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, as tPA is the enzymatic accelerant of fibrinolysis (Fig 1)

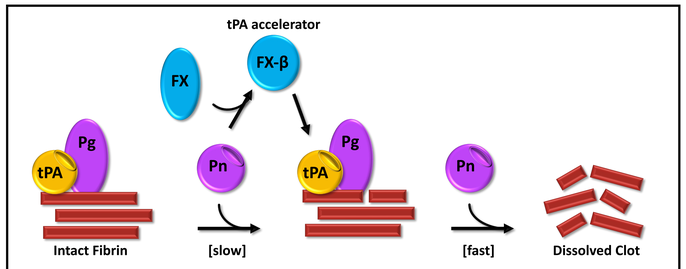


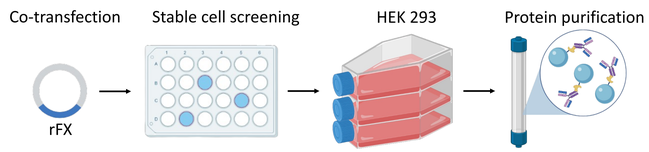
Figure 1. Schematic representation of the fibrinolytic process which breaks down fibrin clots through the cleavage of plasminogen (Pg) into plasmin (Pn) by tPA, which can be accelerated by Pn-cleaved FX, FX- β.

- As an enzyme, rtPA can invoke systemic fibrinolysis and cerebral hemorrhage
- The Pryzdial lab has sought to generate a non-enzymatic alternative, using FX as an accelerator of tPA following plasmin cleavage (Fig 1)
- The current research investigates a recombinant FX (rFX) with two mutations:
  - **K330Q**, preventing cleavage at K330 which correlates to loss of tPA acceleration
  - **S379A**, inhibiting the intrinsic clotting activity of FX

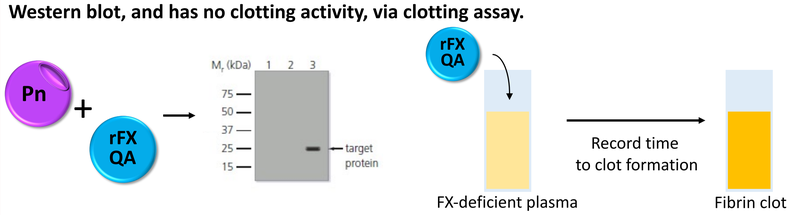
We hypothesize that a non-enzymatic accelerator of fibrinolysis, such as the double mutant rFX, abbreviated QA, will be a safer alternative to rtPA for therapy of heart attacks and stroke.

AIMS AND METHODS

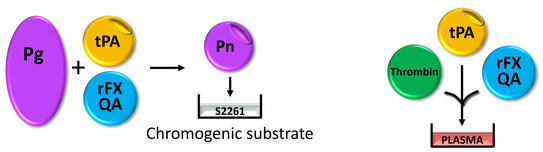
AIM 1: Generate purified recombinant FX (WT, QA) that is calcium-dependent and binds to anionic phospholipid with column chromatography.



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



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



RESULTS: AIM 1

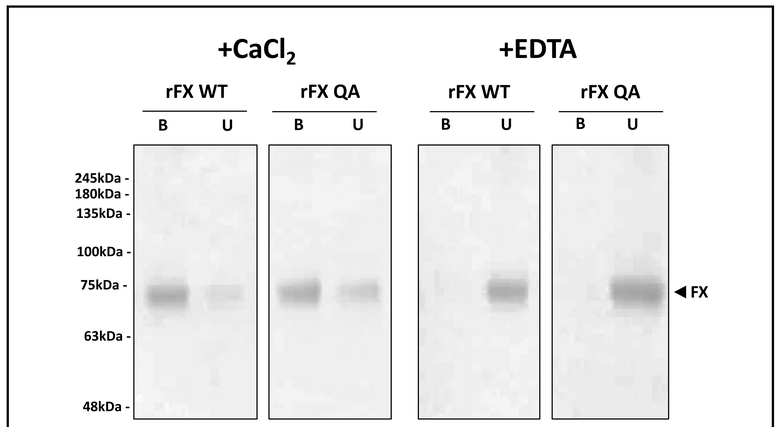


Figure 2. Calcium-dependent binding of rFX WT and QA to anionic phospholipid (aPL) vesicles in the presence of calcium or EDTA, a calcium chelator. After a 10 minute incubation, the samples were centrifuged and the aPL-bound (B) and unbound (U) fractions were probed with α-FX monoclonal antibody.

- When in the presence of calcium, rFX QA follows trends similar to rFX WT, binding to aPL
- When in the presence of EDTA, rFX QA is unable to bind aPL, as expected based on the WT

rFX QA 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

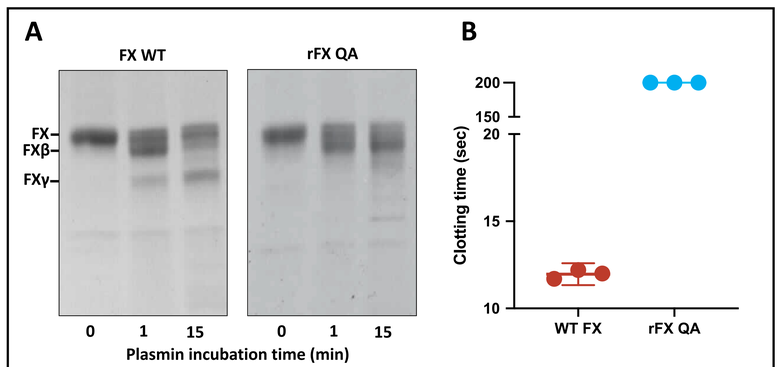


Figure 3. (A) Time course of rFX digestion with 0.1μM plasmin in the presence of aPL and Ca^{2+}. SDS-PAGE transferred to PVDF membrane and probed with α-FX monoclonal antibody. **(B)** Intrinsic clotting assay measuring prothrombin (clotting) time with the addition of tissue factor, FX-deficient plasma,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

- Fig. 3A: rFX QA does not follow the same cleavage profile of its WT counterpart, digesting into the FXβ but not the FXγ species
- Fig. 3B: rFX QA hits the 200 sec assay cut-off time for clotting, surpassing the well-established average of 12 sec (rFX WT)

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

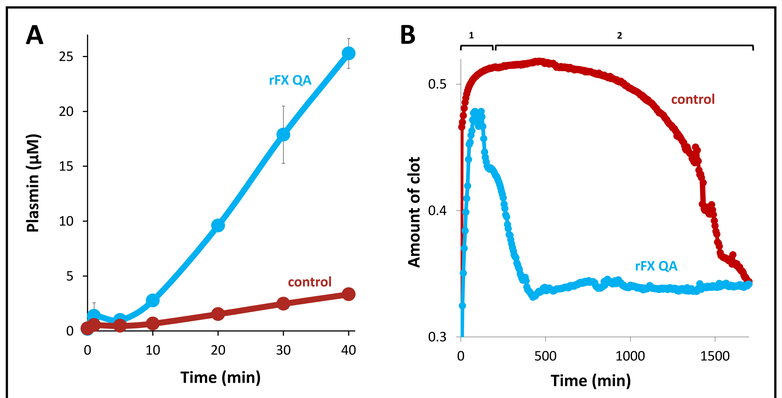


Figure 4. (A) Plasmin generation and **(B)** plasma lysis assays with rFX QA (blue), or no additional cofactors (red) added. Black bars indicate standard deviation of assays run in triplicate. 1, clot formation phase; 2, clot dissolution phase.

- Fig. 4A: rFX QA accelerates the generation of plasmin by tPA more than 10-fold
- Fig. 4B: The peak clot amount in the clot formation phase may suggest that fibrinolysis is accelerated such that it occurs in conjunction with clot formation, when rFX QA is added
- Fig. 4B: rFX QA accelerates fibrinolysis, as indicated by the steep downward slope of the blue line (rFX QA) and maintenance of hemostasis during the clot dissolution phase

rFX QA accelerates plasmin generation and subsequent fibrin clot lysis in plasma, setting the stage for pre-clinical animal studies.

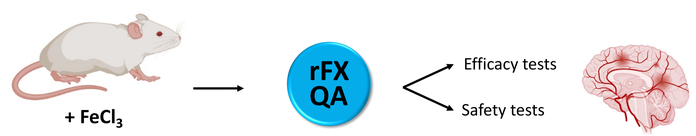
CONCLUSIONS

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



Clot induction in carotid artery by $FeCl_3$

Tail vein injection of rFX QA or rtPA

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