Nanobody-induced stabilization of TAFIa activity improves resistance of hemophilia A clots against premature fibrinolysis

Maarten L.V. Hendrickx[1,2], Annette von Drygalski[1,3], Ann Gils[2], Paul J. Declerck[2] and Laurent O. Mosnier[1]

[1] Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA, [2] Laboratory for Therapeutic and Diagnostic Antibodies, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Belgium and [3] University of California San Diego, Department of Medicine, Division of Hematology/Oncology, San Diego, CA, USA.

Introduction

- In hemophilia, hemostasis with conventional clotting factors is often imperfect, especially in the presence of inhibitory antibodies towards fVIII and fIX.
- (Re)bleeding in hemophilia is thought to be exacerbated by increased fibrinolysis.
- Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) has potent antifibrinolytic effects and its generation is severely impaired in hemophilia.
- The half-life of TAFIa is very short (8 min at 37 °C).
- **Objective:** to identify and characterize a TAFIa stabilizing nanobody, VHH-TAFI-a428 (a428), and to determine the effects of TAFIa stabilization on the regulation of fibrinolysis in hemophilia A.

Results

a428 stabilizes the TAFIa activity

The TAFIa stabilizing properties of a428 were determined in plasma. After 60 min at 37°C, TAFIa stabilization was observed at low concentrations of a428 (fig 1A). Binding of a428 to TAFIa results in a partial inhibition of TAFIa, however the residual TAFIa activity is stabilized (Fig 1B). The half life of the residual TAFIa activity in the presence of a428 was 45.2 min, 6-fold longer than the half-life of TAFIa alone (7 min) (fig 1C). Furthermore, a428 prolonged the half-life of purified TAFIa from 8.7 to 70 min (>8-fold) and an engineered "stable" TAFIa mutant (S305C-T325I-T329I-H333Y-H335Q) from 19 h to >144 h (>7-fold).

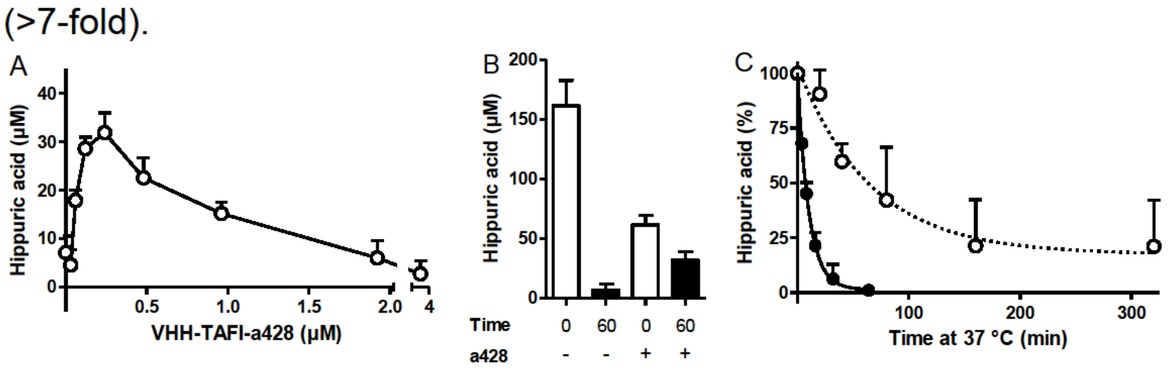


Figure 1: TAFIa activity after activation of TAFI in plasma followed by addition of a428 and subsequently incubation at 37°C for 60 min (panel A). Activity of TAFIa in plasma in the presence (+) or absence (-) of 0.24 µM a428 after incubation at 37°C for 0 or 60 min (open and solid bars) (panel B). Activity of TAFIa in plasma in the presence (dashed line) or absence (solid line) of 0.24 µM a428 after incubation for different time periods at 37°C (panel C) (mean ± SEM, n ≥ 3).

Stabilization of TAFIa in normal plasma

The effect of TAFIa stabilization on clot stability was determined in an *in vitro* clot lysis assay using normal plasma. Addition of a428 to normal plasma followed by thrombin triggered clot formation and t-PA induced lysis resulted in a prolongation of the clot lysis time (CLT) from 153 ± 3 min to 320 ± 32 min (fig 2A). The prolongation of CLT by a428 in plasma was dependent on the concentration of thrombomodulin (TM), i.e. the concentration of TAFIa generated (fig 2B).

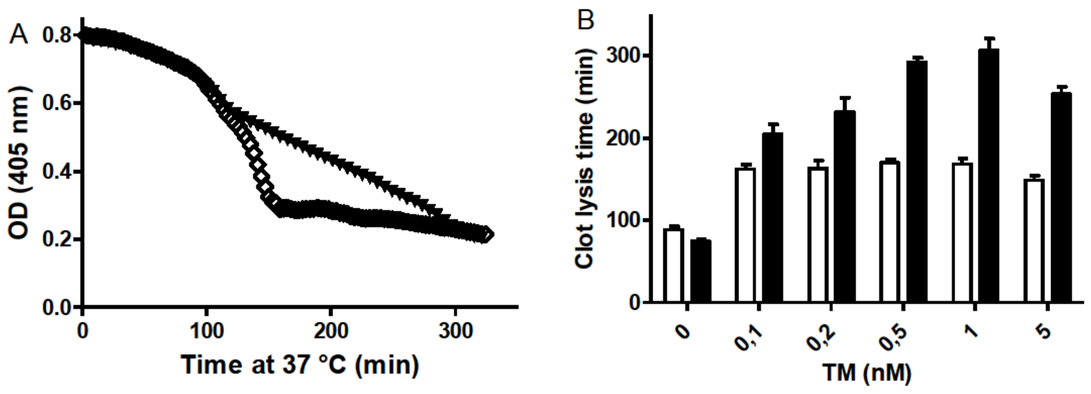


Figure 2: Clot lysis profiles of normal plasma in the absence (open diamonds) or presence of a428 (solid triangles) (panel A). Dose-dependent prolongation of clot lysis times by TM in the presence (black bars) or absence (open bars) of a428 (panel B) (mean ± SD, n ≥ 3).

Stabilization of TAFIa in hemophilia A plasma

Addition of a428 to hemophilia plasma resulted in a prolongation of the clot lysis time from 115 ± 8 min to 166 ± 8 min, similar to that of fVIII reconstituted plasma (173 ± 3 min) (fig 3A). Low concentrations of a428 resulted in a prolongation of clot lysis time while high concentrations inhibited TAFIa and shorten the clot lysis time (fig 3B).

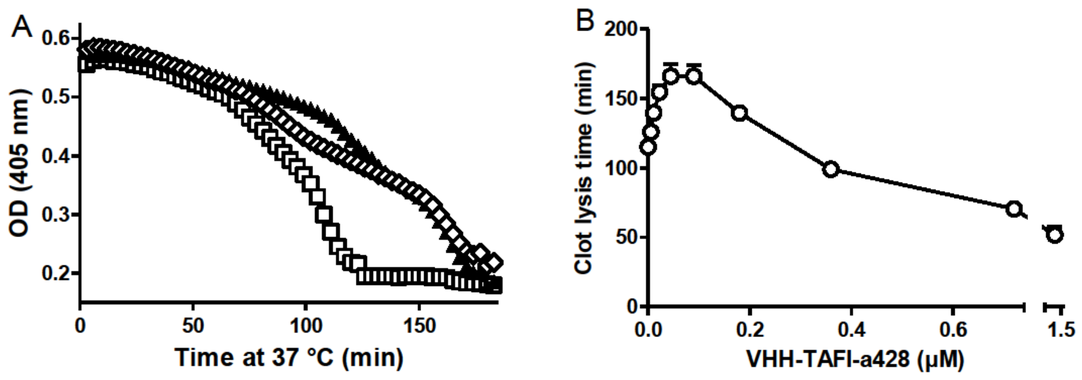
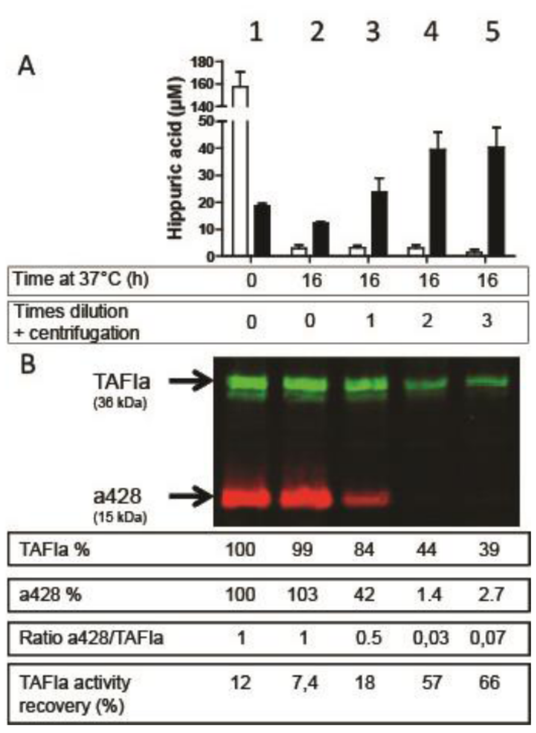


Figure 3: Clot lysis profiles of fVIII deficient plasma in the presence of buffer (open squares), fVIII (2 U/ml, solid triangles) or a428 (open diamonds) (panel A). Clot lysis times of fVIII deficient plasma supplemented with TM (1 nM) in the presence of different concentrations of a428 (panel B) (mean ± SD, n ≥ 3).

Elucidation of TAFIa stabilization by a428

TAFIa forms a reversible complex with a428 but free TAFIa is thermally inactivated to TAFIai (scheme 1). a428 inhibits part of the TAFIa activity and protects TAFIa from thermal inactivation. Dissociation and removal of a428 from TAFIa after 16h at 37°C reveals an increased TAFIa activity indicating that TAFIa bound to a428 is protected from thermal inactivation (fig 4A). Western blot analysis of TAFIa and a428 combined with the TAFIa activity data demonstrate a recovery of 66% of the TAFIa activity in the presence of a428 after 16h at 37°C (fig 4B).



$$\text{TAFIa}_{(36\ kDa)} + \text{a428}_{(15\ kDa)} \leftrightarrow \text{TAFIa-a428}_{(51\ kDa)}$$

$$\downarrow$$

$$\text{TAFIai}_{(36\ kDa)}$$

Scheme 1 (above): Interaction of a428 with TAFIa and TAFIa inactivation to TAFIai.

Figure 4 (left): Panel A: TAFIa activity (in the presence or absence of a428 (full and open bars, respectively)) immediately after activation (column 1), after incubation at 37°C for 16h (column 2) and after 1, 2 and 3 rounds of dilution and subsequent filtration using filters with a 30 kDa cutoff (column 3, 4 and 5) (mean ± SD, n ≥ 3). Panel B: Western blot of TAFIa and VHH-TAFI-a428 after activation (column 1), after incubation at 37°C for 16h (column 2) and after 1, 2 and 3 rounds of dilution and subsequent filtration (column 3, 4 and 5).

Conclusions

- a428 stabilizes the activity of purified TAFIa and TAFIa in plasma.
- Stabilization of TAFIa by a428 prolongs the clot lysis time in normal plasma and normalizes the clot lysis time of hemophilia A plasma.
- These results provide proof of concept that stabilization of TAFIa activity can improve resistance of hemophilia A clots against premature fibrinolysis.
- Development of TAFIa stabilizing agents may provide a novel approach to diminish bleeding in hemophilia.

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Novel therapeutic agents

Maarten Hendrickx

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