Fibrin based *in vitro* assays for evaluation of direct factor XIIIa blockers

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Introduction

Coagulation Factor XIII (FXIII) is considered since many years as scientific qualified target for the development of novel anticoagulants [1]. Since FXIII is a transglutaminase – but not a protease – the enzyme possesses a unique mode of action. Transglutaminases perform cross-linking reactions. Therefore, FXIII is the major factor influencing clot stability, clot maturation and clot lysis (figure 1). After proteolytic activation by thrombin, FXIIIa immediately modifies the soft fibrin clot introducing covalent bonds. First, cross-linking between longitudinal γ-chains of fibrin is catalyzed and subsequently α_2-antiplasmin is incorporated further increasing the resistance against lysis.

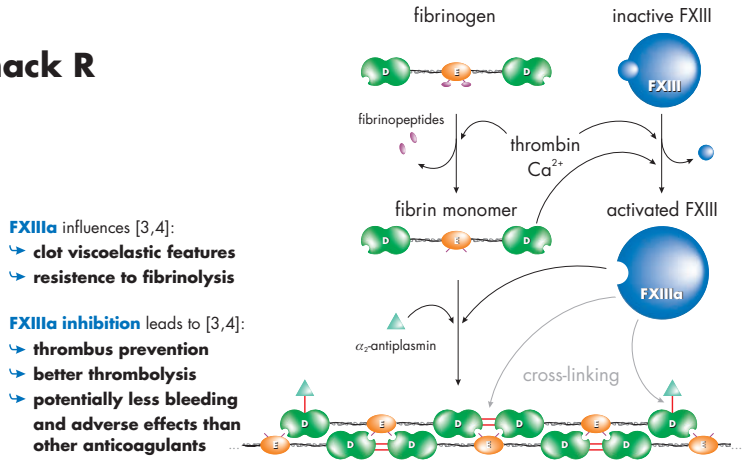

Figure 1: FXIIIa is the major factor influencing clot stability, clot maturation and clot lysis and is therefor a qualified target for anti-coagulation.

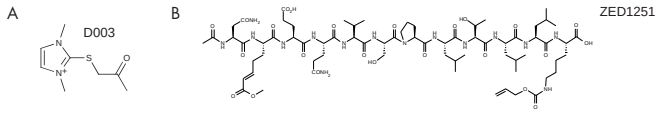

Figure 2: A) Structure of D003. B) Lead-structure of ZED1251 for FXIII-blocker drug development.

Aims

Our aim is to develop small molecule blockers of coagulation Factor XIIIa.

Small molecule compounds addressing Factor XIIIa are rare. Irreversible acting thioimidazole derivatives like D003, developed at Merck Sharp & Dohme [2], are still state of the art. The mechanism based blockers address the active site cysteinyl residue leading to an acetonylation. Due to short plasma half life and low selectivity the preclinical development was discontinued. However, we developed a novel class of transglutaminase inhibitors (figure 2).

The peptidic and peptidomimetic compounds bear a michael acceptor pharmacophore replacing the substrate glutamine. The electrophilic α,β-unsaturated carbonyl compounds are selective for thiol/cysteine dependent transglutaminases.

Method and Results

Inhibition of γ-chain cross-linking

The cross-linking of fibrin γ-chains is the fastest reaction catalyzed by factor XIIIa. Therefore, we developed an *in vitro* assay based on fibrinogen, calcium, thrombin and coagulation factor XIII to determine the efficacy of inhibitors in a more physiological experimental setting.

Figure 3A shows the time dependent cross-linking of fibrin γ-chains. Clotting is initiated by the addition of thrombin. It should be noticed that commercial available fibrinogen preparations contain enough factor XIII for our experiments. To study the progress of the clotting reaction we sampled after indicated time points and separated the proteins using SDS-PAGE followed by western blotting. The fibrin γ-chains were visualized using a monoclonal antibody. Figure 3B shows the cross-linking of fibrin in the presence of novel FXIIIa-blockers. With increasing inhibitor concentration we observed the following three effects:

- Stability of the macroscopic clot decreases: by flicking the reaction tube the clot disrupts into smaller fragments
- More soluble protein compared to controls is detectable in western blots
- Reaction shifts from dimer towards the monomeric fibrin γ-chain: based on the ratio of fibrin monomer vs. dimer we estimated an apparent EC_{50} for inhibition efficacy which turned out to be in good agreement to inhibition kinetics.

The degree of clot cross-linking is dependent on FXIII activity. Thus, the clot lysis rate is a function of FXIII activity. By using FITC-fibrinogen in a whole blood *in vitro* assay, we were able to detect increased clot lysis rates when clot formation was perfomed in presence of FXIII inhibitors (figure 4). These results are in agreement with Mutch *et al.* [5].

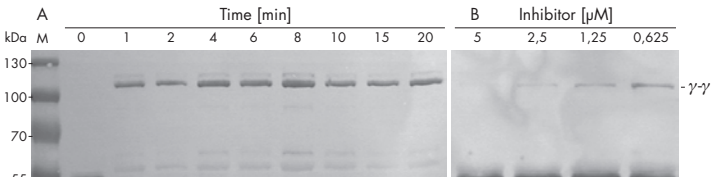

Figure 3: Western Blot and immunostainig against fibrin γ-chain. A) Time dependent cross-linking of fibrin γ-chains. Already after 1 minute there is no monomeric fibrin γ-chain (γ) detectable and after 2 minutes a gel is formed within the tube. Maximum soluble γ-dimer (γ-γ) is reached after approximately 8 minutes. B) Cross-linking of fibrin in the presence of novel FXIIIa-blockers.

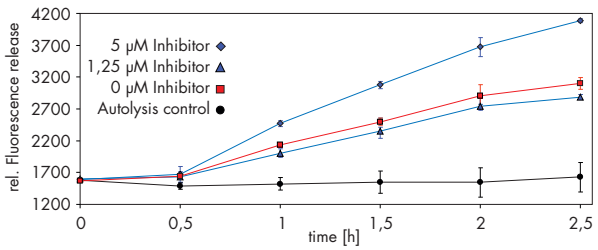

Figure 4: Lysis of blood clots formed in the presence of FITC-fibrinogen. Clot lysis was induced by addition of urokinase.

Conclusion

We found promising novel compounds serving as lead structures for drug able FXIIIa inhibitors. These compounds have the potential to open a novel therapeutic option for thrombosis prophylaxis in risk patients.

Furthermore, fibrin based assays reveal comparable results as in inhibition kinetics.

References

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