provide the basis for a factor-sparing regime that would cut costs and make therapy more widely available. However, before proceeding to advanced trials, safety concerns stemming from the anticoagulant properties of Solulin must be addressed. The effect of overdosing with Solulin was evident herein in dog 4 (Figure 5D), in which a greater than 35nM Solulin decreased clot strength, implying enhanced anticoagulation. To minimize bleeding risk, certain Solulin mutants may be useful in increasing the safety profile of the potential drug. Both oxidized M388-Solulin and F376A-Solulin retain most of their cofactor activity for TAFI activation, but only a small fraction of their cofactor activity for protein C activation. Using these constructs may increase the therapeutic window of Solulin by making it safer at higher doses.

Fibrin formation and deposition is essential for normal hemostasis. Fibrin forms as a direct consequence of thrombin generation, and because this is impaired in hemophilia, so too is fibrin formation. Solulin is a novel compound with potential for the treatment of hemophilia and, perhaps more importantly, illustrates a novel paradigm in which fibrin formation and maintenance is targeted in addition to enhancing thrombin generation. A new paradigm in which fibrin is targeted may result in the development of therapeutics that decrease the incidence of bleeding at trough fVIII:C and make therapies more accessible to those in developing countries.

Acknowledgments

The authors thank Kirsten Christiansen for technical assistance and the staff at the Hemophilia Center, St Thomas’ Hospital, for help in recruiting patients.

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This work was supported by the National Institutes of Health (grant HL46703 project 4 to M.E.N.) and by the Heart and Stroke Foundation of Canada (grant TS575 to M.E.N.). The animal studies were sponsored by PAION Deutschland GmbH. For a portion of this study, J.H.F. was funded by a Heart and Stroke doctoral fellowship and a Queen’s University doctoral field research award. D.L. is the recipient of a Canada Research Chair in Molecular Hemostasis.

Authorship

Contribution: J.H.F. coordinated the study, designed and executed the experiments, analyzed the data, and wrote the manuscript; K.-U.P. coordinated the study, analyzed the data, and contributed to writing the manuscript; C.J.R. performed the experiments, recruited the patients, and edited the manuscript; L.H. and S.P. performed the animal experiments; D.L. coordinated the animal experiments and edited the manuscript; M.E.N. designed the experiments; and B.S. designed the experiments, coordinated the human in vitro studies, and edited the manuscript.

Conflict-of-interest disclosure: J.H.F. has received research funding and speaker honoraria from PAION Deutschland GmbH. K.-U.P. is an employee of PAION Deutschland GmbH, which has a commercial interest in Solulin. B.S. has participated in advisory board meetings for PAION Deutschland GmbH and received a consultancy fee. The remaining authors declare no competing financial interests.

M.E.N. is deceased.

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