Developing a novel double mutant clotting factor X as a thrombolytic therapy

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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)

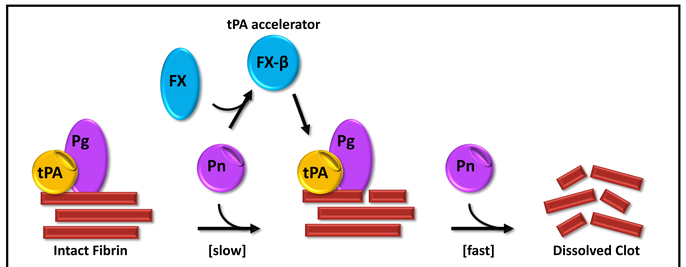


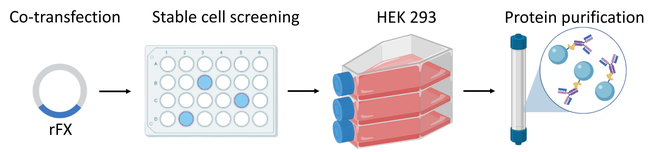
Figure 1. Schematic representation of the fibrinolytic process which breaks down fibrin clots through the cleavage of plasminogen (Pg) into plasmin (Pn) by tPA, which can be accelerated by Pn-cleaved FX, FX- β.

- 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
  - **S195A**, inhibiting the intrinsic clotting activity of FX

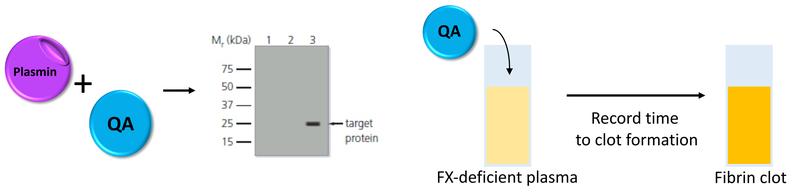
We hypothesize that a non-enzymatic accelerator of fibrinolysis, such as the double mutant rFX, abbreviated QA, will be a safer alternative to rtPA for therapy of heart attacks and stroke.

AIMS AND METHODS

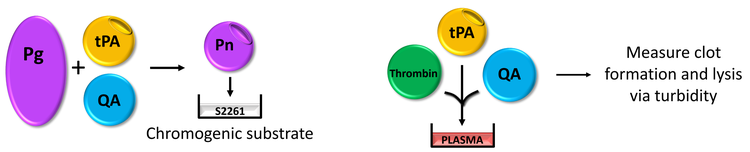
AIM 1: Generate purified recombinant FX (WT, QA) that is calcium-dependent and binds to anionic phospholipid with column chromatography.



AIM 2: Characterize the rFX proteins: ensure that QA does not get cleaved into the 46kDa species, 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.



AIM 1: PROTEIN GENERATION

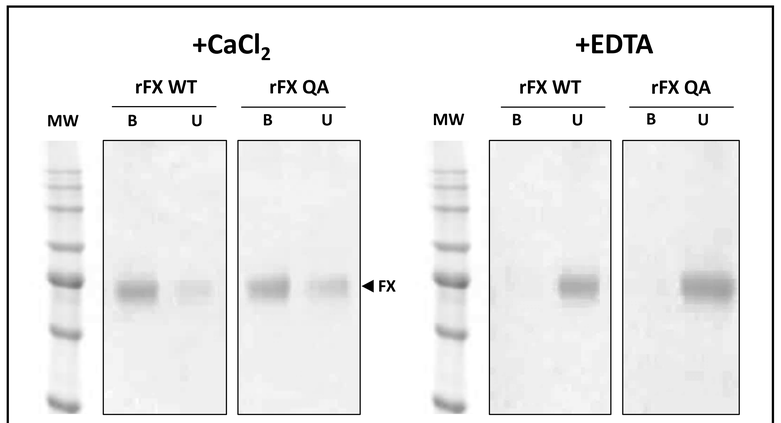


Figure 2. Calcium-dependent binding of WT and QA rFX to anionic phospholipid vesicles in the presence of calcium or EDTA, a calcium chelator. After 10 minute incubation, the samples were centrifuged and the bound (B) and unbound (U) rFX was evaluated. Samples were run non-reduced on 10% SDS-PAGE, transferred to PVDF membrane, and probed with α-FX monoclonal antibody. MW; molecular weight marker.

- 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.

AIM 2: PROTEIN CHARACTERIZATION

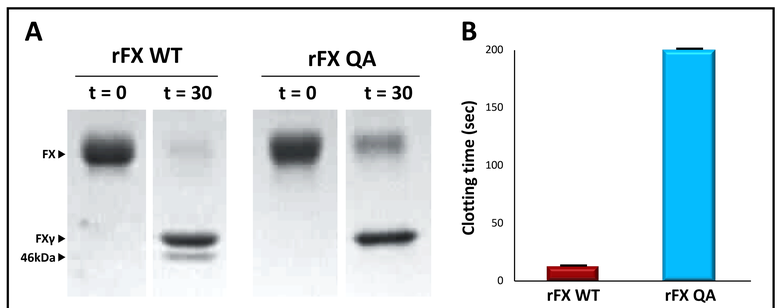


Figure 3. (A) Time course of rFX digestion with 0.1μM plasmin in the presence of aPL and Ca^{2+}. SDS-PAGE transferred to PVDF membrane and probed with α-FX monoclonal antibody. **(B)** Intrinsic clotting assay measuring prothrombin (clotting) time with the addition of tissue factor, FX-deficient plasma,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

- Fig. 3A: Following the expected cleavage profile, plasmin cleaves rFX WT into FXγ and the 46kDa species after a 30 minute incubation
- Fig. 3A: rFX QA does not follow the same cleavage profile of its WT counterpart, digesting more slowly into the FXγ species and not following into the 46kDa species
- Fig. 3B: rFX QA surpasses the 200 sec cut-off time for clotting, surpassing the well-established average of 13 sec (rFX WT)

The K330Q mutation successfully prevents plasmin cleavage of the protein into an inactive 46kDa species. The S195A mutation successfully inhibits the intrinsic clotting activity of FX. This suggests both a longer half-life and increased safety as a therapeutic.

AIM 3: FIBRINOLYTIC ACCELERATION

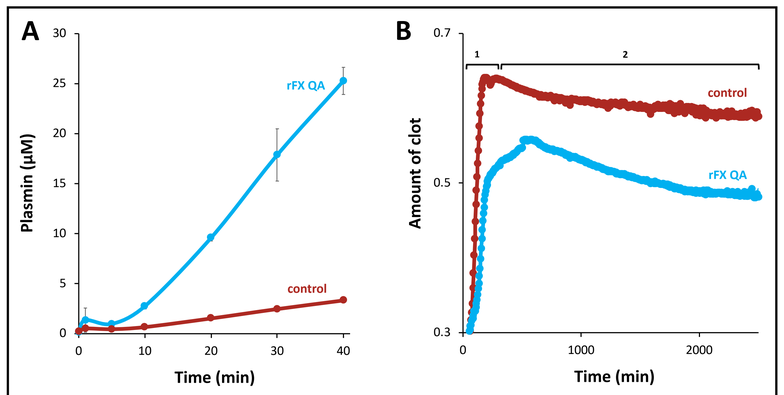


Figure 4. (A) Plasmin generation and **(B)** plasma lysis assays with rFX QA (blue), or no additional cofactors (red) added. Black bars indicate standard deviation of assays run in triplicate. 1, clot formation phase; 2, clot dissolution phase.

- 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
- Fig. 4B: rFX QA accelerates fibrinolysis, as indicated by the downward slope of the blue line (rFX QA) during the clot dissolution phase

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

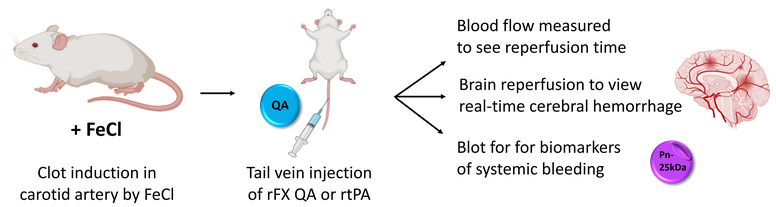
CONCLUSIONS

Summary:

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

Future directions and implications:

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



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