Characterization of antithrombin-specific RNA aptamers for use in anticoagulant therapy

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Johns Hopkins University School of Medicine

What are aptamers?

- Synthetic ssDNA or RNA molecules.
- They bind with high affinity and specificity to their target protein (K_D in the nM to pM range).
- They are similar to monoclonal antibodies.
- They form an elaborate three dimensional structure.

Initial Aptamers (1990)

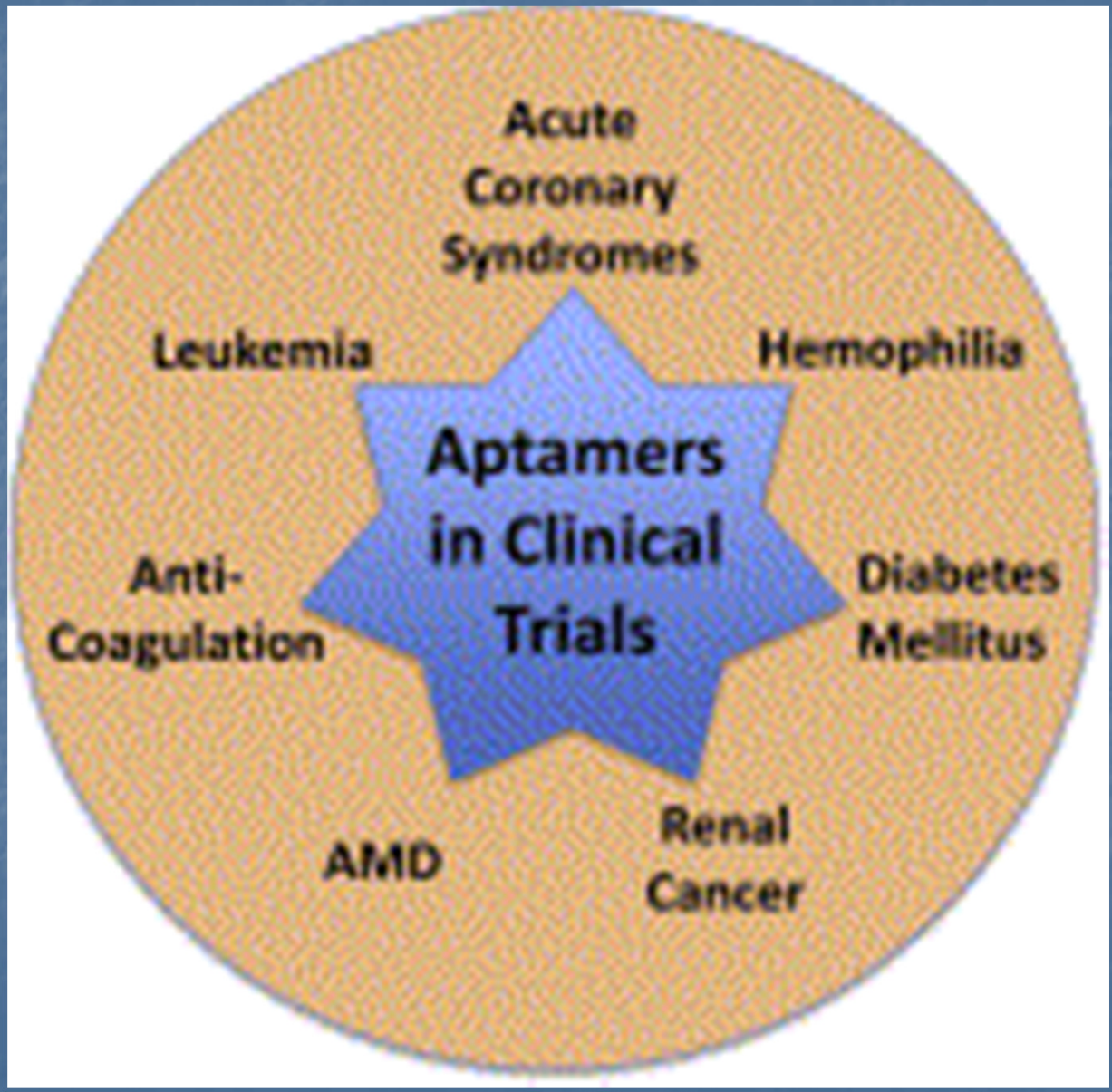
- Tuerk C., Gold L., Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymarase. Science 1990; 249:505-10
- Ellington Ad, Szostak JW. In vitro selection of RNA molecules that bind specific ligands, Nature 1990 346:818-22
- Currently there are over 2000 manuscripts published on aptamers

Aptamers vs. Monoclonal Antibodies

- *In vitro selection*
- Target range (i.e. toxins and other molecules that do not elicit immune responses)
- Low molecular weight mass and structural flexibility
- Low immunogenic potential
- Produced by chemical or enzymatic reactions

Aptamers as tools in diagnostic and analytical applications

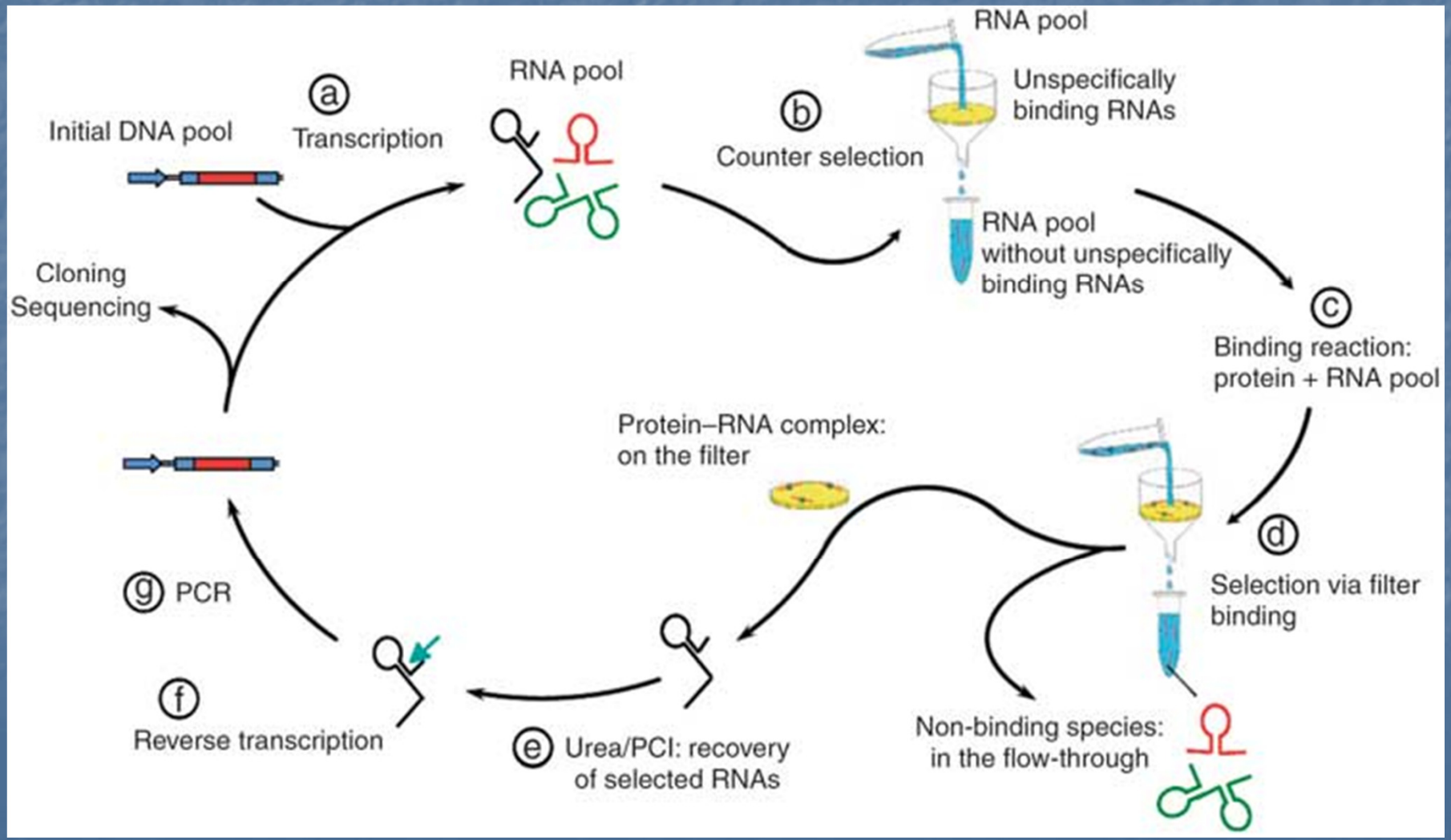
- Affinity Chromatography
- Capillary Electrophoresis
- *In vitro* and *in vivo* diagnostic tools
- Targeting intracellular target molecules
- Drug Discovery
- Therapy
- Protein Purification
- Diagnostics
- ELISA
- Western Blotting


Acute Coronary Syndromes
Leukemia
Hemophilia
Aptamers in Clinical Trials
Anti-Coagulation
Diabetes Mellitus
AMD
Renal Cancer

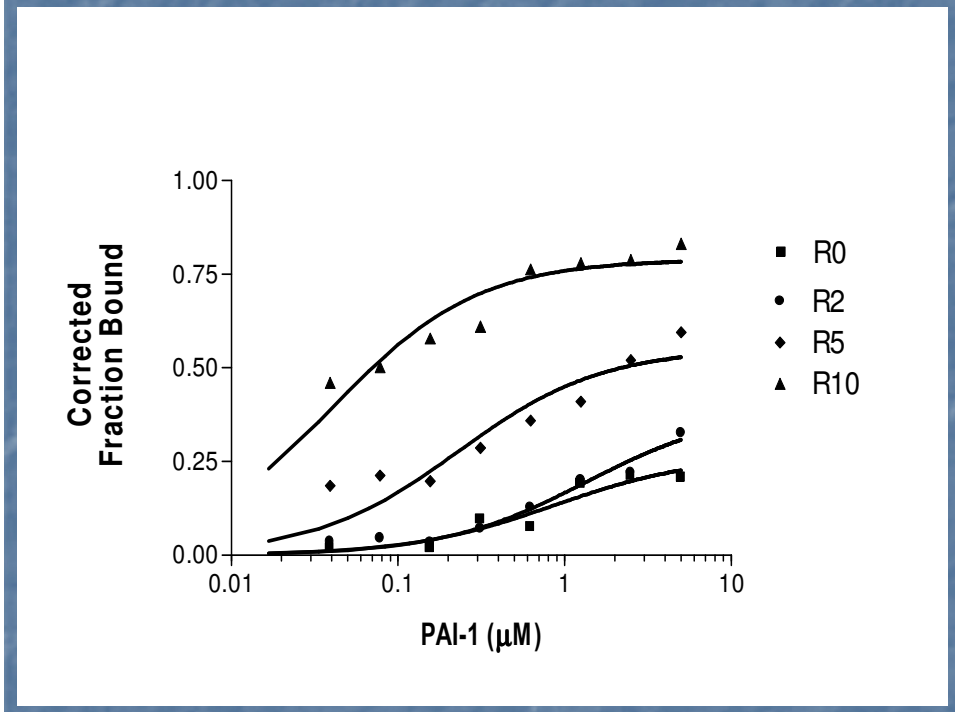
Examples of RNA aptamers in clinical trials

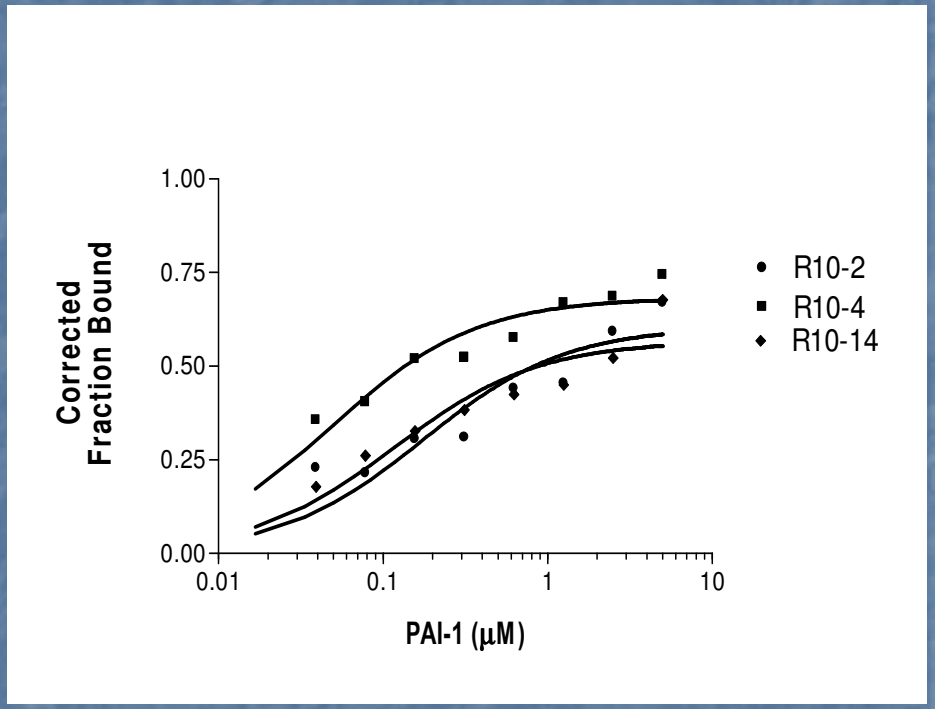
- Macugen (age related macular degeneration/diabetic macular edema/proliferative diabetic retinopathy)
- E10030 and ARC1905 (Neovascular age related macular degeneration-awaiting Phase III)
- RB006 (Coronary artery disease-awaiting Phase II)
- ARC19499 (Hemophilia-Phase I/II)
- AS1411 (Renal cell carcinoma/non-small cell lung cancer – awaiting Phase III

SELEX (Systematic Evolution of Ligands by Exponential Enrichment



Binding Curves of Aptamer Libraries





Goal: To design novel RNA-based anticoagulant aptamers that mimic the activity of LMWHs by accelerating factor Xa inhibition by AT, and design antidote aptamers that will reverse the anticoagulant effect.

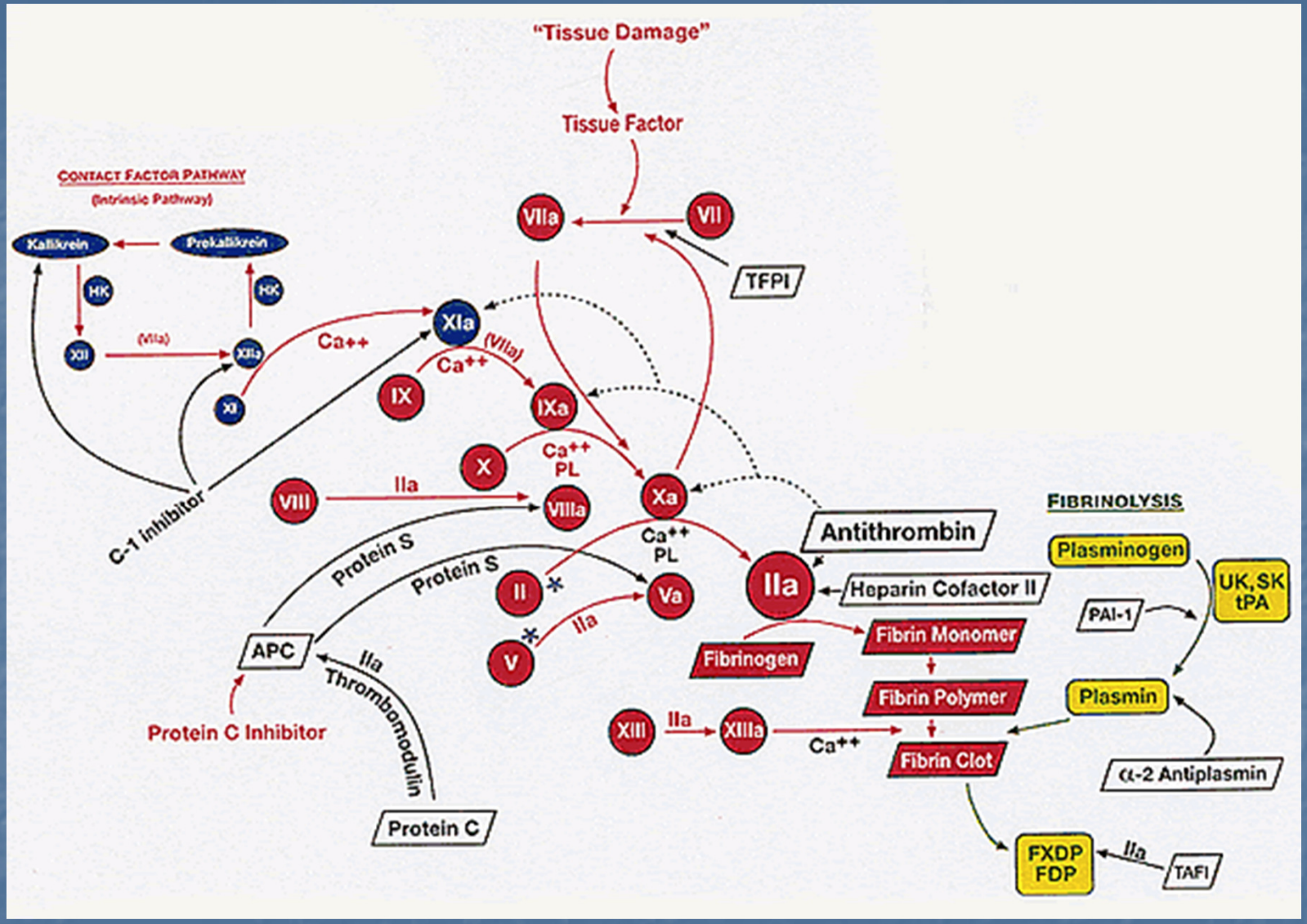


Figure from Enzyme Research laboratories Catalog

Glycosaminoglycan: Heparin

- Heparin accelerates the inhibition of the coagulation proteases thrombin and factor Xa by antithrombin (AT).
- Heparin is an important therapeutic anticoagulant
- Heparin is effective and inexpensive
- Protamine sulfate reverses the effect of heparin

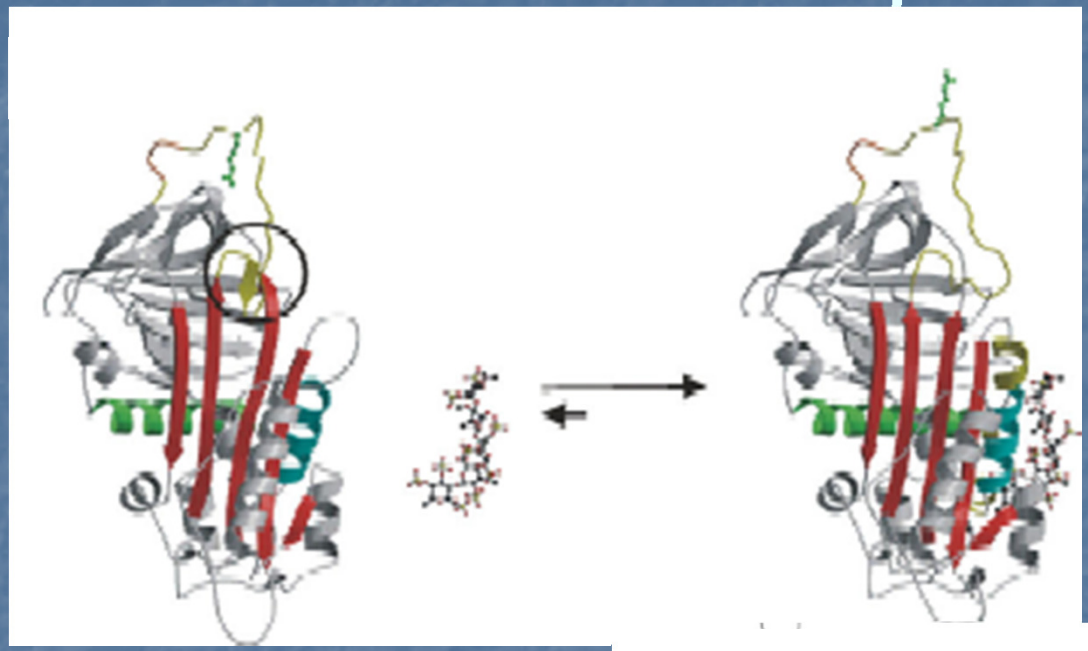
Heparin: Limitations

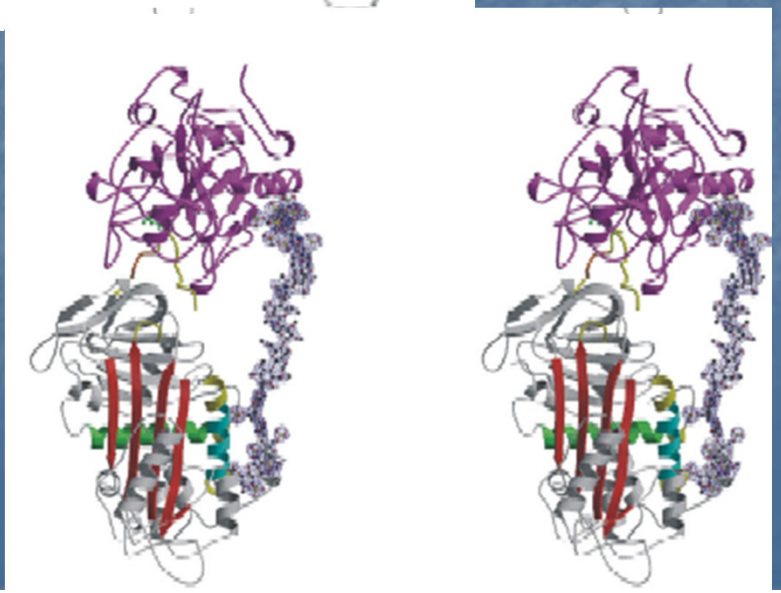
- Patients require constant laboratory monitoring
- It binds non-specifically to other plasma proteins
- It is neutralized by platelet factor-4
- It induces thromobocytopenia in some patients
- Some patients are at risk for recurrent thrombotic events that may be caused by the inability of AT-heparin to inhibit thrombin bound to fibrin

Low Molecular Weight Heparin (LMWH)

- Lower average molecular weight than heparin
- Made by enzymatic or chemical controlled hydrolysis of unfractionated heparin
- No ANTIDOTE

Antithrombin-thrombin/factor Xa-heparin



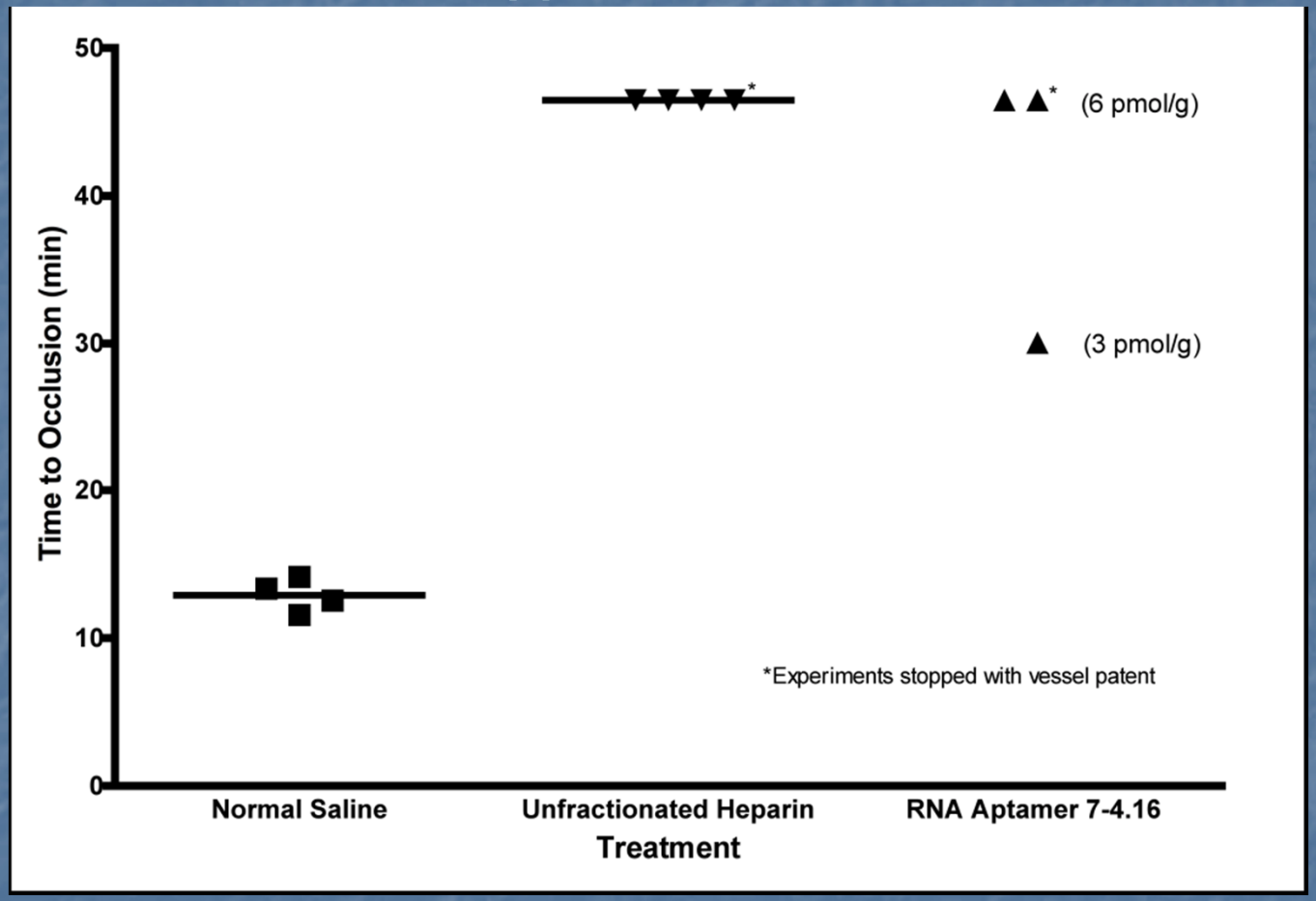


Li et al, 2004, Nature

Effect of RNA Aptamer 7-4.16 to Accelerate AT-Protease Inhibition

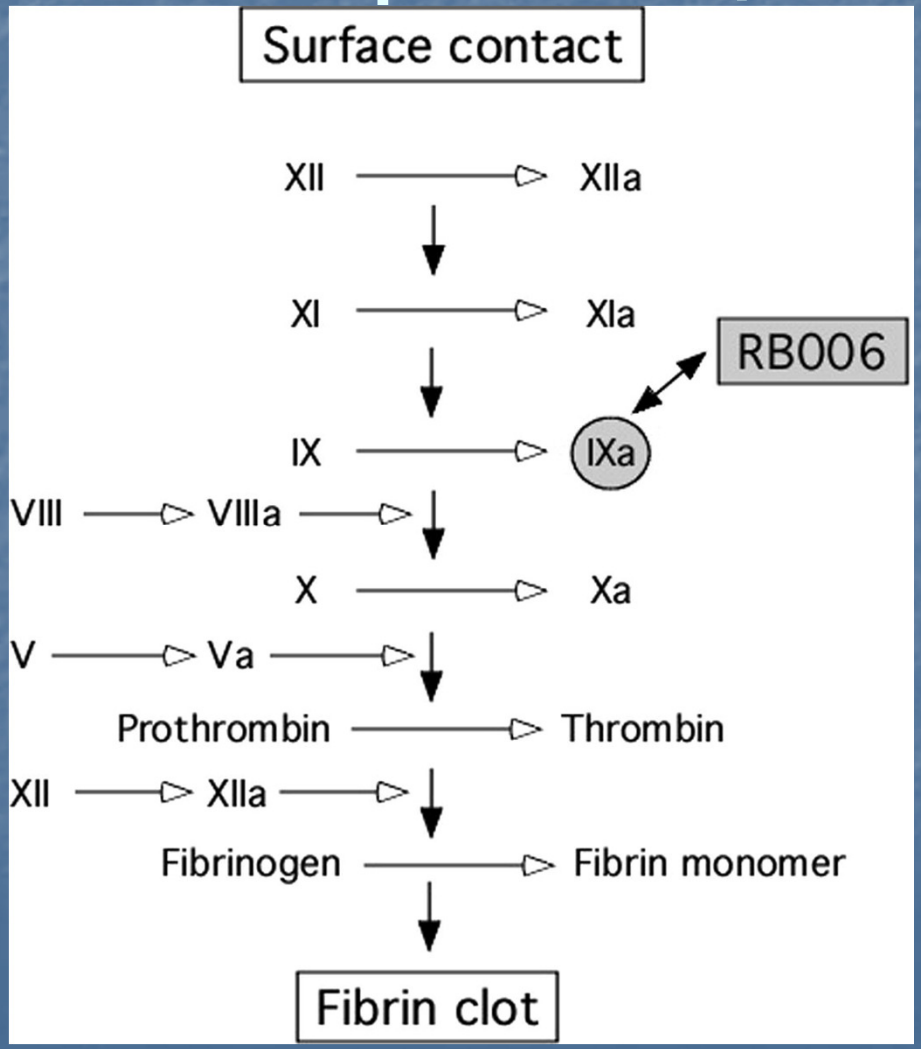
Reaction Condition	Aptamer 7-4.16 or heparin (nM)	K_2 X 10^5 (M^{-1} s^{-1})	Fold-acceleration
AT+ FXa + Aptamer 7-4.16	50	4.2	83
AT+ FXa + Aptamer 7-4.16	500	25	510
AT+ FXa + Heparin	40	20	400
AT+ thrombin + Aptamer 7-4.16	500	0.11	3.4

$FeCl_3$-induced Saphenous Vein Thrombosis in Wild Type C57B6 Mice*



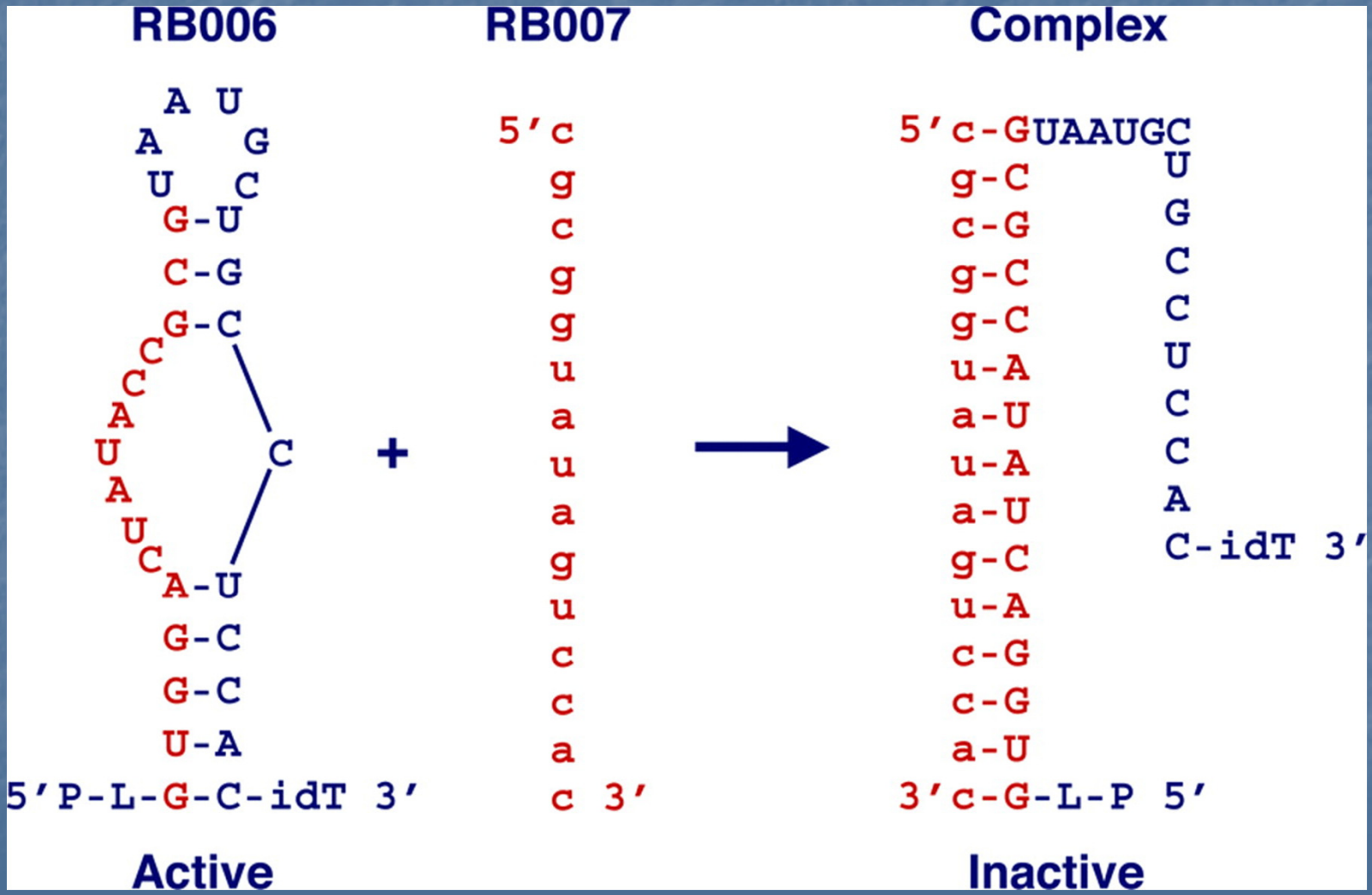
Injury: 10% $FeCl_3$ on 2 x 5 mm filter paper for 2 min, mice treated with saline, unfractionated heparin (300 U/Kg) or RNA aptamer 7-4.16 (3 and 6 pmol/g)

RB006/RB007 – Aptamer/Antidote pair



- Binds to factor IXa.
- It blocks the factor VIIa/Ixa catalyzed conversion of factor X to Xa.
- Anticoagulant
- Awaiting phase III trails (so far the results are very positive).

Antidote controlled aptamer



Oligonucleotides to AT Specific RNA Aptamer

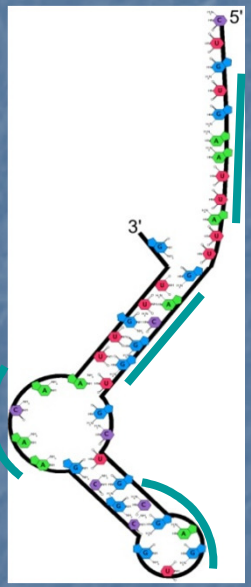


Table 2: Synthesized Complimentary Oligonucleotides to Aptamer 7-4.16

Ap7-4.16: AAGAAGGCGAUAGAAGGCGAUGCGCUGCGAAUCGGGAGCGGCGAUA 3'
ATanti-1:----------CCGCTATCTTCCGC----------
ATanti-2: ----------CCGCTACGCGACGCTT----------
ATanti-3:----------ACGCTTAGCCCTCGCCG----------
ATanti-4:----------CTCGCCGCTAT----

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AT+ FXa + Heparin	40	20	400
AT+ FXa + Aptamer 7-4.16 + **Antidote oligo**	500	8.0	300

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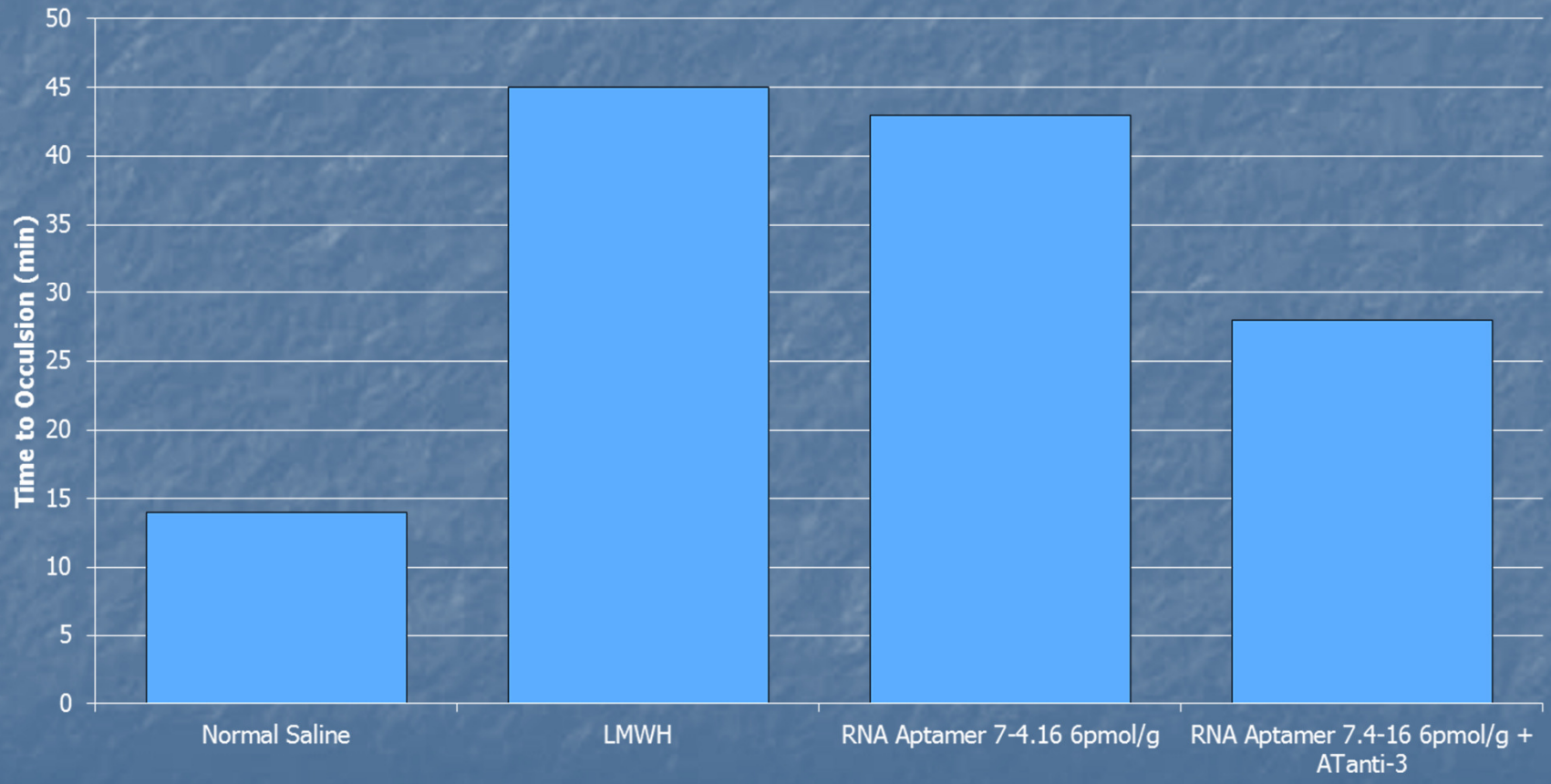


Conclusions (1)

- AT RNA aptamer 7-4.16 accelerates the AT-factor Xa inhibition reaction in vitro and in preliminary studies, promotes an anticoagulant response in vivo using a vascular injury model.
- AT RNA aptamer 7-4.16 does mimic the action of heparin in terms of accelerating the AT-Xa inhibition reaction, but it has no effect to accelerate the AT-thrombin inhibition reaction.
- AT RNA aptamer 7-4.16 may not bind at the heparin-binding site, could it bind at the AT-Xa-specific exosite?
- AT RNA aptamer 7-4.16 binds relatively poorly to AT, and newer RNA aptamers based on this format are needed.
- Antidote oligonucleotide is able to partly reverse the effect of AT RNA aptamer 7-4.16

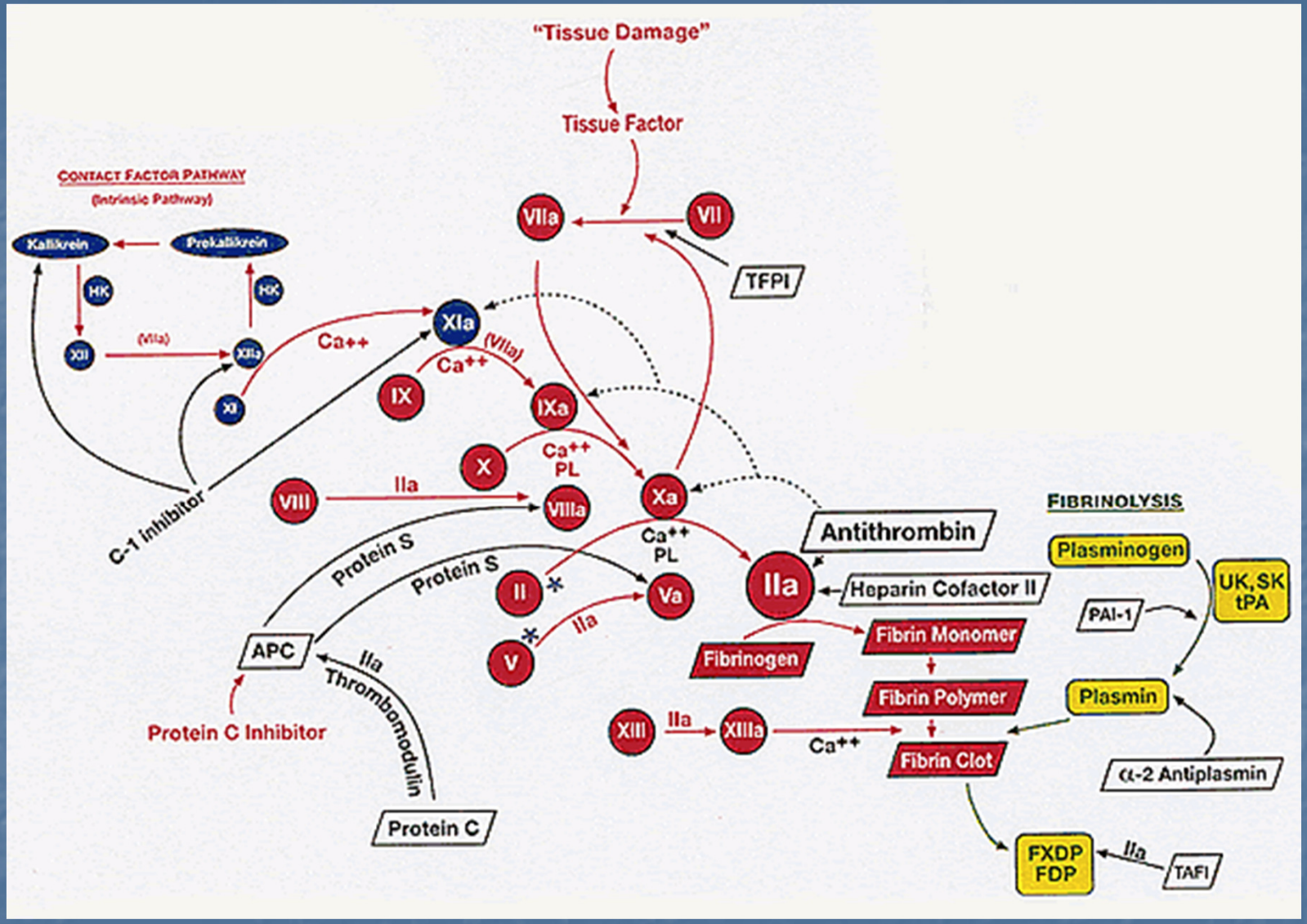


Figure from Enzyme Research laboratories Catalog

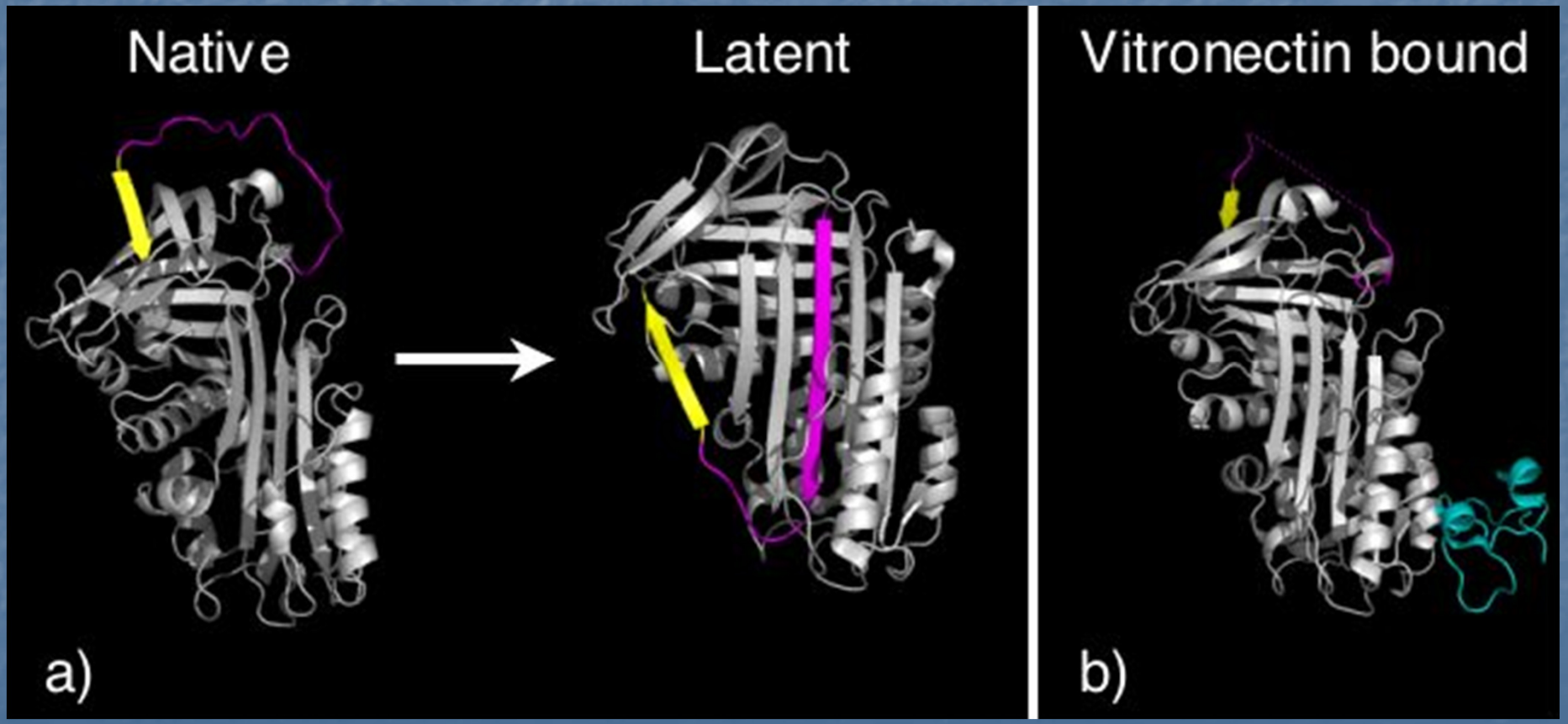
Plasminogen Activator Inhibitor (PAI-1)

- Serine protease inhibitor (Serpin)
- Rapid, specific inhibitor of uPA and tPA
  - Can also inhibit plasmin, trypsin, thrombin, APC
- Short half life, rapidly converted to latent form
- Stabilized by binding to vitronectin (VN)
- Normally expressed in liver, SMC, adipocytes, platelets, endothelial cell, fibroblast
- Pathological conditions– tumor cells, endothelial cells, cardiovascular disease, obesity, metabolic syndrome

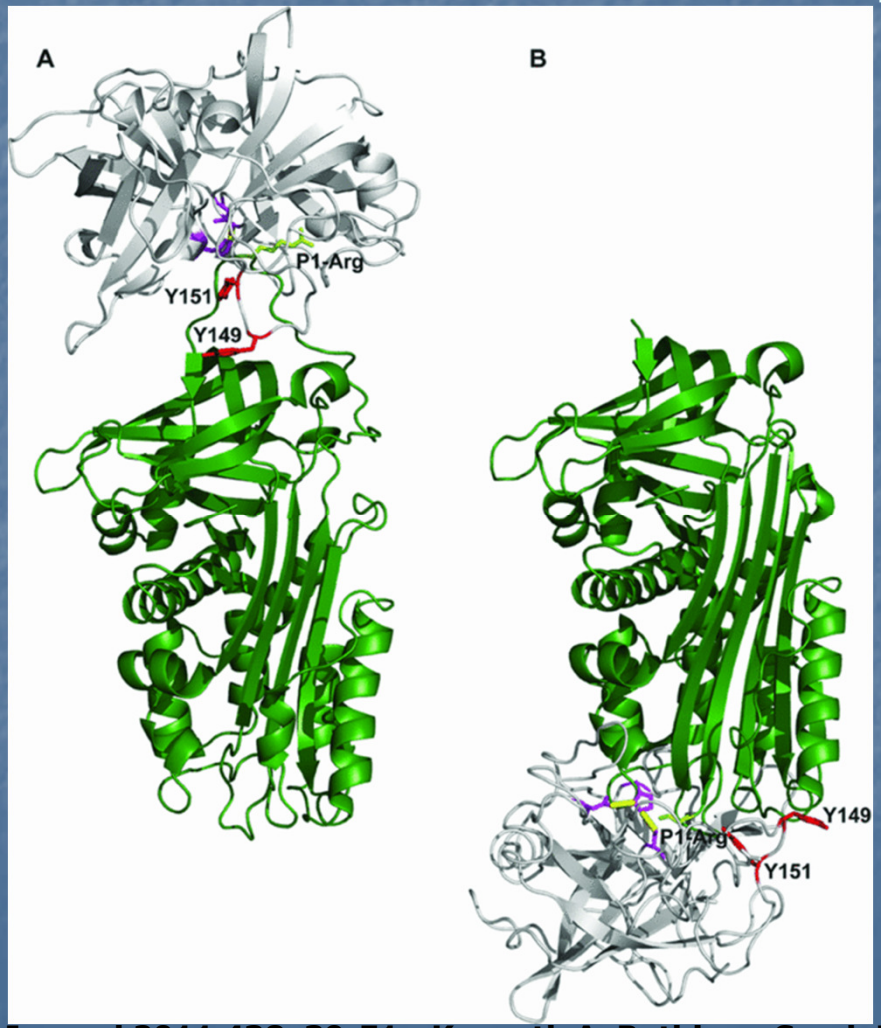
Develop RNA aptamers to the various functional domains of PAI-1

- Vitronectin binding domain
- Heparin binding domain
- Lipoprotein-related protein binding domain
- Reactive Center loop region

PAI-1's different conformations



Negative SELEX Method using tPA-PAI-1 complex



Biochemical Journal 2011 438, 39-51 - Kenneth A. Botkjaer, Sarah Fogh and others.

PAI-1 aptamer sequences from negative selection

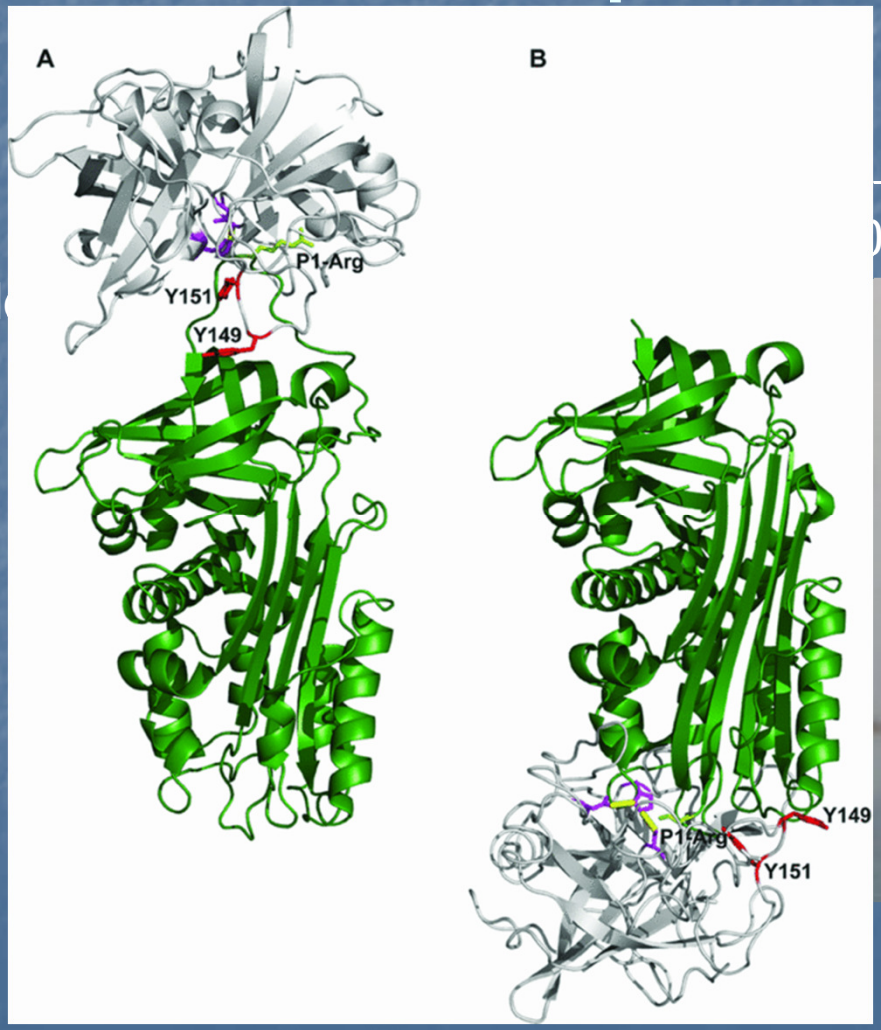
Aptamer clone ID	**Sequence (variable region)**	Frequency in Library	Kd (to wild-type PAI-1)
R10-14	UCACCAGCGCUCUACGAACCCCGCAUUCCCAGUUGCUACA	67%	28 nM
R10-2	CACACGAGGCAAGUGGCCUGCAUAACGUAGGCGUCGAGUA	17%	172 nM
R10-4	CCAGGCGUCUCACUCGUUACGCUAUCGUUGCGUACUUCUG	17%	53 nM

PAI-1 antagonist

- Monoclonal antibodies
- Peptides
- Low molecular weight inhibitors (PAI-039)
- Chemical suppressors
- Aptamers

R10-4 Disrupts PAI-1/tPA complex

tPA/PAI-1 compl



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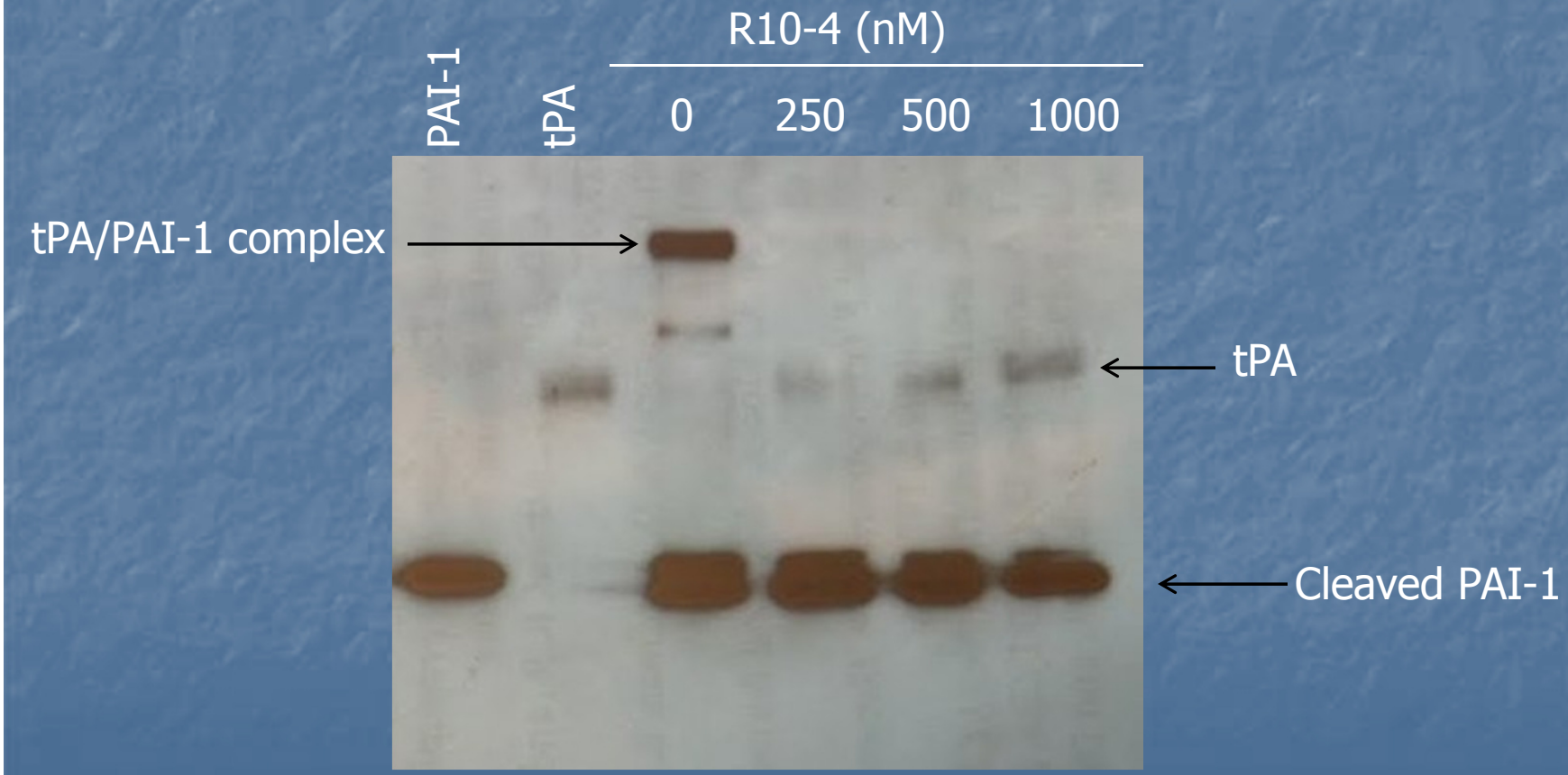
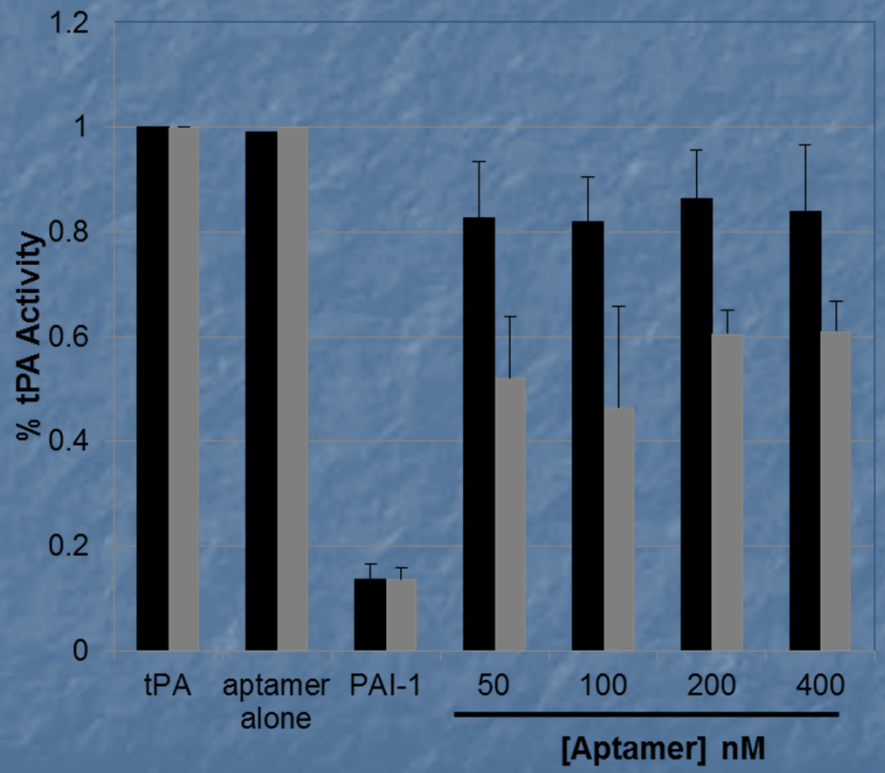
Cleaved PAI-1

R10-4 Disrupts PAI-1/tPA complex



R10-4 inhibits PAI-1's ability to inhibit tPA



Conclusions

- We have developed RNA aptamers that disrupts PAI-1's antiproteolytic activity.
- R10-4 prevents PAI-1 from interacting with tPA.
- R10-4 converts PAI-1 to a substrate
- R10-4 is less effective at inhibiting PAI-1 in the presence of vitronectin
- R10-4 is able to increase fibrinolysis

Acknowledgements

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- Laura Gray (UNC-Chapel Hill)