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## **ORAL ABSTRACTS**

## 321.COAGULATION AND FIBRINOLYSIS: BASIC AND TRANSLATIONAL

## Design of a Superior Factor VIIIa Mimetic By Coupling a Membrane Binding Domain to a Factor IXa Binding Antibody Fragment

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Emicizumab is a bispecific antibody that augments Factor X (FX) activation by factor IXa (FIXa) in the absence of factor VIIIa (FVIIIa) by binding and approximating the substrate FX and protease FIXa. Clinical use of this FVIIIa mimetic significantly reduces bleeding episodes in Hemophilia A (HA) patients. Membrane dependent assembly of FVIIIa-FIXa-FX is an absolute requirement for the intrinsic tenase activity. In contrast, emicizumab lacks any membrane binding ability itself and exhibits significant FXa generation even in the absence of membranes. This major distinction likely allows FVIIIa to function at subnanomolar concentrations to greatly accelerate the activation of FX while only much lower enhancements of rate are achieved at several 100-fold higher concentrations of emicizumab. We sought to test whether the addition of a membrane binding feature in the monospecific FIXa binding fragment of emicizumab could alone approximate the cofactor activity obtained with the bispecific antibody.

We first generated a single chain antibody fragment (scFv) of the FIXa binding arm of emicizumab by joining the variable heavy chain (V<sub>H</sub>) with the variable light chain (V<sub>L</sub>) to produce V<sub>H</sub>9V<sub>L</sub>. In line with previous observations that FIXa one arm antibodies of emicizumab that can only bind FIXa and impart a modest cofactor mimetic activity, V<sub>H</sub>9V<sub>L</sub>, facilitated FIXa-mediated FX activation at a much lower rate in comparison to bispecific emicizumab. The scFv, V<sub>H</sub>9V<sub>L</sub>, was used as a template to introduce membrane binding properties.

The Factor V C2-domain, which binds membrane, was fused at the C-terminus of V  $_{\rm H}$ 9V  $_{\rm L}$  via a repeating Gly  $_{\rm 4}$ Ser flexible linker of ~ 5.7 nm to allow the V  $_{\rm H}$  and V  $_{\rm L}$  domains of the scFv to potentially orient correctly at the membrane surface. The resulting construct V  $_{\rm H}$ 9V  $_{\rm L}$ C2 showed a substantial amplification in cofactor mimetic activity as compared to V  $_{\rm H}$ 9V  $_{\rm L}$ . *In vitro* kinetic analyses with purified proteins and synthetic phospholipid vesicles containing phosphatidylcholine (PC) and phosphatidylserine (PS) (PC:PS, 75:25 %) showed approximately 22-fold faster activation of FX by V  $_{\rm H}$ 9V  $_{\rm L}$ C2 as compared to V  $_{\rm H}$ 9V  $_{\rm L}$ . Remarkably, despite its inability to bind FX, cofactor mimetic activity of V  $_{\rm H}$ 9V  $_{\rm L}$ C2 was about two-fold higher than that of emicizumab. Potent enhancement of FX activation by V  $_{\rm H}$ 9V  $_{\rm L}$ C2 observed with purified proteins was also reflected in thrombin generation assays (TGA). V  $_{\rm H}$ 9V  $_{\rm L}$ C2 added at 100 nM brought thrombin generation into the reference range in congenital FVIII-deficient HA plasma supplemented with 4  $\mu$ M PC:PS and triggered with either low tissue factor (0.1 pM), or factor XIa (0.1 nM).

The role of C2 domain-mediated increase in cofactor activity of V<sub>H</sub>9V<sub>L</sub>C2 was further assessed by using an antibody fragment (E <sub>9</sub>scFv) that inhibits the binding of factor V to membranes. Increasing concentrations of E <sub>9</sub>scFv caused a dose-dependent reduction in the rate of FXa formation catalyzed by the complex of V<sub>H</sub>9V<sub>L</sub>C2: FIXa (20nM:25nM). Inhibition saturated at ~85% at 100 nM E <sub>9</sub>scFv, indicating a substantial contribution of membrane binding to the cofactor mimetic function of V<sub>H</sub>9V<sub>L</sub>C2. To evaluate whether V<sub>H</sub>9V<sub>L</sub>C2 can restore clotting in HA plasma with FVIII inhibitors, normal pooled plasma was supplemented with a FVIII neutralizing antibody to mimic FVIII inhibitor plasma. Addition of V<sub>H</sub>9V<sub>L</sub>C2 to this plasma restored clotting time to the levels shown by normal pooled plasma, suggesting that V<sub>H</sub>9V<sub>L</sub>C2 can bypass FVIII inhibitor activity.

The engineered scFv with a factor V C2 domain fusion, V<sub>H</sub>9V<sub>L</sub>C2, demonstrates that mere addition of a membrane binding feature to the FIXa arm of the emicizumab surpasses the mimetic activity achieved by a bispecific antibody that exploits both bridging and allostery to be an effective cofactor. Such a construct also avoids the burden of productive assembly from a combination of three separate polypeptide chains, as in a functional bispecific antibody. Replicating the membrane binding feature into bispecific FVIIIa mimetic antibodies could further amplify function and more closely resemble FVIIIa in both activity and membrane dependent regulation. Our results provide surprising insights into how a single chain, monospecific, membrane-anchored FVIIIa mimetic enhances the catalytic activity of FIXa.

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