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Fibrinogen targeted Affimers can successfully modulate fibrin clot lysis in the presence of plasminogen inhibitor

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Background

Cardiovascular disease (CVD) remains the main cause of morbidity and mortality in the developed world.

Fibrin clot structure and fibrinolysis can determine predisposition to CVD and manipulating the prothrombotic environment can reduce the risk of vascular events.

Current treatment strategies are only partially effective at reducing the thrombotic milieu, and frequently associated with adverse events secondary to bleeding.

Identification of new therapeutic targets is necessary to reduce thrombosis potential in high risk subjects

Aims

The aim of the research was to utilise an artificial binding protein (Affimer) phage display library to screen fibrinogen for binding peptides that could interfere with clot lysis, in particular interaction with plasminogen inhibitor (PI).

ELISA & SPR

- A phage display library consisting of two loops of 9 amino acids, constrained in a protein scaffold was used to screen against fibrinogen (library size: 10^{10} Affimers).
- High affinity binding Affimers were released by the addition of excess PI.
- To determine if the Affimers were acting at functionally relevant sites, fibrinogen-binding Affimers were tested in turbidimetric assays using plasma and purified systems.
- Time from full clot formation to 50% lysis was taken as clot lysis time (LT).
- In a plasma system, Affimer/fibrinogen molar ratio was kept at 5:1, whereas this ratio was reduced to 2:1 in the purified system.
- Real time lysis of mature clots was performed using confocal microscopy and the effects of Affimers on clot structure was analysed using scanning electron microscopy.

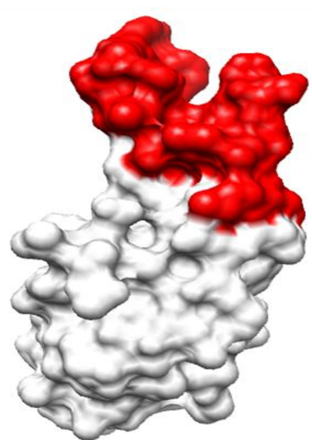

Figure 1. Crystal structure of Affimer scaffold protein with two regions containing variable amino acid loops in red.

Effect on clot structure

- Clots prepared from normal pooled plasma and pre-incubated with either buffered saline, Affimer A2 or Affimer G4 and viewed under a scanning electron microscope.
- Fibre diameter was significantly affected by the addition of Affimers.
- Control clot fibre diameter was (mean±SEM) 103±1.89nm which was reduced to 90.2±1.86 nm by Affimer A2 and increased to 124.6±1.76nm by Affimer G4.

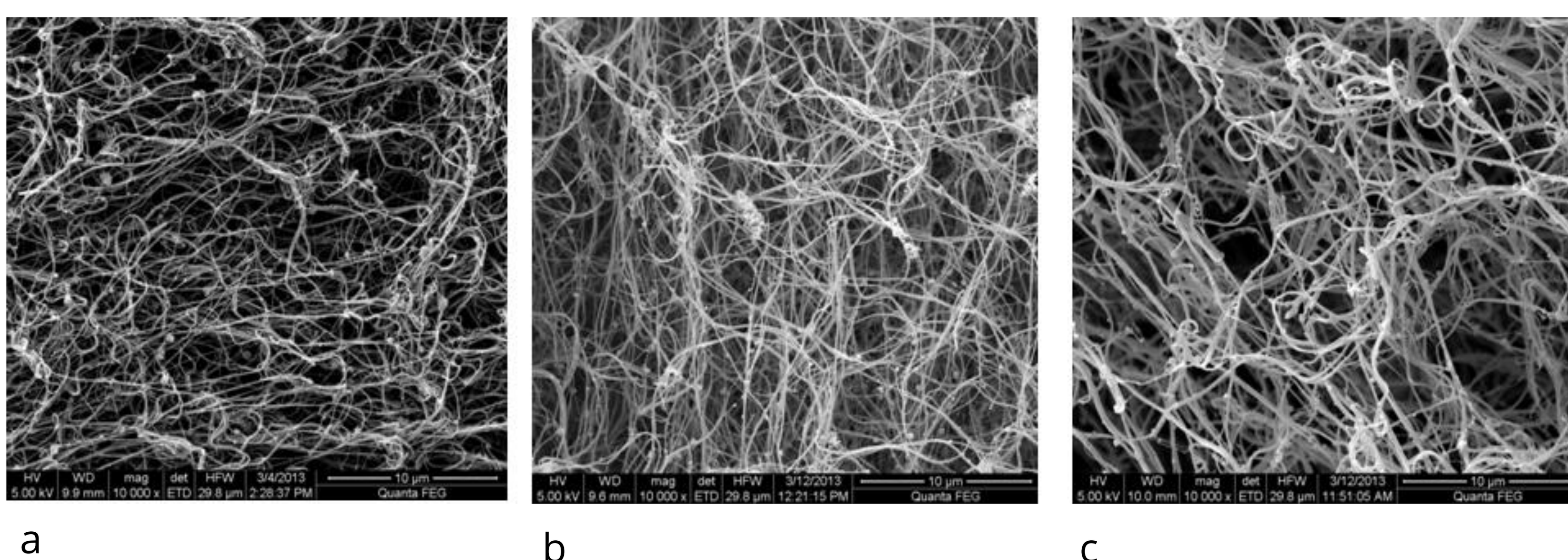

Figure 4. The effects of Affimers on fibrin network structure. Scanning electron micrographs of clots prepared from normal pooled plasma with the addition of a) buffered saline b) Affimer A2 and c) Affimer G4

Results

Numerous Affimers were seen to bind fibrinogen and therefore only those with the highest binding affinities were tested (n=8).

Plasma – LT was 840 sec with 4 Affimers increasing this by a mean of 894 sec (range 144-1872). One Affimer abolished clot lysis, another reduced clot lysis by 48 seconds whereas two had no effect.

Similar results were observed for the real time lysis of mature clots following the addition of Affimers, A2, G2 and G4 to plasma (figure 2)

We then tested Affimers that prolonged, reduced and had no effect on plasma clot lysis in a purified system, both in the presence and absence of PI.

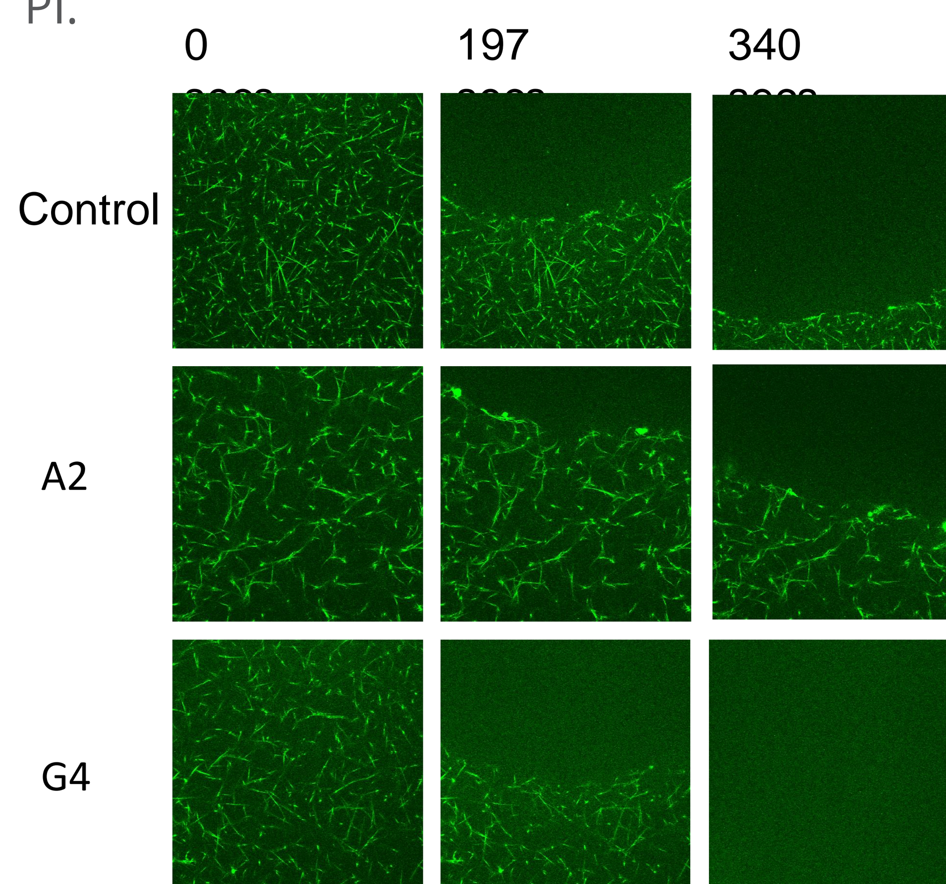

Figure 2. Real time lysis of mature clots prepared from normal pooled plasma and incubated with Affimer A2 or G4 or buffered saline as a control. A lysis mix was added to the edge of the clot and the time taken for the lysis front to dissolve the visualised clot as lysis time (LT). In the absence of Affimers LT (mean±SEM) was 463±21.5 secs, which was increased to 567.7±51.5 secs by Affimer A2. The addition of Affimer G4 reduced LT to 308±38.2 secs. Affimer G2 had no significant effect on LT (data not shown)

Purified system

In the absence of PI -

- Affimer A2 prolonged clot lysis from (mean±SEM) 606±43 to 993±85 sec ($p<0.05$)
- Affimers G2 and G4 had no significant effect at 708±26 and 672±17 sec, respectively.

In the presence of PI –

- A2 prolonged LT from1440±111.5 sec to 1880±122 sec
- G4 reduced LT to 940±71 sec ($p<0.05$).
- G2 had no significant effect on PI-mediated prolongation of clot lysis (1188±71 sec).

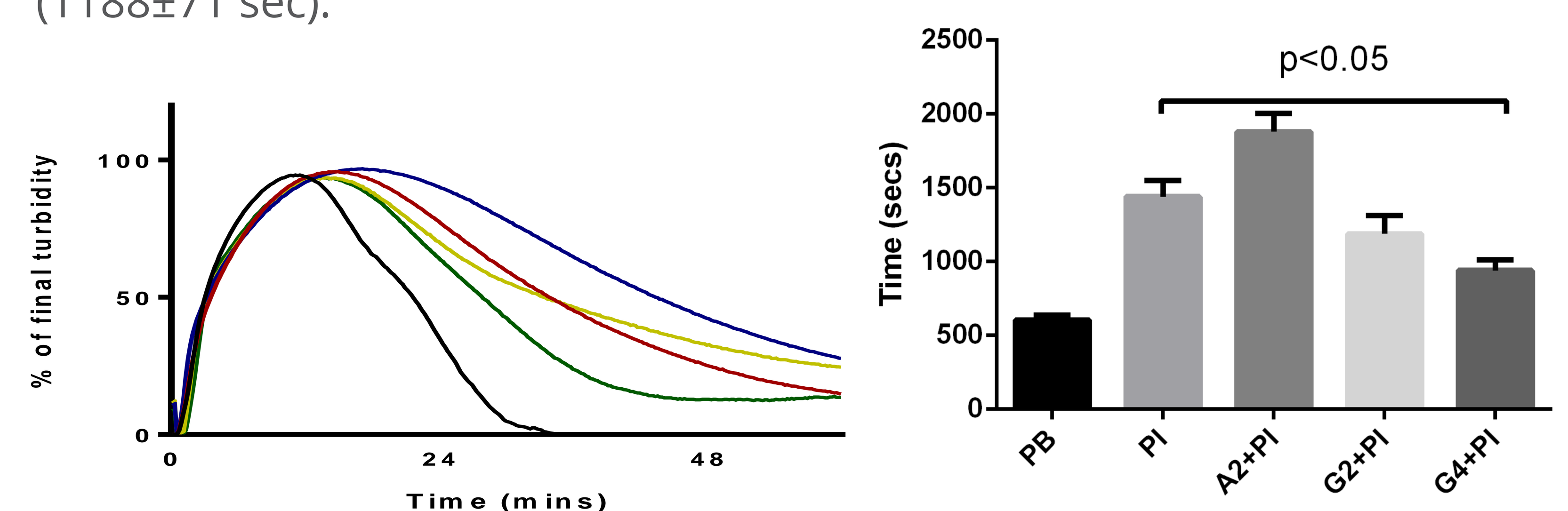

Figure 3. a) Effects of Affimers on fibrinolysis during clot formation using commercial fibrinogen in the presence and absence of PI. Black line, fibrinogen only; red line, addition of PI; blue line PI & Affimer A2; yellow line PI & Affimer G2; green line, PI & Affimer G4. b) In the presence of PI, Affimer A2 prolongs PI induced LT, G4 reduces PI induced LT G2 had no significant effect.

Summary

The use of a phage display system allows a novel and rapid screening of proteins with numerous random peptides for the investigation of coagulation protein interactions.

In this instance the interactions between fibrinogen and PI were analysed.

Two Affimers were discovered that bind to fibrinogen at functionally active sites and were capable of enhancing and reducing the effects of PI respectively.

These Affimers could be the focus of novel therapeutic agents for thrombotic disorders and may even be beneficial in those with bleeding tendencies.

For further information regarding Affimer technology, please contact chris.miller@avacta.com or visit www.avacta.com