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Supramolecular anticoagulants with on-demand reversibility

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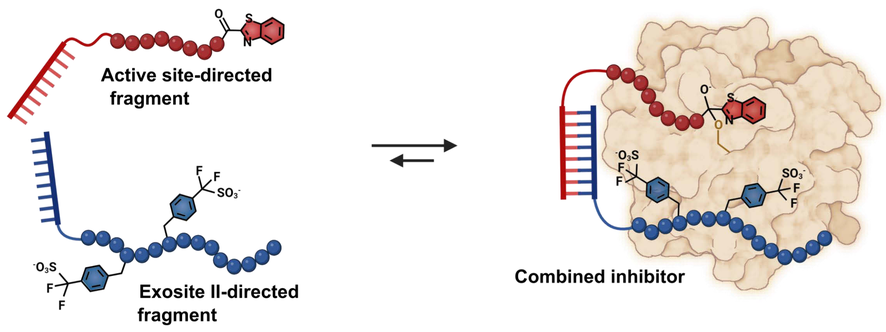
1- Introduction

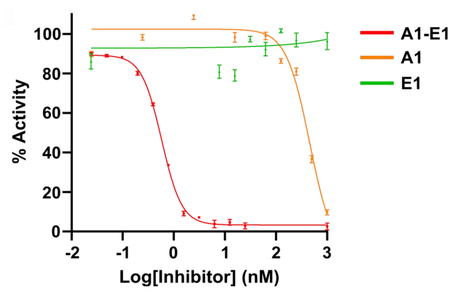


Drugs are administered at a dosing schedule set by their therapeutic index and termination of action is achieved by the clearance and metabolism of the drug. In some cases, such as anticoagulants, it is important to achieve a fast reversal of the drug's action. We report a general strategy to achieve on-demand reversibility by leveraging a supramolecular assembly of drug fragments. The action of the bivalent drug is reinforced by the hybridisation of peptide nucleic acids (PNAs) between two fragments, yielding a potent bivalent direct thrombin inhibitor (K_i 74 pM). The inhibitor, which binds to the active site and exosite II of thrombin (an essential enzyme in the blood coagulation pathway) was evaluated *in vitro* and *in vivo.* Furthermore, our findings indicate that the inhibition, which relies on the hybridisation of the two fragments, can be quickly reversed by the simple addition of a PNA antidote.

2- Initial Validation

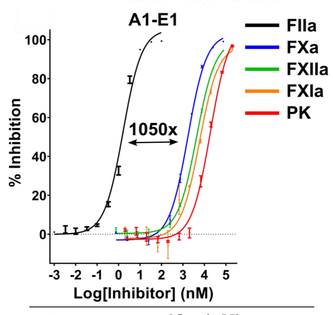
Inspired by proteins from blood-sucking insects that bridge multiple binding sites on thrombin, we designed two peptide fragments that bind to the active site (**A1,** sequence from *Hyalomin1*) and to the exosite II (**E1**, sequence from Tsetse Thrombin Inhibitor (TTI)) linked to complementary PNA strands. Alone, they show little inhibition (IC_{50} > 450 nM), but together they selectively inhibit thrombin (FIIa) (IC_{50} = 578 pM).

Fragments alone vs Supramolecular assembly



		IC_{50} (nM)	K_i (nM)
A1-E1	Combined inhibitor	0.578 (R^2=0.999)	0.074
A1	Active site binder alone	455.9 (R^2=0.983)	58.7
E1	Exosite 2 binder alone	>1000	

Selectivity Assays

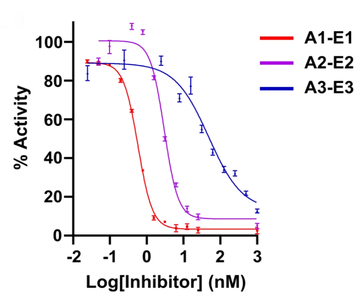


Protease	IC_{50} (nM)
FIIa	1.61 (R^2=0.990)
FXa	1690 (R^2=0.997)
FXIIa	4310 (R^2=0.999)
FXIa	5850 (R^2=0.983)
PK	18680 (R^2=0.991)

3- Structure Activity Relationship (SAR)

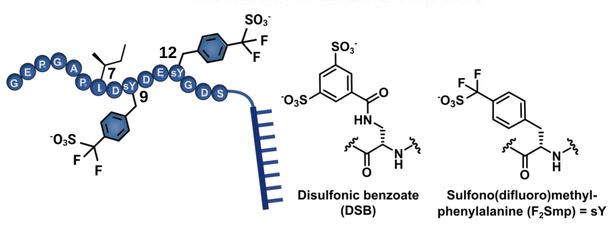
To further validate binders, SAR was performed by:
(1) Varying the PNA length (stronger vs weaker supramolecular tether)
(2) Varying negative charge on exosite II binding fragment which interacts with the positively charged exosite.
(3) Varying isoleucine which fills a hydrophobic pocket (identified by an Ala scan) with a range of unnatural hydrophobic amino acids

Variation of PNA length



	PNA length	IC_{50} (nM)	K_i (nM)
A1-E1	8	0.578 (R^2=0.999)	0.074
A2-E2	6	2.991 (R^2=0.981)	0.385
A3-E3	4	47.98 (R^2=0.970)	6.178

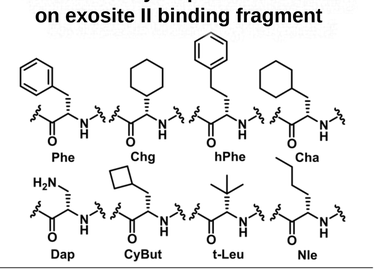
Variation of negative charges on exosite II binding fragment



	sY9	sY12	IC_{50} (pM)	K_i (pM)
A1-E1	sY	sY	578 (R^2=0.999)	74
A1-E4	sY	DSB	293 (R^2=0.991)	38
A1-E5	DSB	sY	589 (R^2=0.991)	76
A1-E6	DSB	DSB	1330 (R^2=0.998)	171

	Peptide Sequence	IC_{50} (pM)	K_i (pM)
A1-E1	GEPGAPIDsYDEsYGDS	578 (R^2=0.999)	74
A1-E7	GEPGAPIDsYDEsYGsYS	229 (R^2=0.988)	29
A1-E8	GEPGAPIsYDsYEsYGDS	1782 (R^2=0.991)	229

Variation of hydrophobic residues on exosite II binding fragment

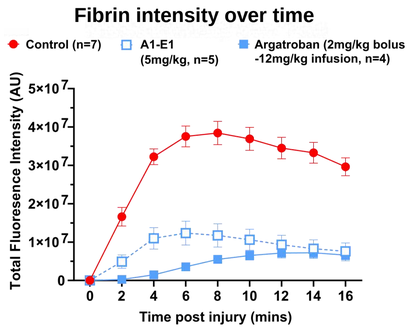


	Ile7	IC_{50} (pM)	K_i (pM)
A1-E1	Ile	578 (R^2=0.999)	74
A1-E9	Phe	540 (R^2=0.978)	70
A1-E10	Chg	1180 (R^2=0.990)	152
A1-E11	hPhe	813 (R^2=0.992)	105
A1-E12	Cha	812 (R^2=0.982)	105
A1-E13	Dap	3806 (R^2=0.961)	490
A1-E14	CyBut	403 (R^2=0.994)	52
A1-E15	t-Leu	308 (R^2=0.998)	40
A1-E16	Nle	309 (R^2=0.996)	40

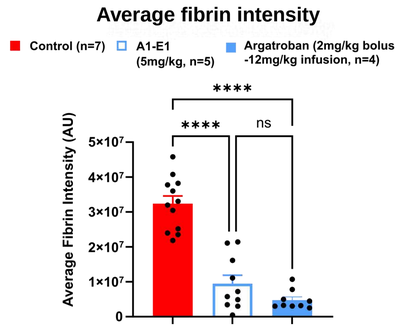
4- Anti-coagulant activity *In Vivo*

Our supramolecular drug (**A1-E1**) was tested *in vivo* via a needle injury mouse model. The combined inhibitor was compared to Argatroban (a clinically approved small molecule drug). **A1-E1** decreased fibrin intensity and thrombus size at a similar level to Argatroban (at 21.5-fold lower dose).

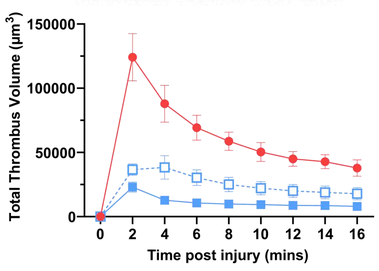
Fibrin intensity over time



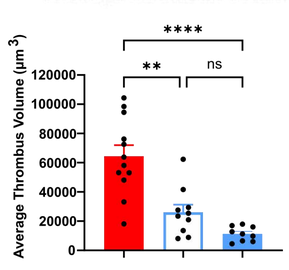
Average fibrin intensity



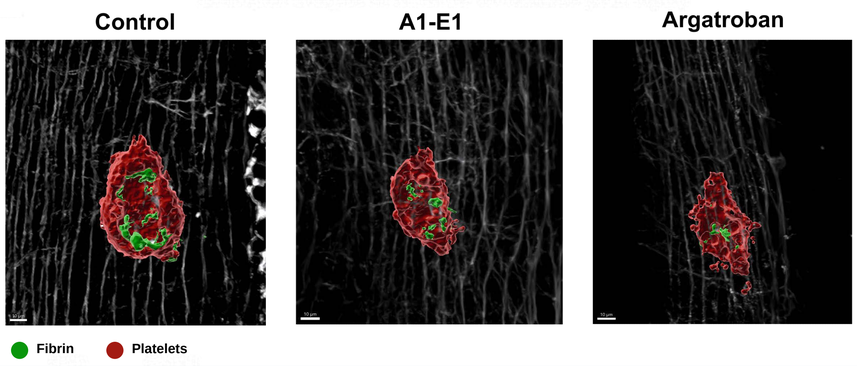
Thrombus volume over time



Average thrombus volume

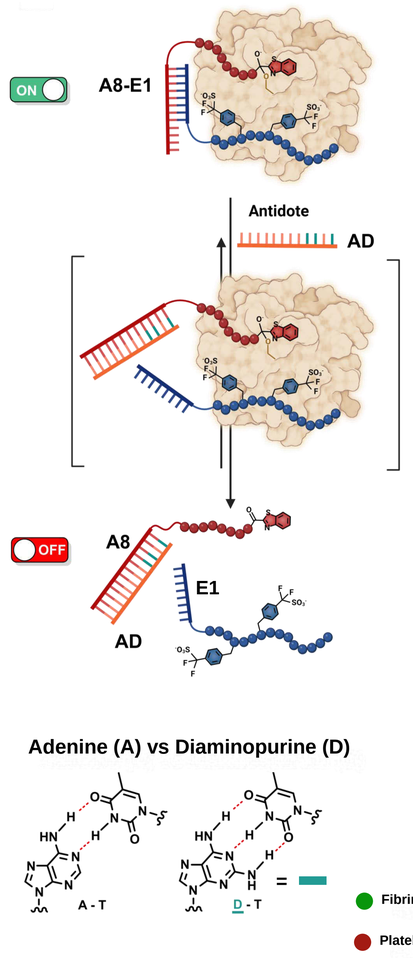


Exemplar images of thrombi 15 minutes post-injury



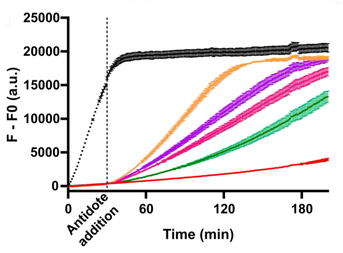
5- Reversal of inhibition *In Vitro* and *In Vivo*

Since inhibition of thrombin is dependent on the hybridisation of the two fragments, reversing its effect is possible by breaking the PNA interaction. A toe-hold sequence (4-mer) was added to one of the fragments (**A8**) and a 12-mer PNA antidote (**AD**) which incorporates diaminopurine instead of adenine (3 hydrogen bonds versus 2) was able to restore clotting activity.
This was first shown *in vitro* (fluorogenic substrate assay and fibrinogen assay) where just 1 equivalent of antidote restored activity.
The reversal was also tested *in vivo* (needle injury mouse model) where 5 equivalents of antidote effectively restored blood coagulation.

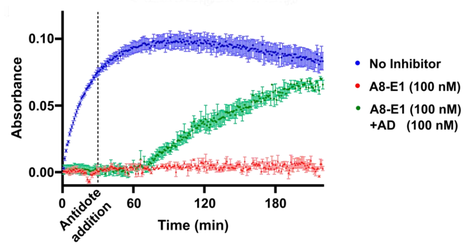


Adenine (A) vs Diaminopurine (D)

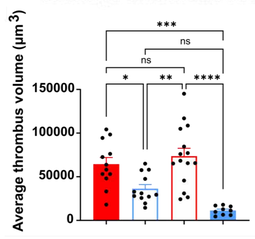
Reversal of inhibition Fluorogenic substrate assay



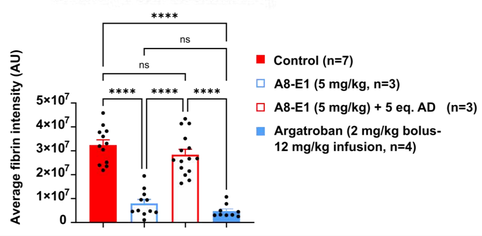
Reversal of inhibition Fibrinogen assay



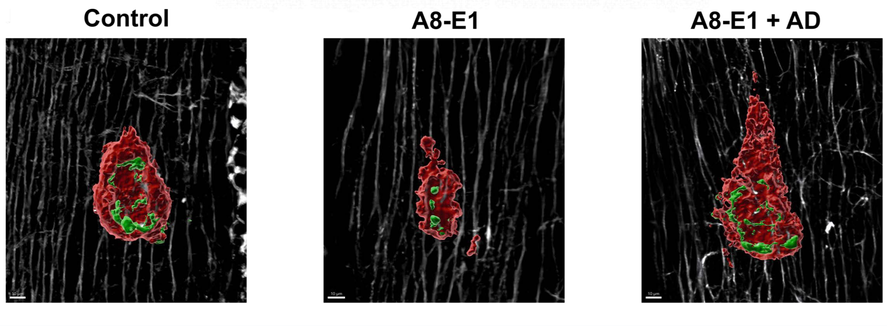
Average thrombus volume



Average fibrin intensity



Exemplar images of thrombi 15 minutes post-injury



6- Conclusions

We have designed bivalent thrombin inhibitors which rely on supramolecular assembly for potent inhibition. The combined inhibitor displays 800-fold gain in activity relative to the individual fragments. The drug was evaluated *in vitro* and *in vivo* and showed effects similar to that of a clinically approved drug (Argatroban). The effect could be rapidly reversed by the simple addition of a short PNA-based antidote. This technology was applied to anti-coagulants but provides a general strategy for the development of reversible drugs based on bivalent binding.

If you want to learn more – read the paper here:

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