Development of double mutant clotting factor X as a novel thrombolytic agent

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INTRODUCTION

- Heart attack and stroke are the leading causes of death and are caused by blood clots
- Recombinant (r) tissue plasminogen activator (tPA) is used to "bust" these clots (Fig 1)

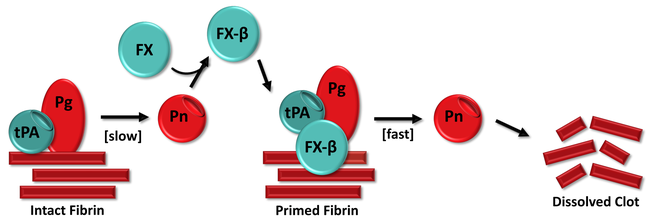


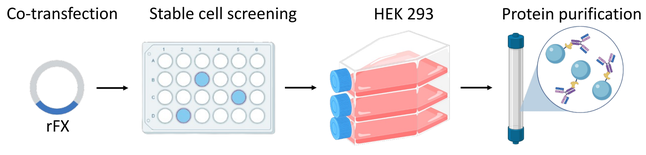
Figure 1. In the fibrinolytic process, the cleavage of plasminogen (Pg) into plasmin (Pn) by tPA can be accelerated by Pn-cleaved FX, FX-β.

- As an enzyme, large doses of rtPA invoke systemic fibrinolysis and cerebral hemorrhage
- We have generated a non-enzymatic alternative, using FX as an accelerator of tPA following plasmin cleavage (Fig 1)
- The current research investigates a double mutant rFX with inhibited clotting function (i) and plasmin cleavage-resistant (c) mutants (rFXic)

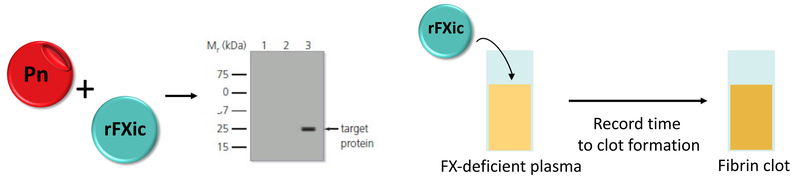
We hypothesize that rFXic will be a safer alternative to rtPA, and that the γ-carboxyglutamic acid (Gla)-domain of FX, enabling binding to anionic phospholipid membrane and fibrin, is key to localized thrombolytic activity.

AIMS AND METHODS

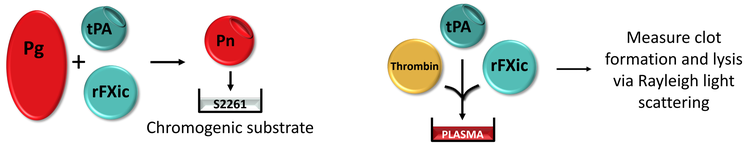
AIM 1: Generate recombinant FX (wild-type, rFXic) that is carboxylated and binds to anionic phospholipid in a calcium-dependent manner.



AIM 2: Characterize the rFX proteins: ensure that rFXic does not get cleaved into FXγ, via Western blot, and has no clotting activity, via clotting assay.



AIM 3: Test rFXic for tPA acceleration and enhancement of fibrinolysis via thrombin generation and plasmin lysis assays.



RESULTS: AIM 1

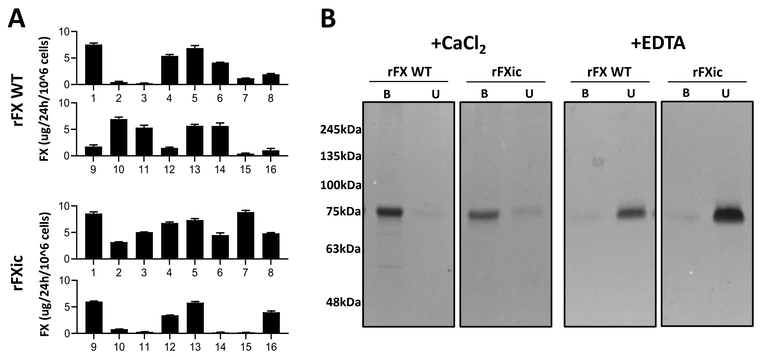


Figure 2. (A) Multiple clones of rFX were generated and tested for rFX productivity levels via ELISA. **(B)** Calcium-dependent binding of rFX WT and rFXic to anionic phospholipid (aPL) vesicles in the presence of calcium or EDTA, a calcium chelator, was measured in aPL-bound (B) and unbound (U) fractions.

- Clones producing >5ug rFX/mL/24hrs were chosen for production
- Fig. 2A: Both rFX WT and rFXic bind to aPL in the presence of calcium; EDTA dissociates both rFX WT and rFXic from aPL

rFXic binds anionic phospholipids (aPL) in a calcium-dependent manner, which indicates that it will localize to the site of a clot, in which aPL is exposed.

RESULTS: AIM 2

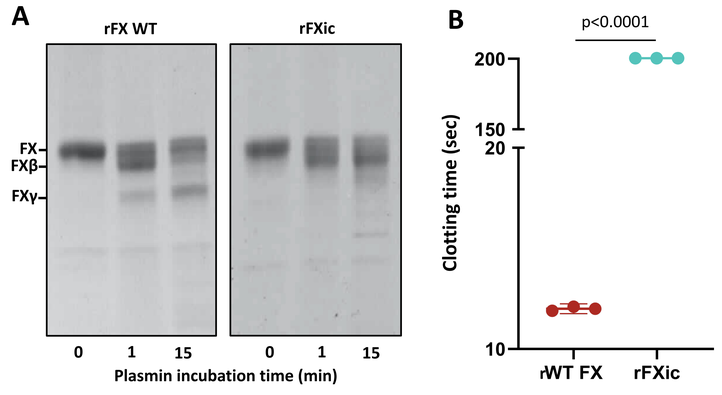


Figure 3. (A) rFX digestion with 0.1μM plasmin in the presence of aPL and Ca^{2+}. SDS-PAGE transferred to PVDF membrane and probed with α-FX monoclonal antibody. **(B)** Intrinsic clotting assay measuring prothrombin time initiated by tissue factor in FX-deficient plasma supplemented with either rFX WT (red) or rFXic (blue).

- Fig. 3A: rFXic does not follow the same cleavage profile of its WT counterpart, digesting into the FXβ but not the FXγ species
- Fig. 3B: rFXic does not generate a clot before the 200sec assay cut-off time, surpassing the well-established average of 12sec (rFX WT)
- These data suggest that both the K330Q and S379A mutations were successful

The K330Q mutation successfully prevents plasmin cleavage of the protein into the inactive FXγ. The S379A mutation successfully inhibits the intrinsic clotting activity of FX. This suggests both a longer half-life and increased safety as a therapeutic.

RESULTS: AIM 3 + 4

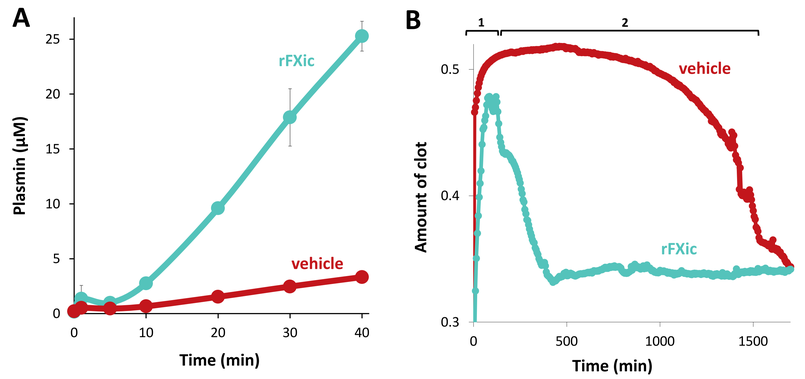


Figure 4. (A) Plasmin generation and **(B)** plasma lysis assays with rFXic (teal), or no additional cofactors (red) added. Black bars indicate standard deviation of assays run in triplicate. 1, clot formation phase; 2, clot dissolution phase.

- Fig. 4A: rFXic accelerates the generation of plasmin by tPA more than 10-fold
- Fig. 4B: rFXic accelerates fibrinolysis in plasma when added during clot initiation

rFXic requires the Gla domain to accelerate plasmin generation and subsequent fibrin clot lysis in plasma, setting the stage for pre-clinical animal studies.

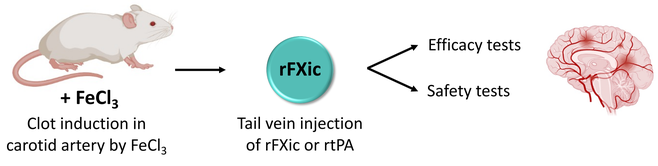
CONCLUSIONS

Summary:

- The gold-standard treatment for heart attack and stroke causes cerebral hemorrhage in 6% of patients, suggesting a non-enzymatic alternative would improve outcome
- A double mutant FX, rFX QA, binds anionic phospholipids in a calcium dependent manner to localize its accelerant activity to the site of a clot
- rFX QA does not degrade to a known inactive species, has no residual clotting activity, and accelerates fibrinolysis in plasma

Future directions and significance:

- Further *in vitro* and *ex vivo* studies, including a mouse model of carotid thrombosis, are anticipated to show that QA is a safer alternative to rtPA



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