Modular construction of DNA aptamers for human thrombin

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Aptamers – Molecular Recognition Elements (MoRE) made of Nucleic Acids

Aptamers are oligonucleotides that share some attributes of monoclonal antibodies due to complex 3D structure

"Chemical Antibody" for Theranostics (*Thera*py&Diag*nostics*)

030213-141013 PubMed: aptamer + review = 37 refs (370 Total)

Mode of Application:

1: Yadav SK, Chandra P, Goyal RN, Shim YB. A review on determination of steroids in biological samples exploiting nanobio-electroanalytical methods. Anal Chim Acta. 2013 Jan 31;762:14-24.

2: Wang T, Ray J. Aptamer-based molecular imaging. Protein Cell. 2012 Oct;3(10):739-54.

3: Sundaram P, Kurniawan H, Byrne ME, Wower J. Therapeutic RNA aptamers in clinical trials. Eur J Pharm Sci. 2013 Jan 23;48(1-2):259-71.

4: Xing H, Wong NY, Xiang Y, Lu Y. DNA aptamer functionalized nanomaterials for intracellular analysis, cancer cell imaging and drug delivery. Curr Opin Chem Biol. 2012 Aug;16(3-4):429-35.

5: Pednekar PP, Jadhav KR, Kadam VJ. Aptamer-dendrimer bioconjugate: a nanotool for therapeutics, diagnosis, and imaging. Expert Opin Drug Deliv. 2012 Oct;9(10):1273-88

6. Mishra S, Kim S, Lee DK. Recent patents on nucleic acid-based antiviral therapeutics. Recent Pat Antiinfect Drug Discov. 2010 Nov 1; 5(3): 255-71.

Field of Application:

1: Binning JM, Leung DW, Amarasinghe GK. Aptamers in virology: recent advances and challenges. Front Microbiol. 2012;3:29.

2: Hu M, Zhang K. The application of aptamers in cancer research: an up-to-date review. Future Oncol. 2013 Mar;9(3):369-76

3: Yang Y, Ren X, Schluesener HJ, Zhang Z. Aptamers: Selection, Modification and Application to Nervous System Diseases. Curr Med Chem. 2011 18(27):4159-68.

4: Haberland A, Wallukat G, Schimke I. Aptamer binding and neutralization of β1-adrenoceptor autoantibodies: basics and a vision of its future in cardiomyopathy treatment. Trends Cardiovasc Med. 2011 Aug;21(6):177-82.

5: Vavalle JP, Cohen MG. The REG1 anticoagulation system: a novel actively controlled factor IX inhibitor using RNA aptamer technology for treatment of acute coronary syndrome. Future Cardiol. 2012 May;8(3):371-82.

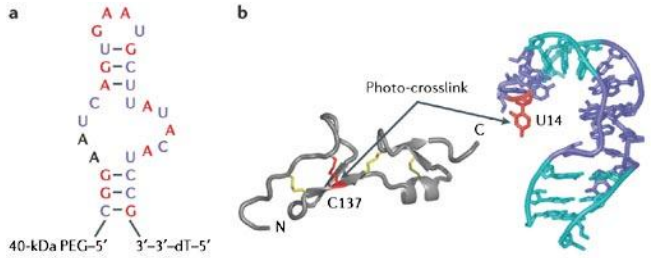
The success story:

ANGIOGENESIS - new vessels are created from pre-existing vasculature.

Increased rates of angiogenesis are associated with several disease states:

- cancer
- age-related macular degeneration (AMD)
- psoriasis
- rheumatoid arthritis
- diabetic retinopathy

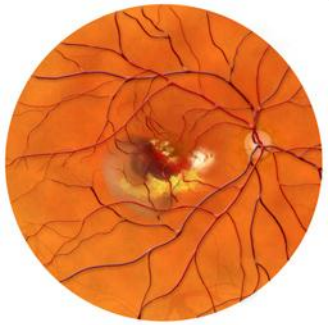
Treatment options for AMD
have been limited with photodynamic therapy



Commercial VEGF inhibitors/drugs are:

- RNA **APTAMER** pegaptanib (Macugen, Eyetech Parm/Pfizer)
-partial and full length **ANTIBODIES** ranibizumab (F_{ab}, *Lucentis, $1,600*), and bevacizumab (*Avastin, $40*), Genentech
- VEGF receptor trap - fusion protein aflibercept
- small interfering RNA-based therapies bevasiranib and AGN 211745, sirolimus
- and tyrosine kinase inhibitors, including vatalanib, pazopanib TG 100801, TG 101095, AG 013958, and AL 39324

1994 - (NeXstar Pharma) - 2004 (FDA)



Therapies have met with great success in reducing the vision loss associated with neovascular AMD

Retina. 2013 Feb;33(2):397-402. Intravitreal pegaptanib sodium (macugen) for treatment of myopic choroidalneovascularization: a morphologic and functional study.

http://www.regadobio.com/

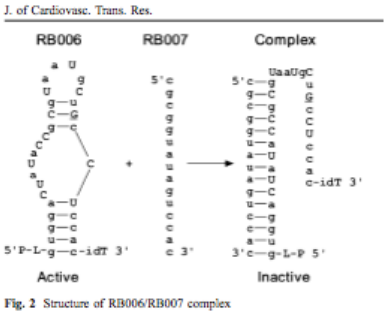


Fig. 2 Structure of RB006/RB007 complex

REGADO BIOSCIENCES, INC.
ENROLLS FIRST PATIENT
IN **PHASE 3** TRIAL OF REG1"REGULATE-PCI"
TO STUDY REG1 IN PATIENTS UNDERGOING
PERCUTANEOUS CORONARY INTERVENTION
BASKING RIDGE, N.J.,
Sept. 17, 2013 /PRNewswire/

APTO-PHARM

TIDES, May 2013, Boston

Regado Biosciences, Inc. (Nasdaq: RGDO) - discovery and development of novel, first-in-class, actively controllable antithrombotic drug systems for acute and sub-acute cardiovascular indications, today announced the enrollment of the first patient in its REGULATE-PCI clinical trial. REGULATE-PCI is **Phase 3,** PROBE design (Prospective, Randomized, Open-label, Blinded-Endpoint) superiority study comparing the effects of Regado's REG1 to **bivalirudin** in patients undergoing percutaneous coronary intervention (PCI) electively or for the treatment of unstable angina (UA) or non-ST elevated myocardial infarction (N-STEMI). REGULATE-PCI, if successful, will serve as the basis for product registration applications throughout the world. Led by co-PIs, Drs. J. H. Alexander of Duke University Medical Center, A. M.Lincoff of The Cleveland Clinic and R. Mehran of Mount Sinai School of Medicine, REGULATE-PCI is expected to enroll **13,200** patients at approximately **500** investigational sites worldwide. The primary endpoint of the trial is efficacy compared to bivalrudin based on a composite of death, nonfatal myocardial infarction (MI), nonfatal stroke and urgent target lesion revascularization through day three. The principal secondary endpoint is safety compared to bivalrudin as measured by major bleeding events through day three. The trial is powered to show superiority in efficacy and non-inferiority in safety against bivalirudin. If successful, REGULATE-PCI will become the cornerstone of Regado's international new drug applications, expected to be filed in **early 2016**. The first of three key interim analyses in the trial will occur after enrollment of the first 1,000 patients and is expected to occur during the second quarter of 2014.

Next – NOXXON?

MAKING APTAMERS

APTO-PHARM

SELEX - Systematic Evolution of
Ligands by EXponential enrichment

L. Gold,1990

SELEX –*in vitro* selection of RNA/DNA
of single stranded oligo combinatorial libraries
for molecules which bind a target.
Winners, not champs

The molecules are coined **APTAMERS** [aptus (lat) – to correspond, to fit)

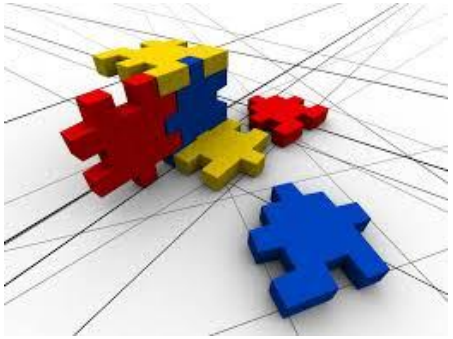
APTAMERS are single-stranded oligos with 3D structure that binds to the target with high affinity and specificity, and possibly modulate target function.

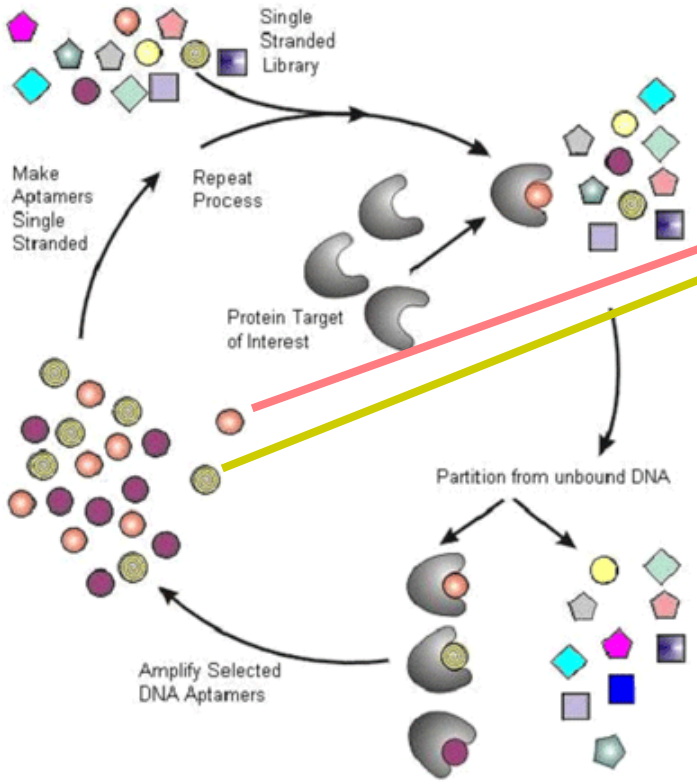
Goal of SELEX – to fish out aptamers, and to make large amount of aptamers by chemical synthesis

SELEX

Chemical synthesis and selection of aptamers out of 4^n sequences

(ie 10^{14} for pegaptanib)





SELEX: Systematic Evolution of Ligands by Exponential Amplification
Tuerk, C. & Gold, L. (1990) Science 249, 505-510

Families of aptamers with repertoire of affinities

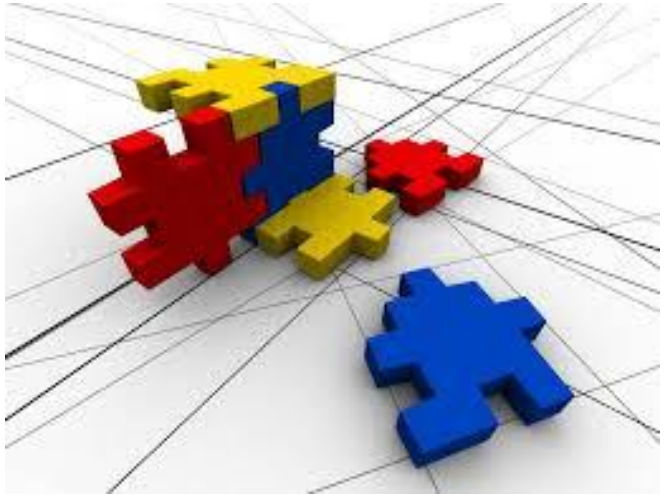
Winners, but not champs

Aptus: "to fit"

mer : "smallest unit of repeating structure"

APTO-PHARM

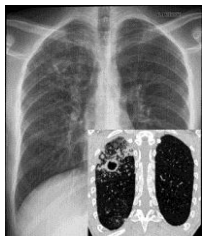
Aptamers could be developed for different targets, both LMW and HMW:



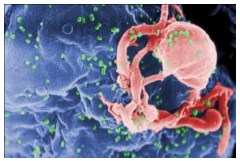
- Toxins
- Proteins
- Viruses
- Pathogenic microorganisms
- Cancer cells

Aptamer targets

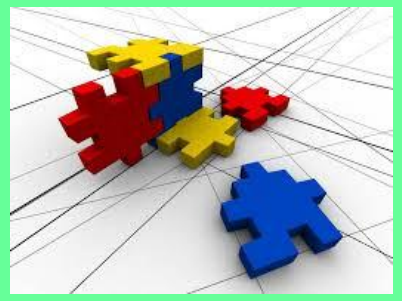
Oncology



Mycobacterium tuberculosis



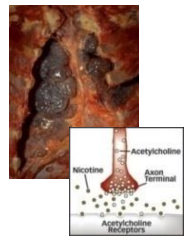
Viruses

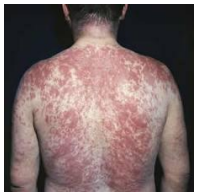


Aptamers under development

Acetylcholine

(nicotine)





Allergy

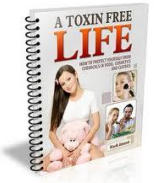
Prions

Photodynamic and Radiotherapy

Alzheimer's disease

Thrombin

LMW comps & drugs



KNOWING APTAMERS

APTAMERS vs/and	ANTIBODIES
High affinity and specificity for the target	High affinity and specificity for the target
In vitro chemical protocols	*In vivo* biological protocols
Binding parameters could be modified	Possibility for changes of binding parameters
Reversible temperature denaturing	Irreversible temperature denaturing
Extended storage time	Limited storage time
Low immunogenity	High immunogenity
Availability of specific ANTIDOTE	NO rational ANTIDOTE

Aptamers have some potential advantages

Then why the progress slow?

APTO-PHARM

A key attribute of THERAPEUTIC APTAMERS is the ability to tailor the pharmacokinetic profile by modulating the degree of metabolic stability, renal clearance and rate of distribution

Good safety margins between the pharmacologically effective dose and toxicologically established no-adverse-effect levels

Several Aptamers are on Clinical Trials
Then why the progress slow?

http://clinicaltrials.gov <aptamer>: 27/21 entries (oct, 2013/feb, 2012)
Why the progress is slow?

Dual Paradigm of Drug Design

- I. Small – CHEMICALS, Low molecular weight

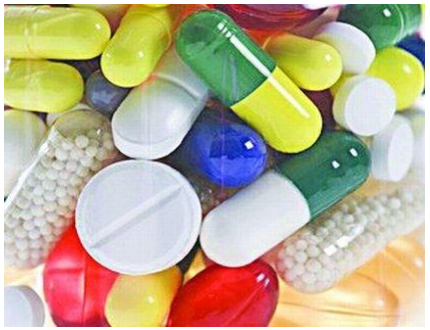
  Creation of combinatory library of synthetic and natural CHEMICAL COMPOUNDS

  Selection by activity

  Chemical synthesis

PLUS – Better distribution

MINUS – Less specificity



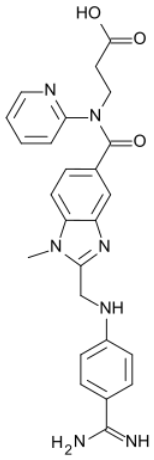
- II. Large – BIOLOGICS, High molecular weight

  Identification of proteins with defensive functions (Ig, IFN, GF)

  Making genetic engineering constructs Biotechnological synthesis.

MINUS – Slow distribution

PLUS – High specificity

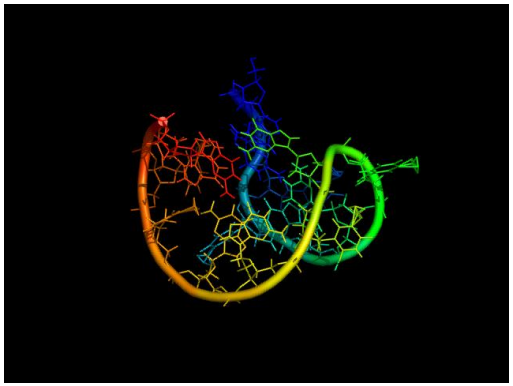


Triple Paradigm of Anti-Thrombin Drug Design

I. CHEMICALS

Dabigatran

(Pradaxa)



APTAMERS as the third paradigm

Intermediate size status in attempt

to combine the best of both groups

Peptamer: Hirulog (20 aa) (*Bivalirudin,* Angiomax, Angiox)

II. BIOLOGICS

Leeches > hirudin (65 aa)

Prophylactics and Treatments of Thrombosis

1. Thrombolytics – dissolve thrombi

   (Streptokinase (SK), Urokinase (UK),

   Tissue plasminogen activator (tPA)

2. Anti-aggregants – inhibits platelet aggregation

   (aspirin, Thienopyridines – Clopidogrel (Plavix),

   IIb-IIIa glycoproteins antagonists

3. ANTI-COAGULANTS – inhibit fibrin formation:

Non-direct anticoagulants (warfarin)

Direct anti-coagulants:

Heparin and derivatives (thrombin, 10a)

Enoxaparin (10a)

Rivaroxaban (10a)

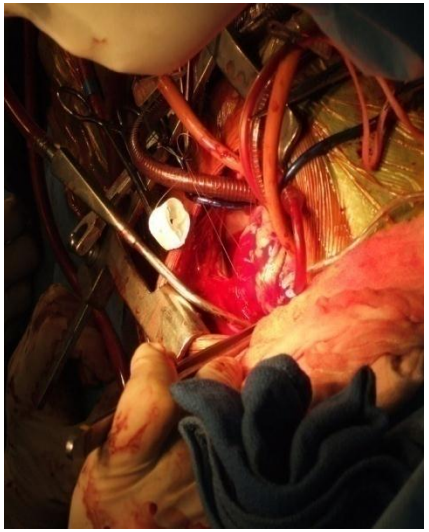
Direct thrombin inhibitors:

monovalent – chemicals (dabigatran)

bivalent - biologics (bivalirudin)

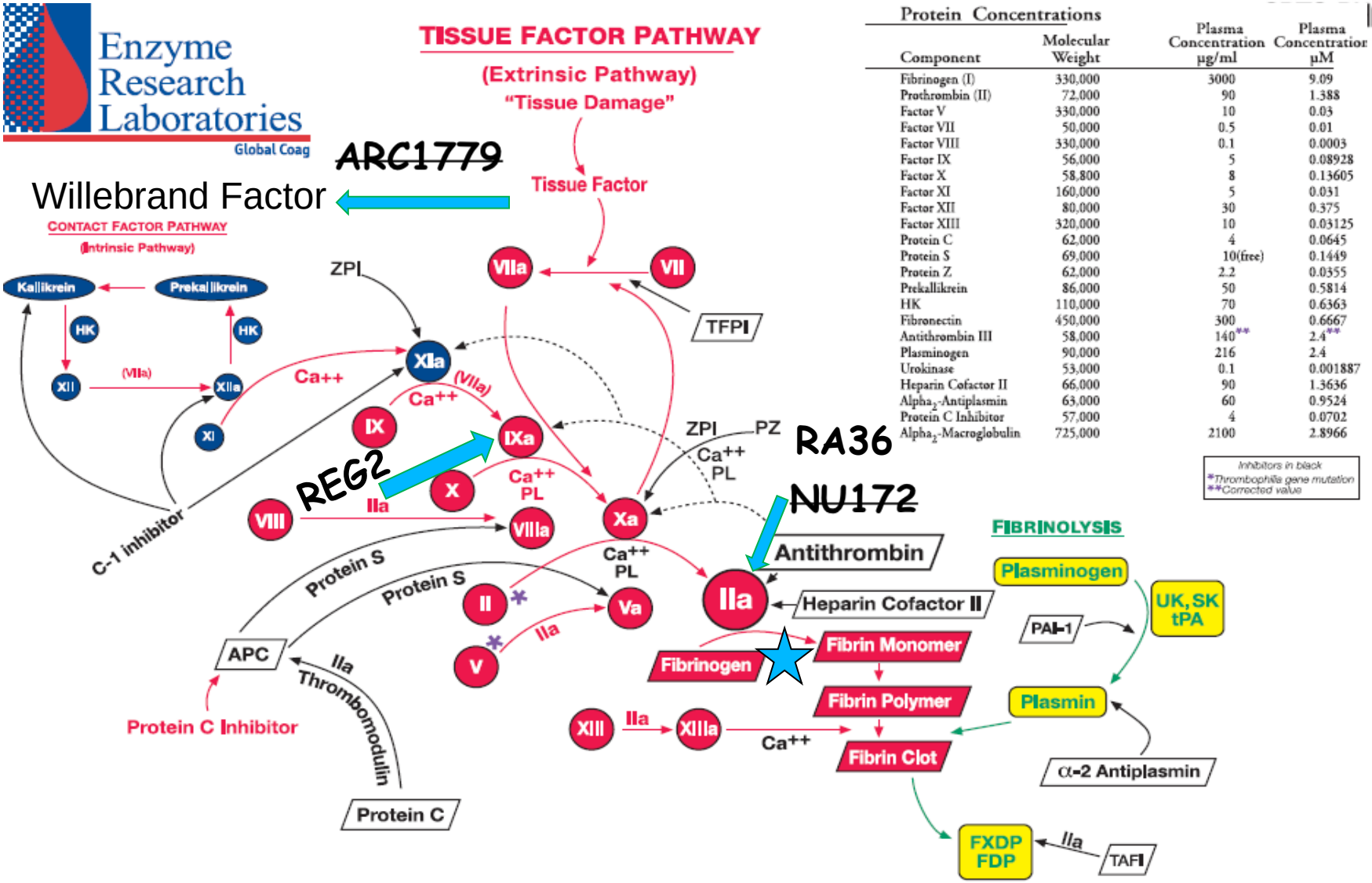
Primary challenges of anti-thrombin aptamer applications

to reduce
cerebral embolization
after
carotid endarterectomy



to reduce
post-operational bleeding
after
coronary artery bypass surgery

Coagulation Cascade and Aptamers



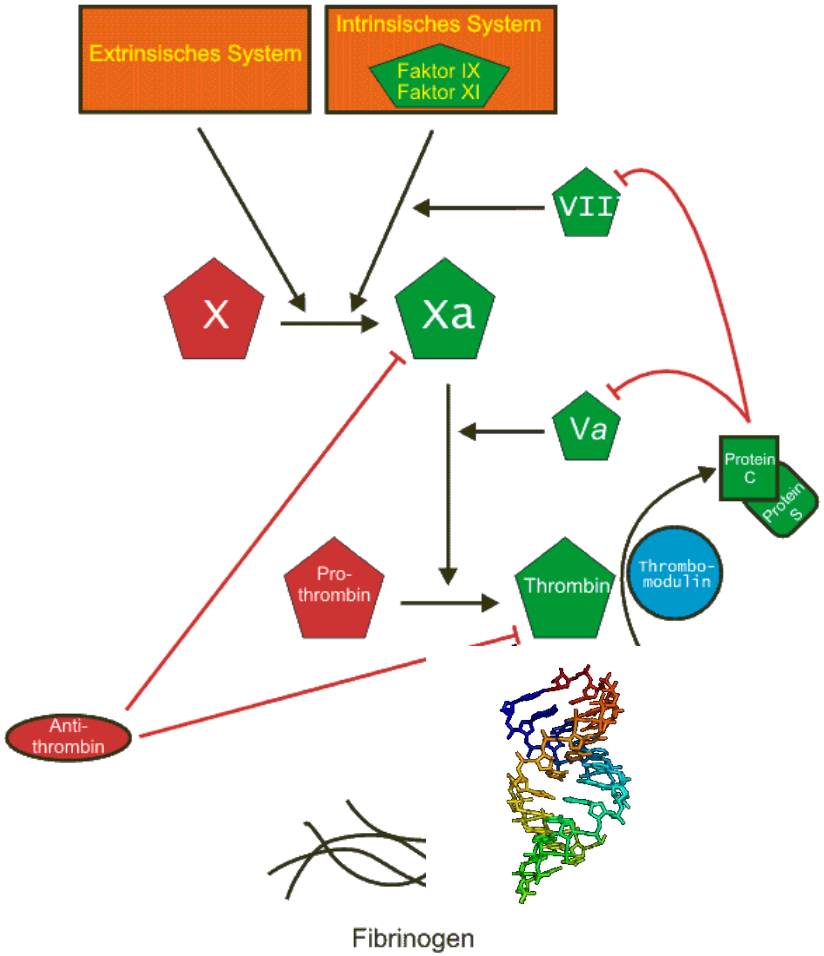
Protein Concentrations

Component	Molecular Weight	Plasma Concentration µg/ml	Plasma Concentration µM
Fibrinogen (I)	330,000	3000	9.09
Prothrombin (II)	72,000	90	1.388
Factor V	330,000	10	0.03
Factor VII	50,000	0.5	0.01
Factor VIII	330,000	0.1	0.0003
Factor IX	56,000	5	0.08928
Factor X	58,800	8	0.13605
Factor XI	160,000	5	0.031
Factor XII	80,000	30	0.375
Factor XIII	320,000	10	0.03125
Protein C	62,000	4	0.0645
Protein S	69,000	10(free)	0.1449
Protein Z	62,000	2.2	0.0355
Prekallikrein	86,000	50	0.5814
HK	110,000	70	0.6363
Fibronectin	450,000	300	0.6667
Antithrombin III	58,000	140**	2.4**
Plasminogen	90,000	216	2.4
Urokinase	53,000	0.1	0.001887
Heparin Cofactor II	66,000	90	1.3636
$Alpha_2$-Antiplasmin	63,000	60	0.9524
Protein C Inhibitor	57,000	4	0.0702
$Alpha_2$-Macroglobulin	725,000	2100	2.8966

Inhibitors in black
*Thrombophilia gene mutation
**Corrected value

Unique opportunity – fast antidot

Aptamer blocks thrombin, and antidot blocks aptamer, restoring coagulation



Fibrin mesh for clotting

4 steps of making useful aptamer

I. Selecting primary aptamer families

Gold Rush (Missing links):

Understanding aptamers

Designing aptamers

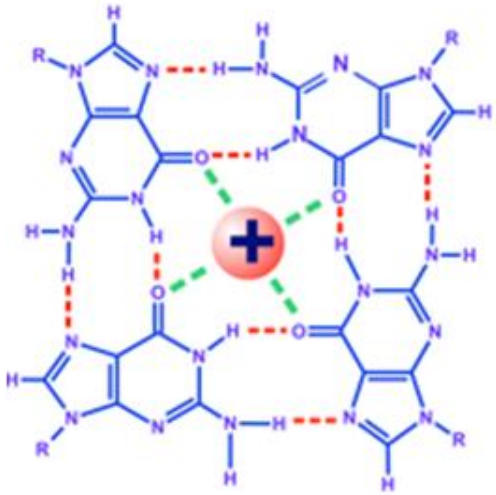
II. Adjusting aptamers to the target

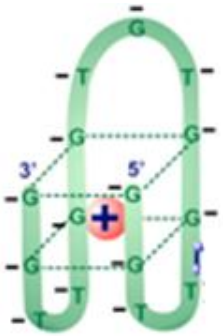
III. Making aptamers stable/durable

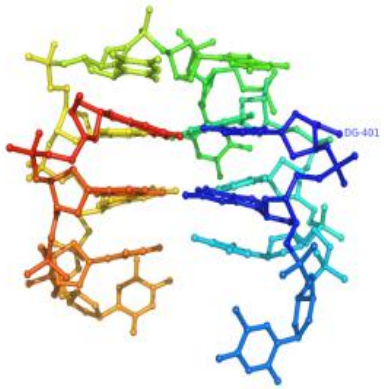
IV. Solving specific tasks

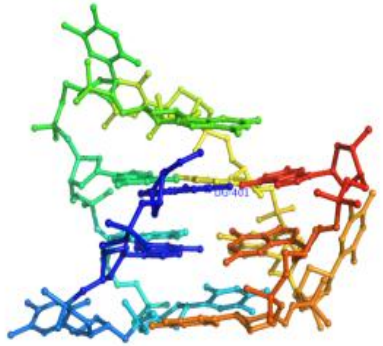
V. Making therapeutic aptamer

G-quadruplex structure of 15-mer (15TGT) - chair minimal DNA aptamer, pharmacophore





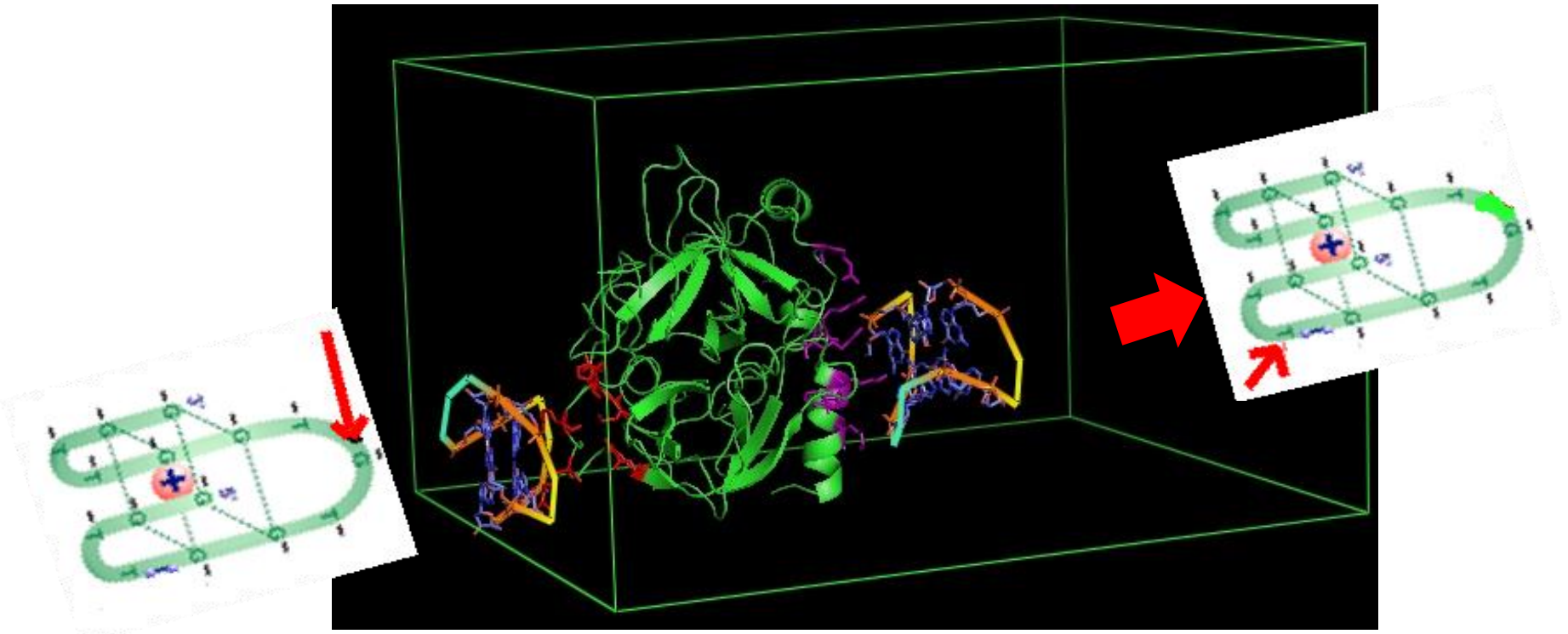




Cation (+) in the center

X-Ray of the complex of thrombin with 15TGT

APTO-PHARM



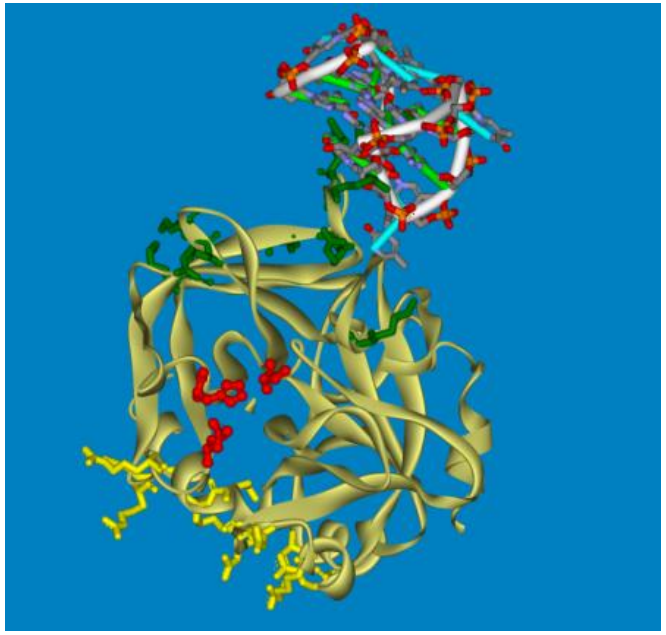
Problem # 2

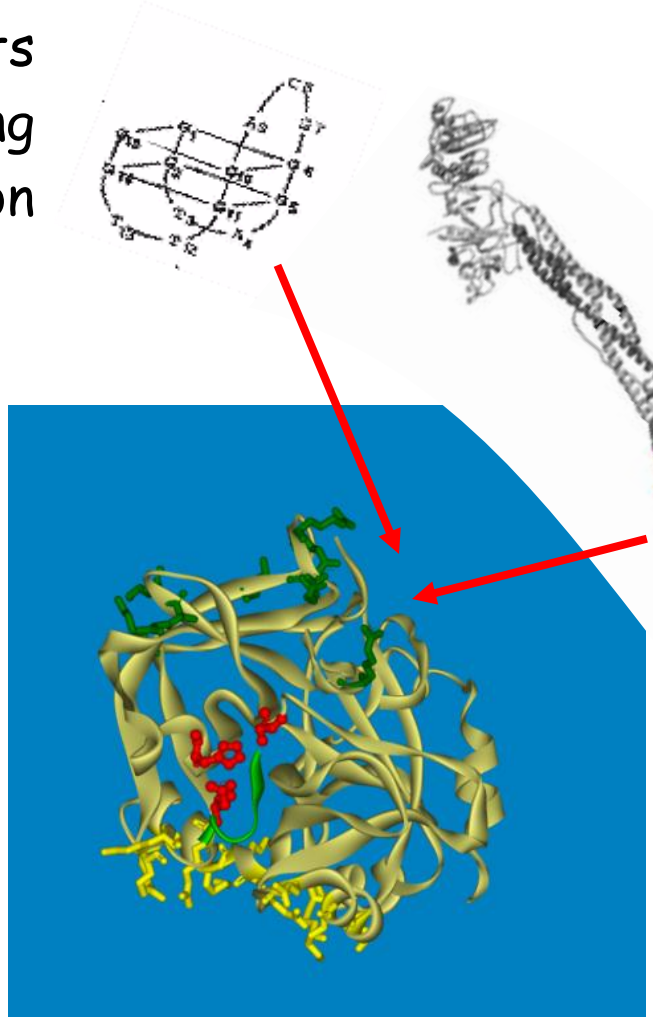
Which loop (TT or TGT) is a pharmacophore?

DNA aptamer and Thrombin

APTO-PHARM

2) Aptamer inhibits fibrinogen binding and clot formation





1) Thrombin hydrolyzes fibrinogen which yields clot formation

Question # 3: Is it a competitive binding/inhibiting?

Understanding Aptamers
Lack of Structure -Function Relationship

APTO-PHARM

15-mer TGT (USA)

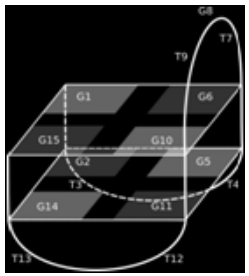
Ki 14,7 nM (RF)

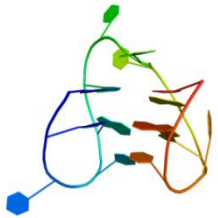
31-mer TGT (Japan)
Ki 0,3 nM (RF)

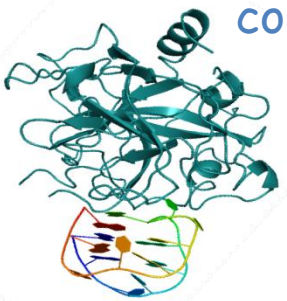
26-mer NU 172 (USA)
Ki 0,3 nM (RF)

3D: X-ray, NMR

No 3D: just 1D







complex

?

MD: rational drug design

Apto-Pharm Ltd

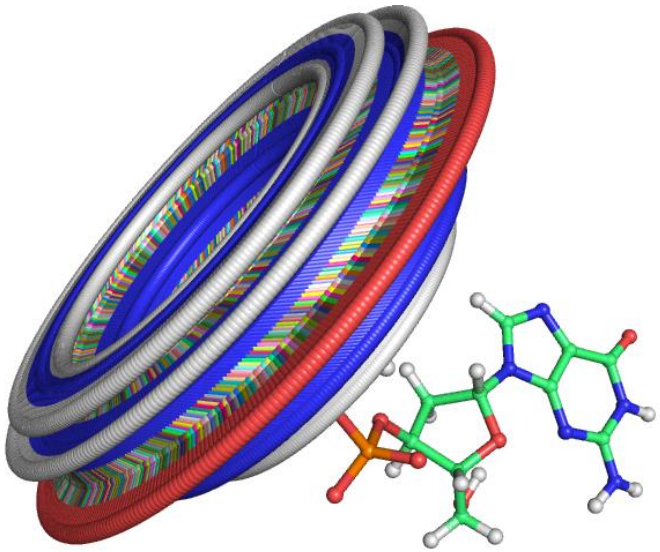
RA-36 (RF)

Ki 7,5 nM (RF)

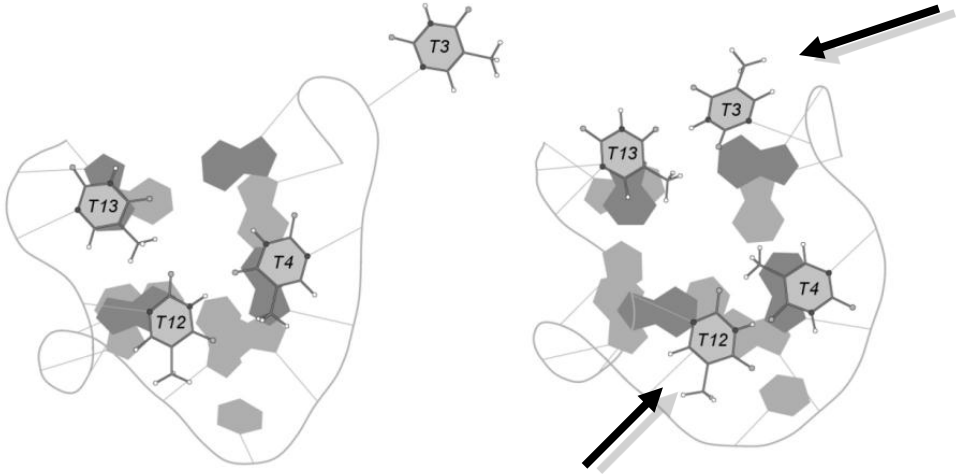
DNA aptamer modelling with molecular dynamics using super-computer

APTO-PHARM

'Lomonosov' Top 31 (06/2013)
Cores: 78,660; Rmax (901.9 TFlop/s)
http://www.top500.org/system/177421

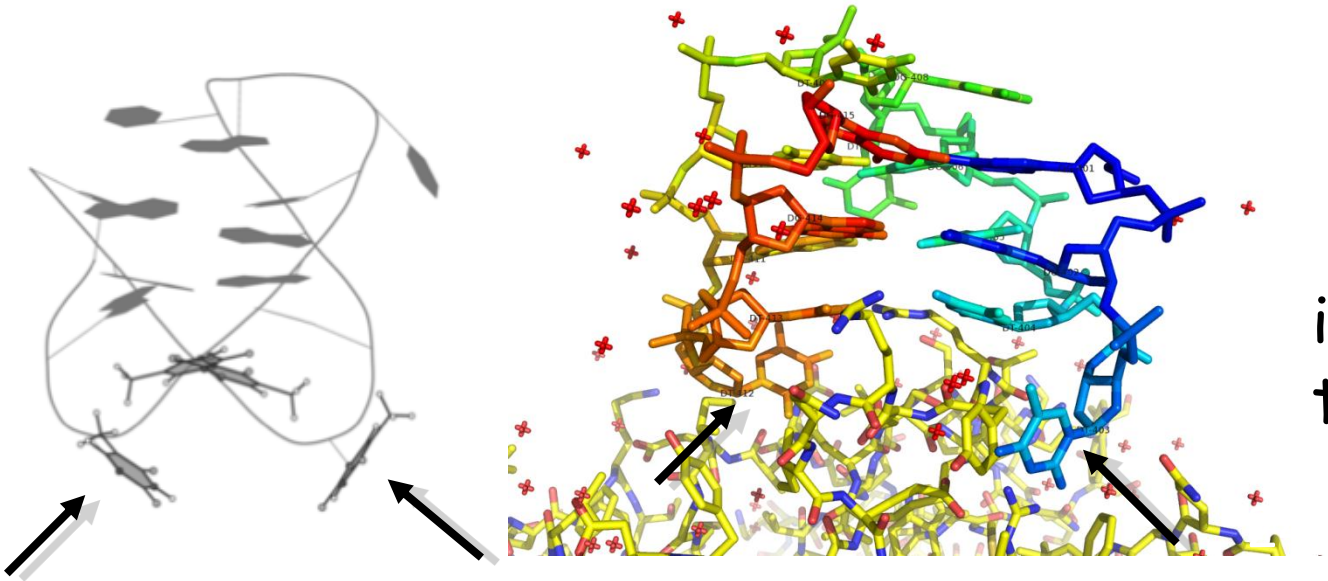


NO conventional force field parameters were available till now
Porting of **new Force Fields** into Gromacs



Bottom view of
2 TT base pairs of
2 TT loops

NMR model of 15TGT during MD
in the new force field



T12 and T3
interact with
the thrombin

TGT upper loop vs TT bottom loops

Loops vs cation

MD: 60-80 ns frame

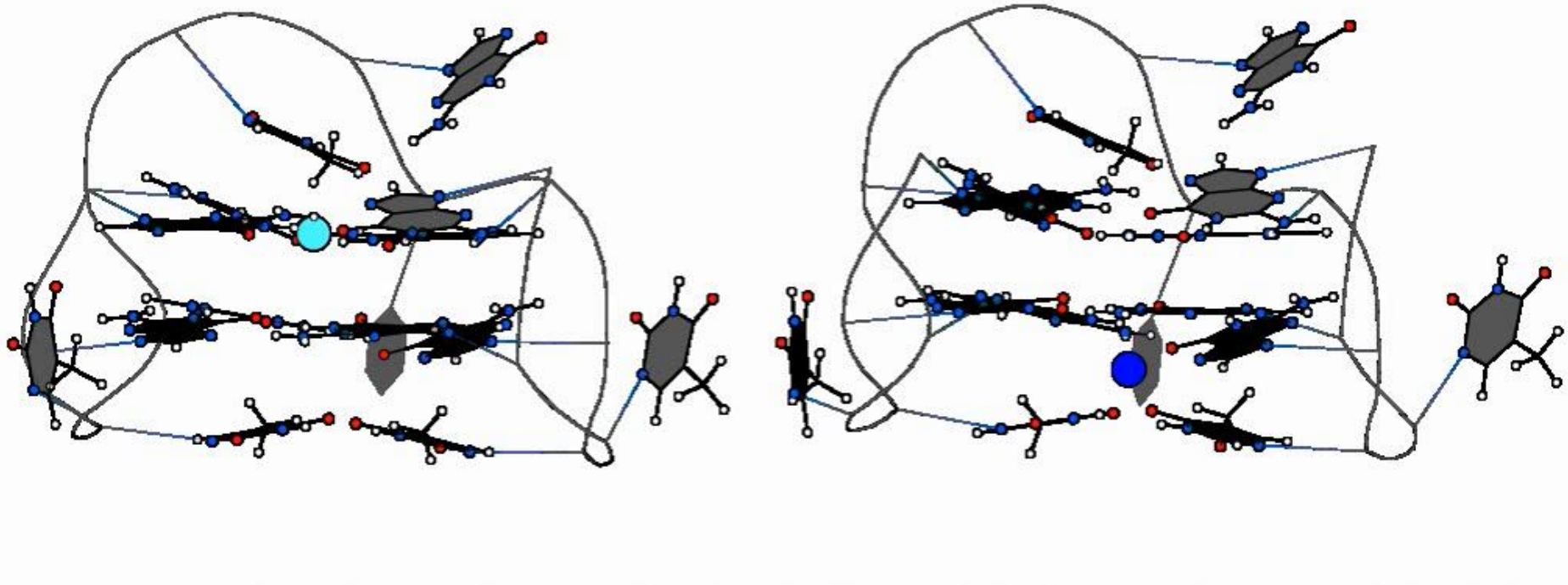
Na^+ movement into G-quadrulpex through TT loops

Most of the time Na^+ sits in the center of G-quadruplex

ps scale

APTO-PHARM

Aptamer – cation QM/MM simulation

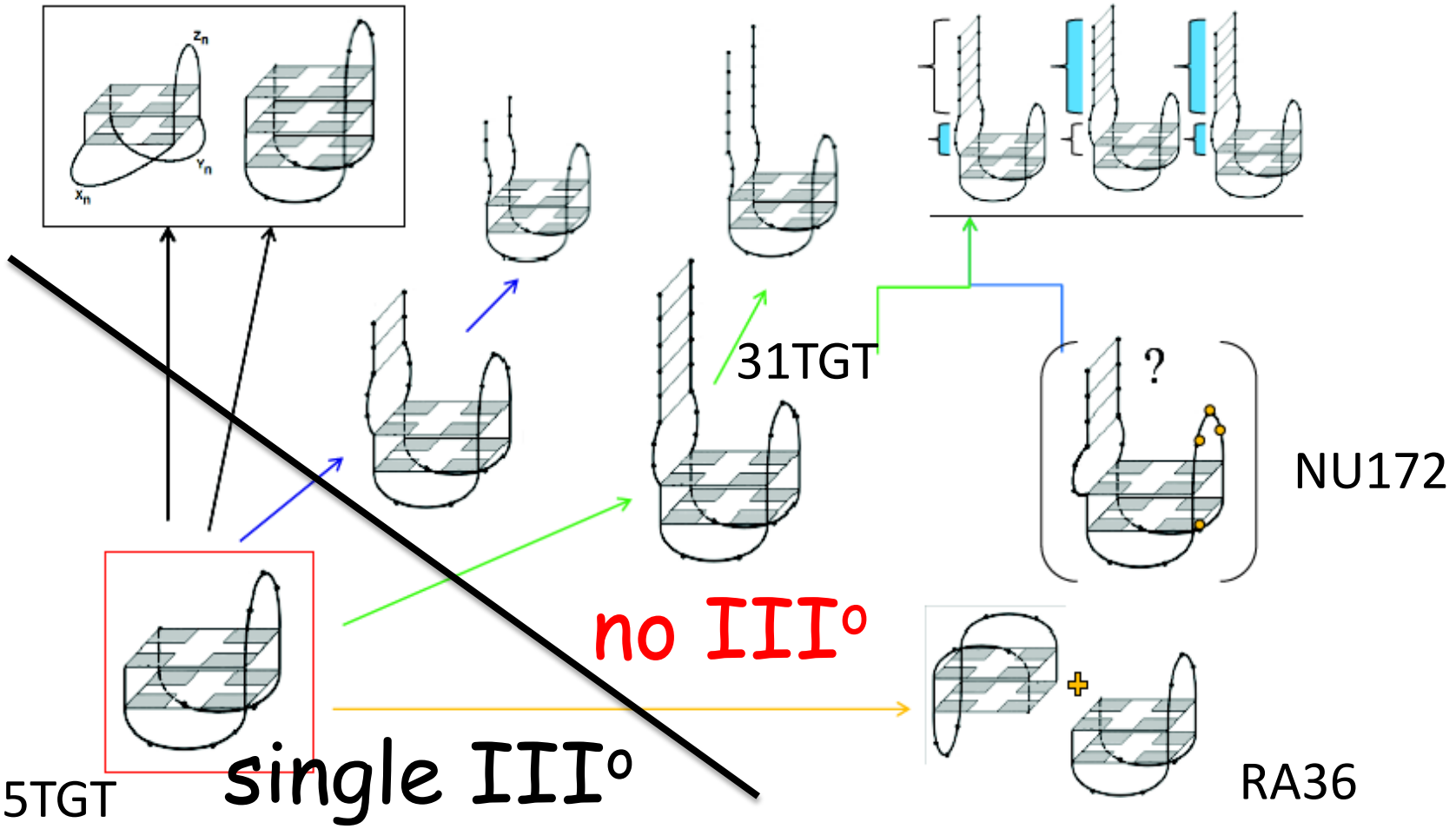


QM/MM simulation approach for proteins:
Martin Karplus, Michal Levitt, Arieh Warshell,
Nobel Prize in Chemistry 2013, Oct 9

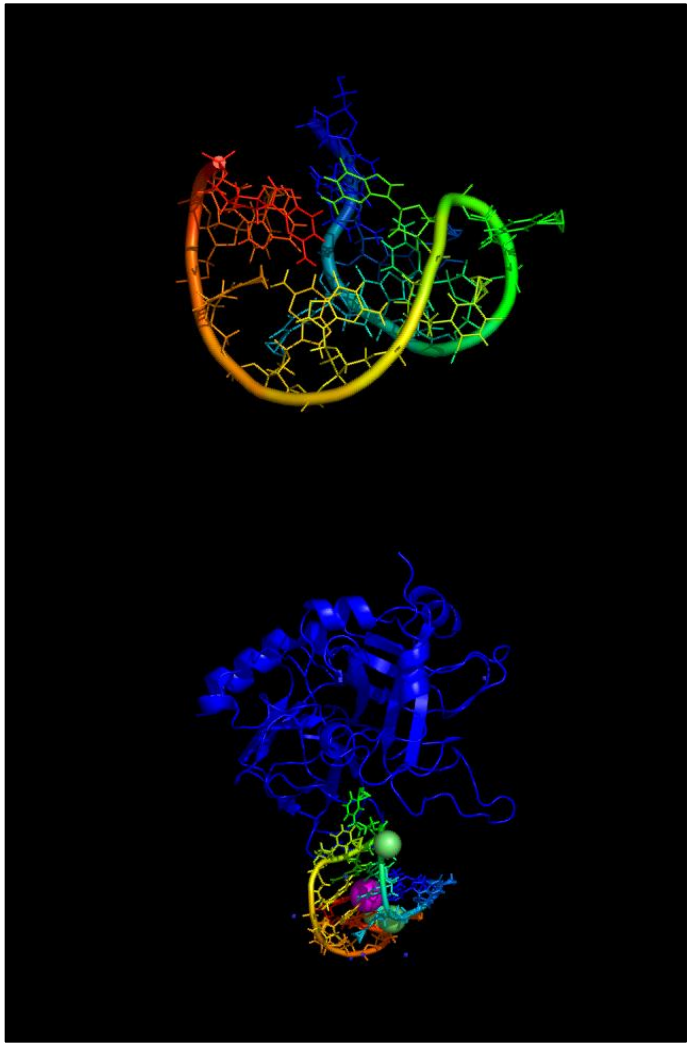
Functional Aptamers: I° & Coagul Activity

APTO-PHARM

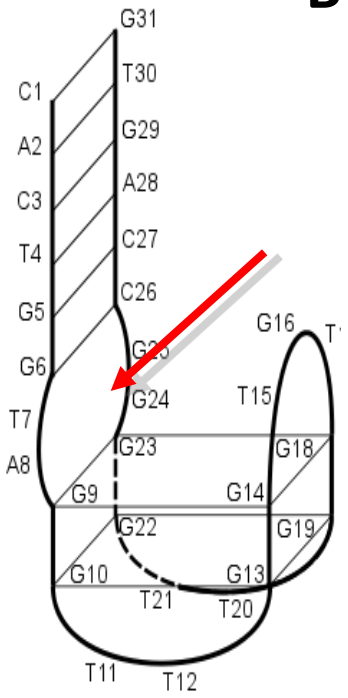
Just putative structures
of 15TGT pharmacophore extentions



Structure:
MD for exploring and design of extended pharmacophore structures [G-quadruplex + Duplex?]



A



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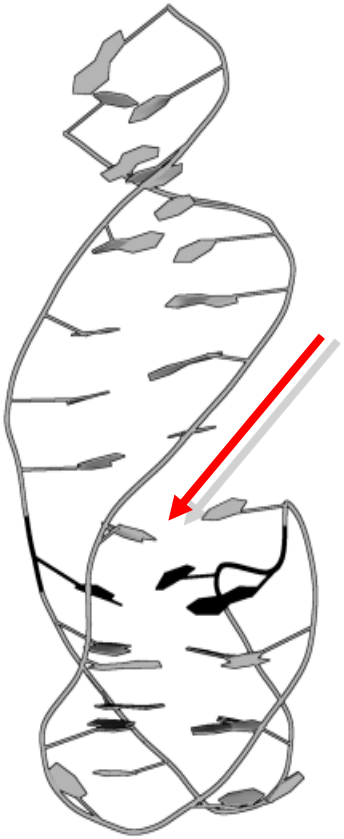
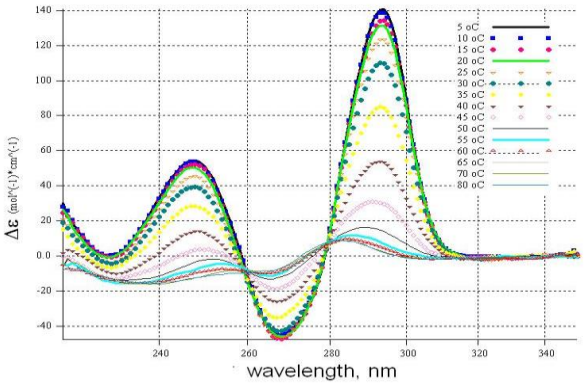
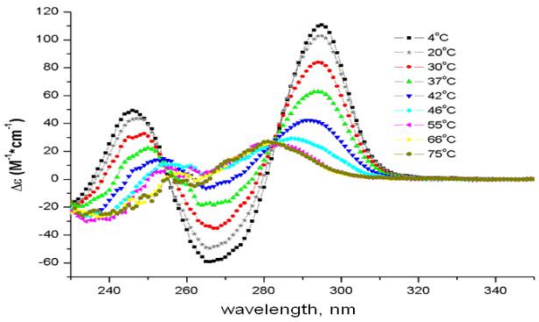


Structure, 3D Assembly

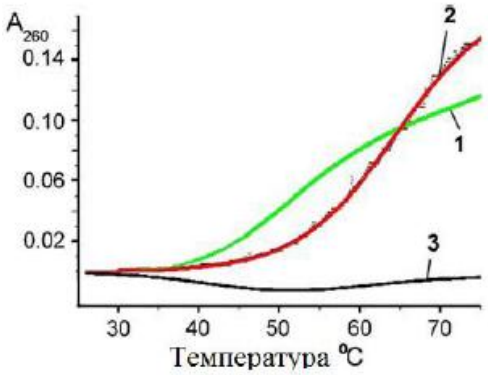




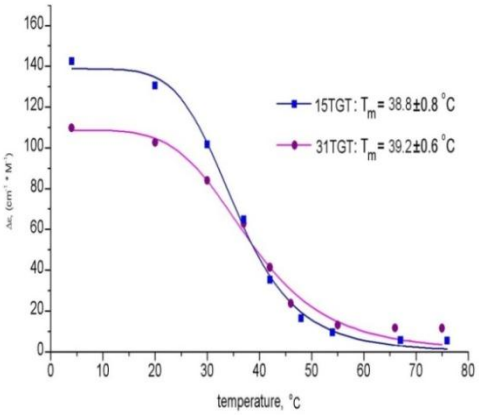
G-quadruplex has
the same
thermal stability
within 15TGT and 31TGT

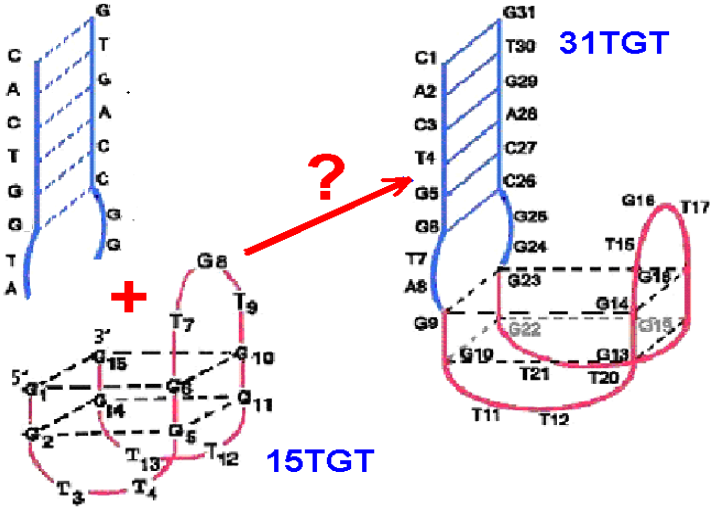
Duplex is stable enough

UV melting
of 15TGT
and 31TGT
(260 nm)



УФ-плавления (260 нм) аптамера
31TGT (1), шпильки (2), и 15TGT (3)

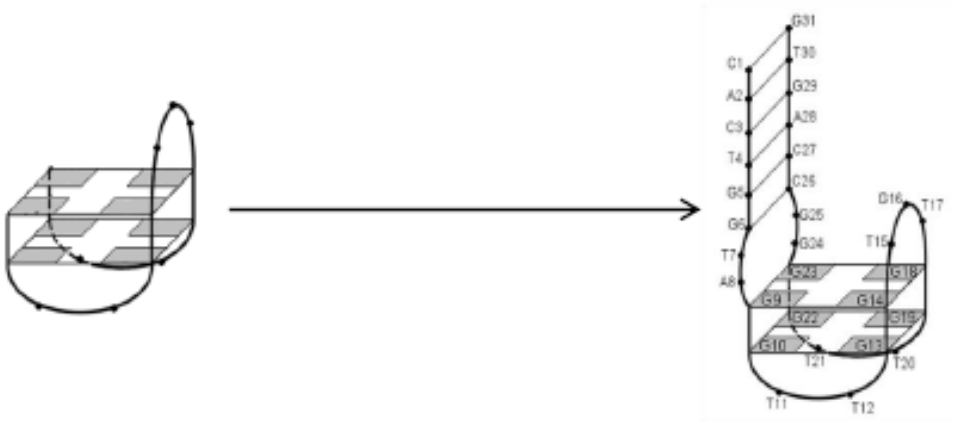


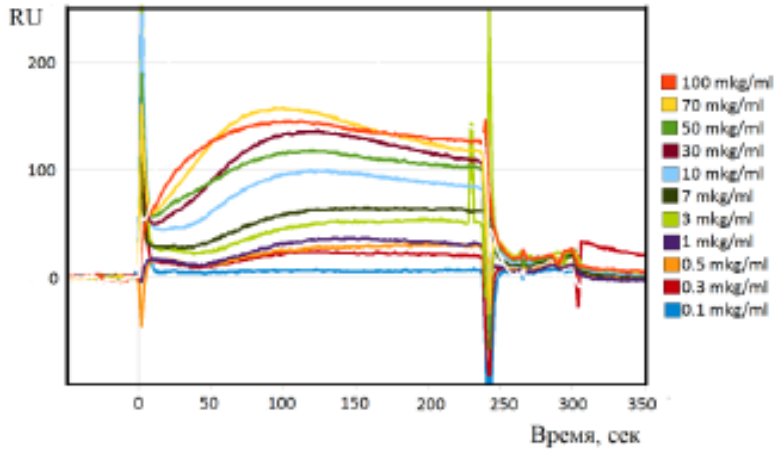


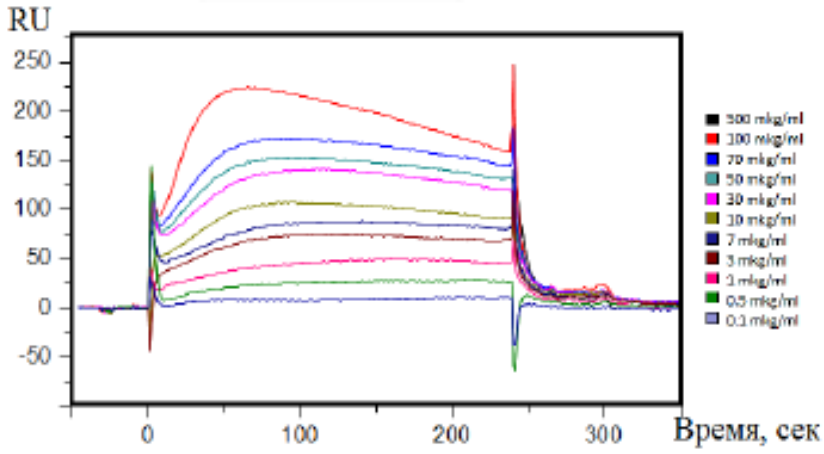
CD melting of 15TGT and 31TGT (294 nm)

Affinity. Kinetics of Interactions: thrombin + 15-mer or 31-mer Surface Plasmon Resonance

APTO-PHARM

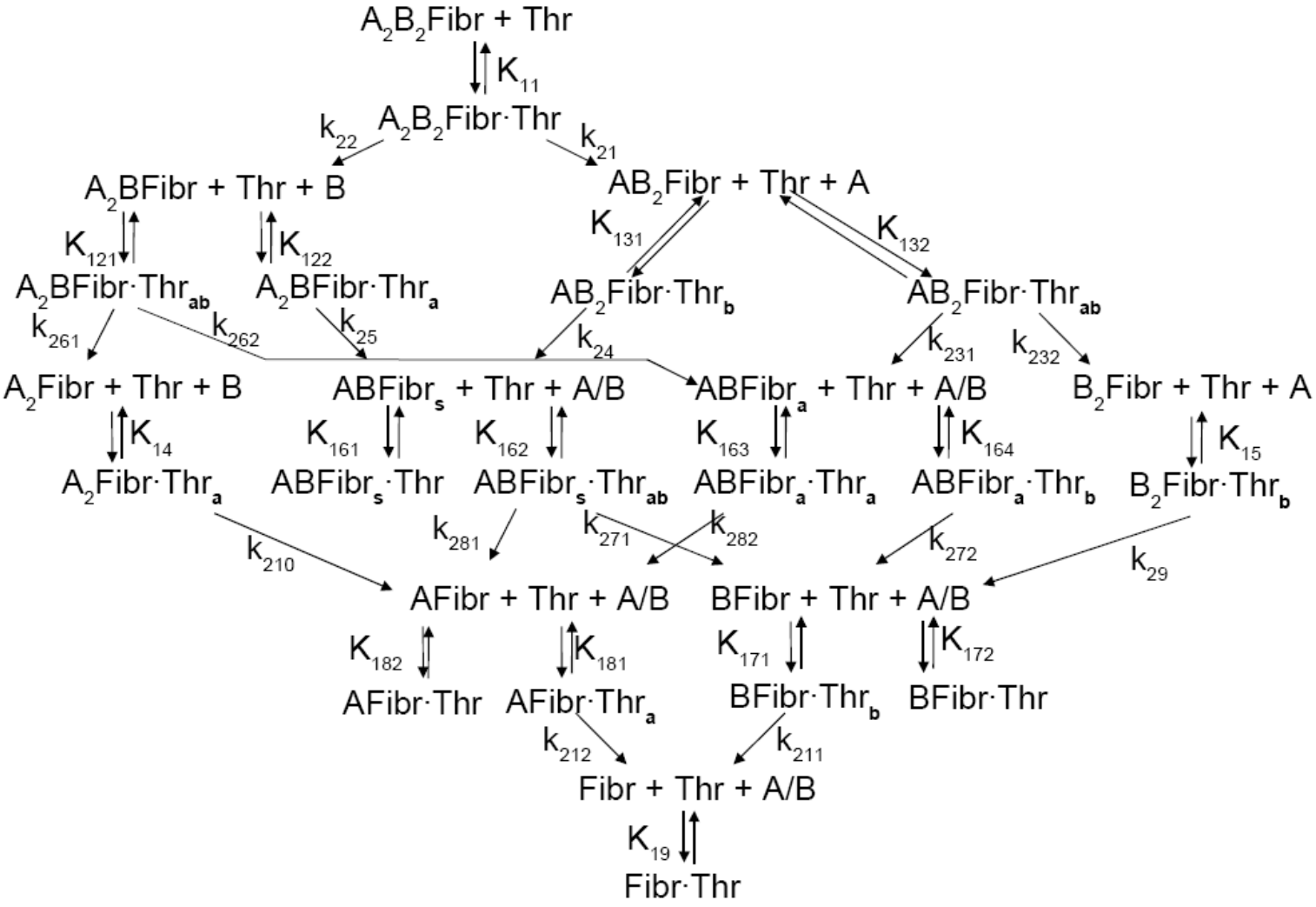




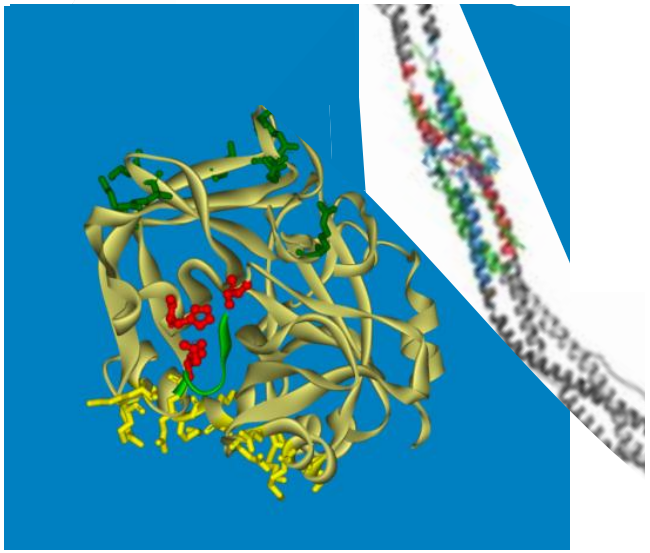


K_{on} different, K_{of} similar

Function:
Kinetics of Fibrinogen Hydrolysis with Thrombin



Thrombin hydrolyzes fibrinogen, and fibrin aggregates. Models, AFM, optical density measurements



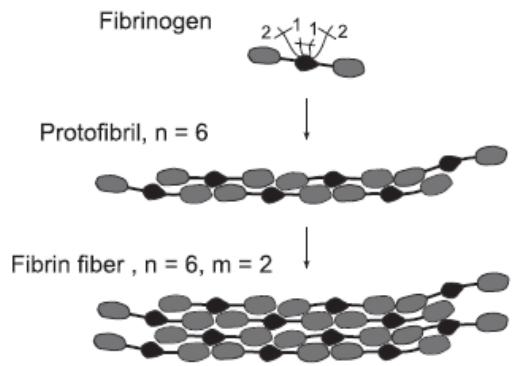


Fig.1. Scheme of fibrin association. E-domains are gray, and D-domains are black. Thrombin cleaves two fibrinopeptides A (1) and two fibrinopeptides B (2). Hydrolysis products associate in two-stranded protofibrils and their associates, fibers. Here n is the number of fibrin molecules in the protofibril, and m is the number of protofibrils in the fiber.

In the case of mixed inhibition type with $\alpha \neq \beta \neq 1$, Eq. (7) becomes

$$IC = B + \frac{1 - B}{1 + AK_I C_I^0}, \tag{8}$$

where $A = \alpha\beta$ and $B = \frac{1}{\alpha\beta}\frac{1+\alpha K_0 C_F^0}{1+K_0 C_F^0}$ allows estimating the parameters α and β from the dependencies of B on the fibrinogen concentration.

In the case of uncompetitive inhibition with $\alpha = \beta < 1$, the parameters in Eq. (8) are $A = \alpha^2$ and $B = \frac{1}{\alpha^2}\frac{1+\alpha K_0 C_F^0}{1+K_0 C_F^0}$.

In the case of competitive inhibition with $\alpha = 0$, Eq. (7) becomes

$$IC = 1 + \frac{K_I}{1 + K_0 C_F^0} C_I^0. \tag{9}$$

In the case of noncompetitive inhibition with $\alpha = 1$ and $\beta = 0$, Eq. (7) becomes

$$IC = 1 + K_I C_I^0. \tag{10}$$

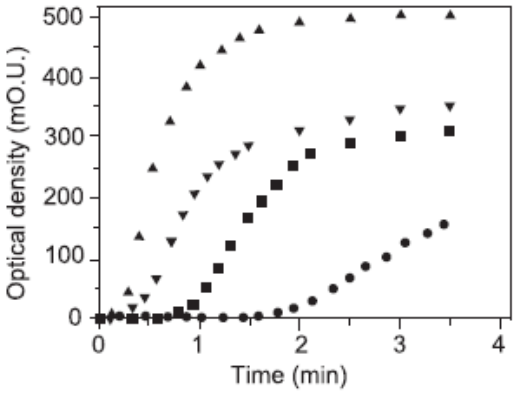
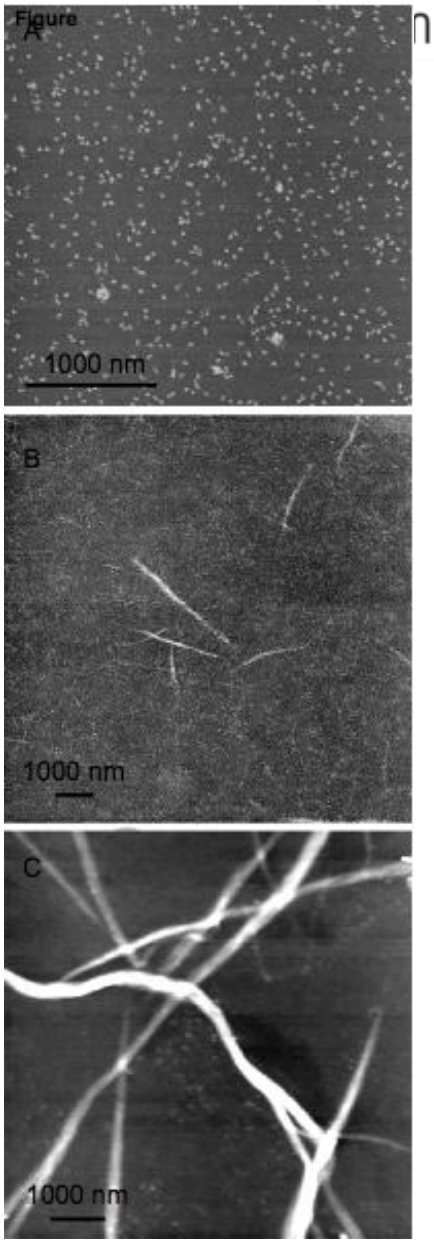


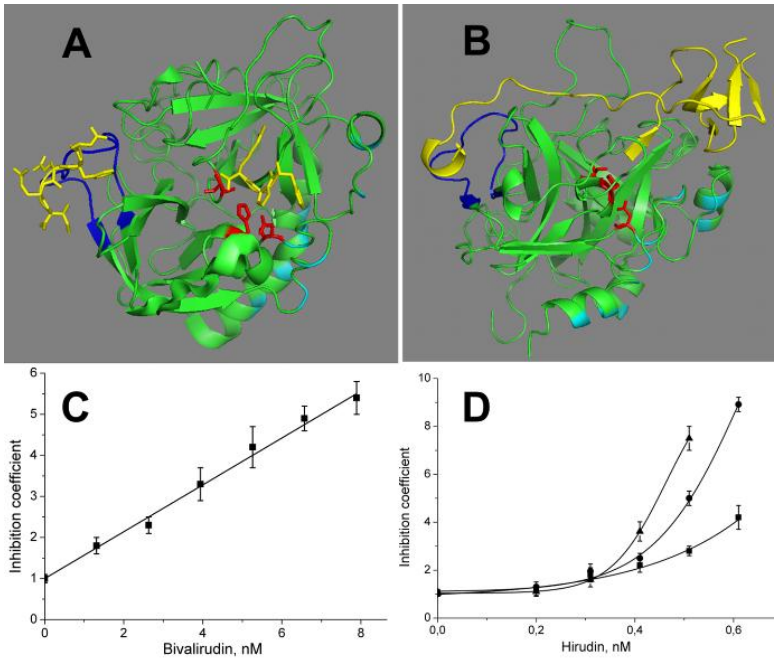
Fig.5. Turbidimetric curves of fibrin association with different thrombin concentrations: 15 nM (▲), 7.0 nM (▼), 3.6 nM (■), and 1.2 nM (●).



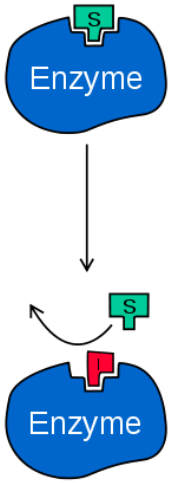
Possibility of calculations of both Ki and Inhibition Type

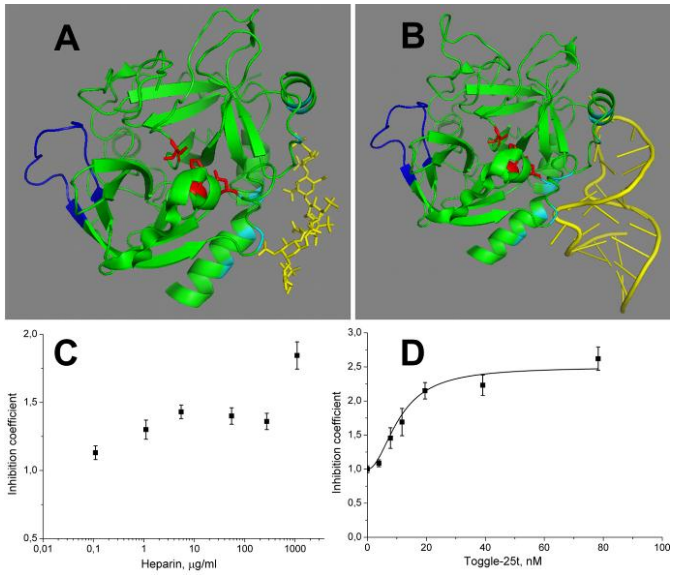
Examples:

bivalirudin
hirudin
heparin
aptamer

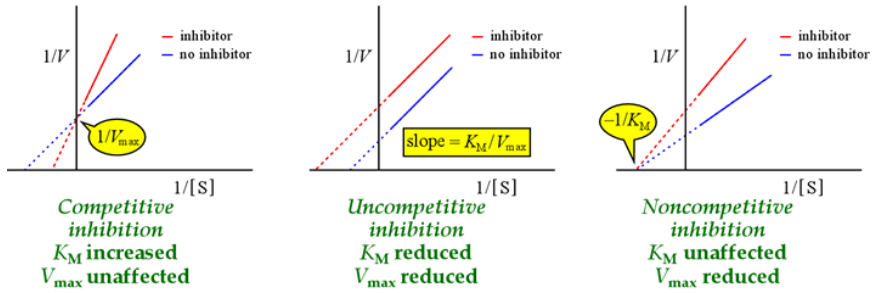


Aptamer	Inhibition type	Inhibition constant, nM
15TGT	Non-competitive	14,7±1,0
31TGT	Competitive	0,34±0,10
NU172	Competitive	0,29±0,06
RA-36	Non-competitive	7,5±0,3





The Lineweaver-Burk plots for inhibition



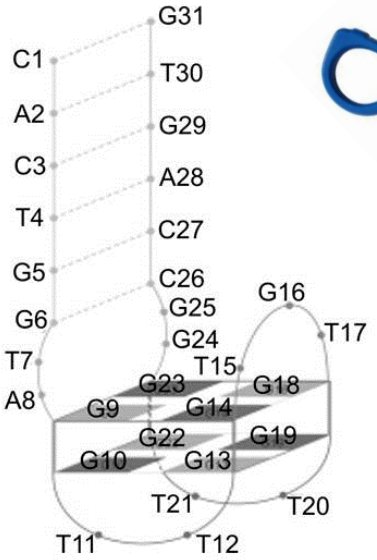
Apta-nano-Lego

APTO-PHARM

J

J

j



31TGT

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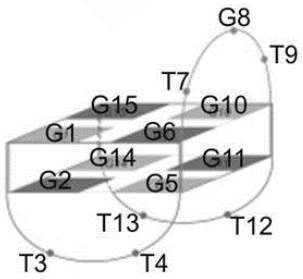

15TGT

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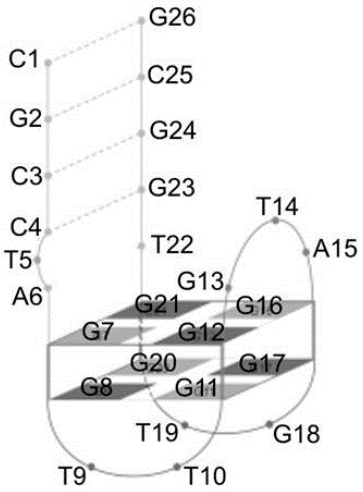
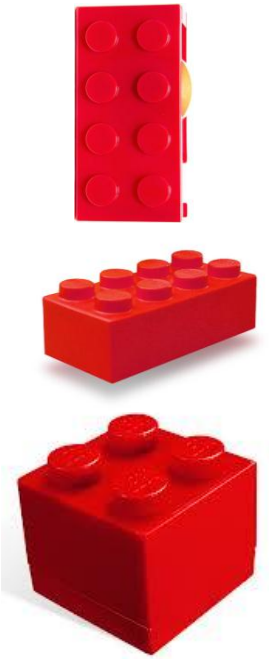


NU172

a

a

a



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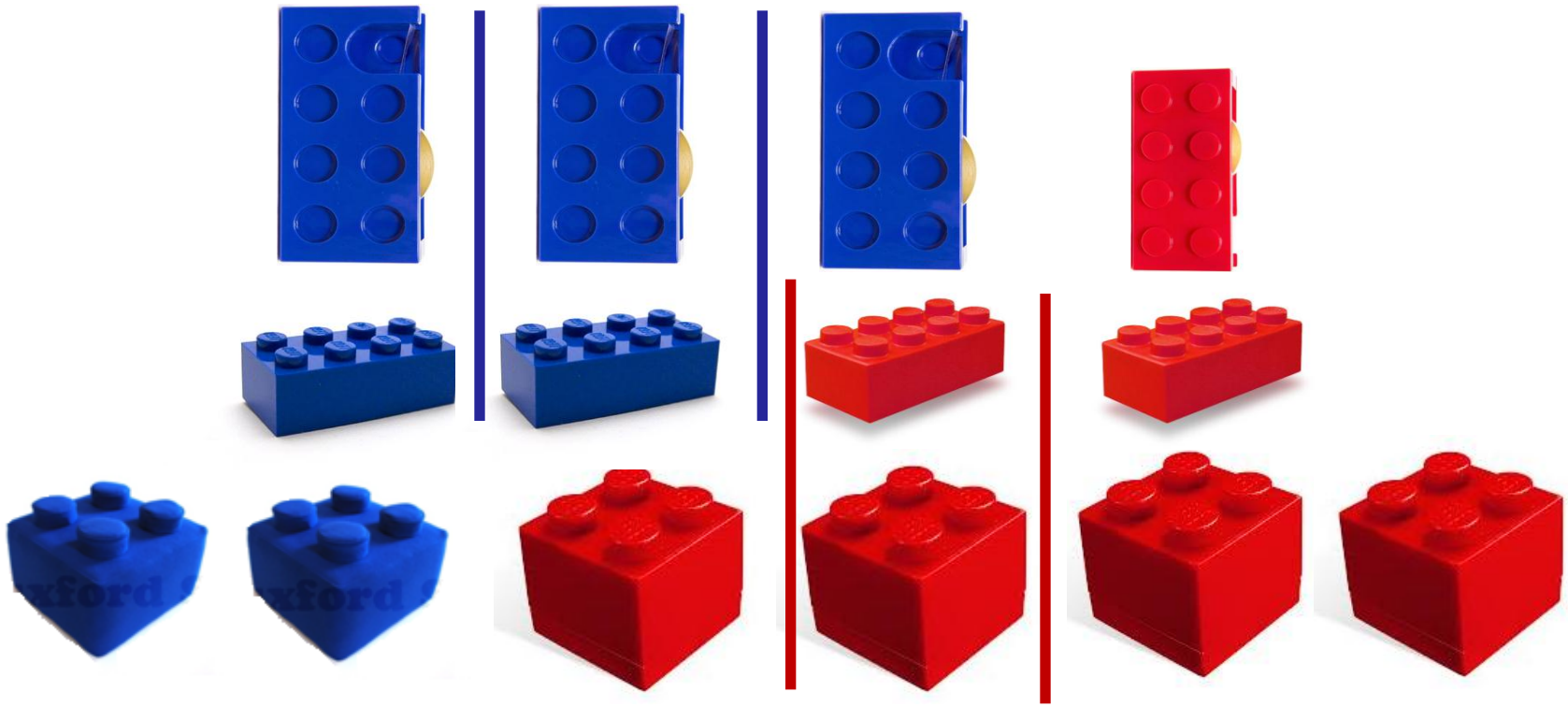
Apta-nano-Lego

APTO-PHARM

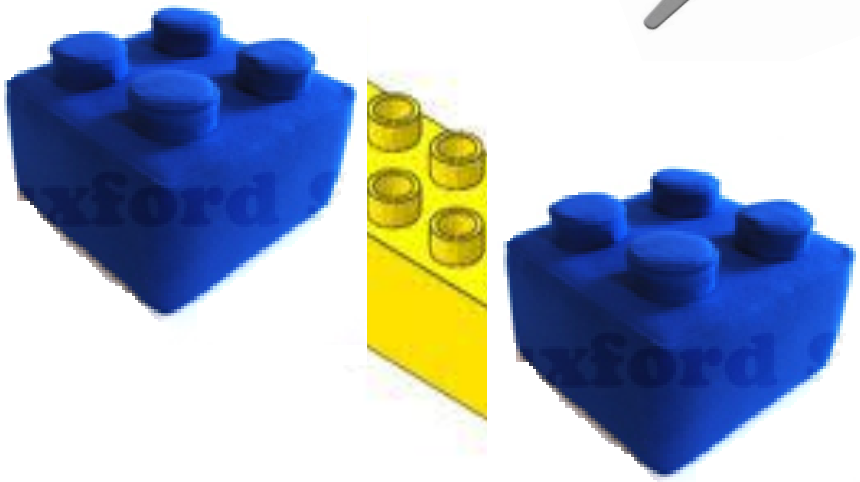
15TGT 31TGT NU172

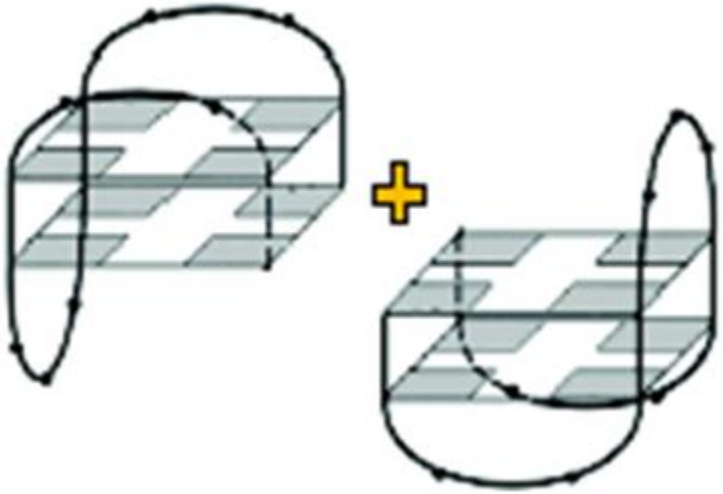


Ki, nM 14,7 0,3 1,2 1,3 0,3 12

Making multi-nano-tools
Bivalent Aptamer , RA-36
Apto-Pharm

APTO-PHARM



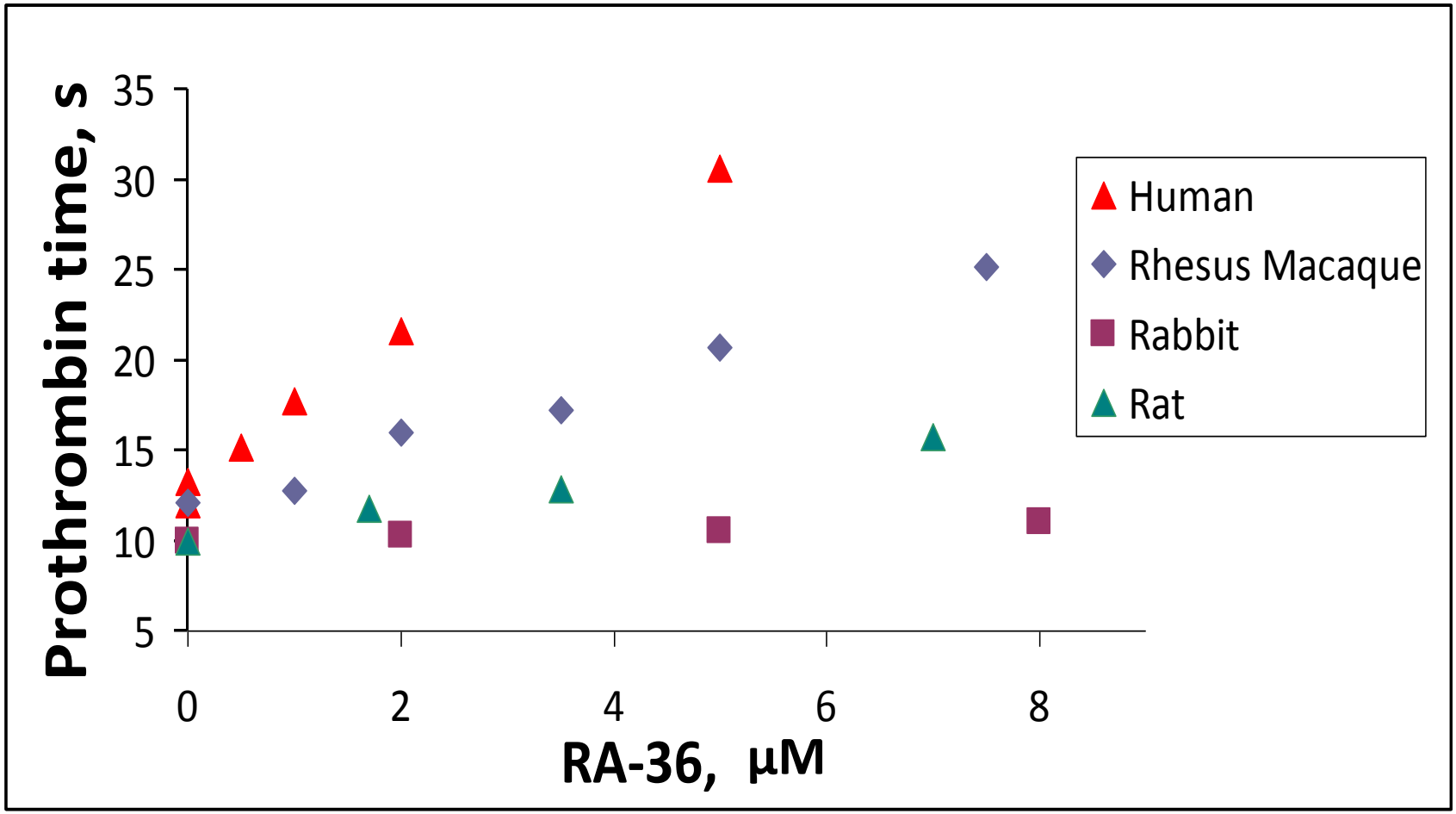


Ki, nM 7,5

The inhibiting type
not like for extended aptamers

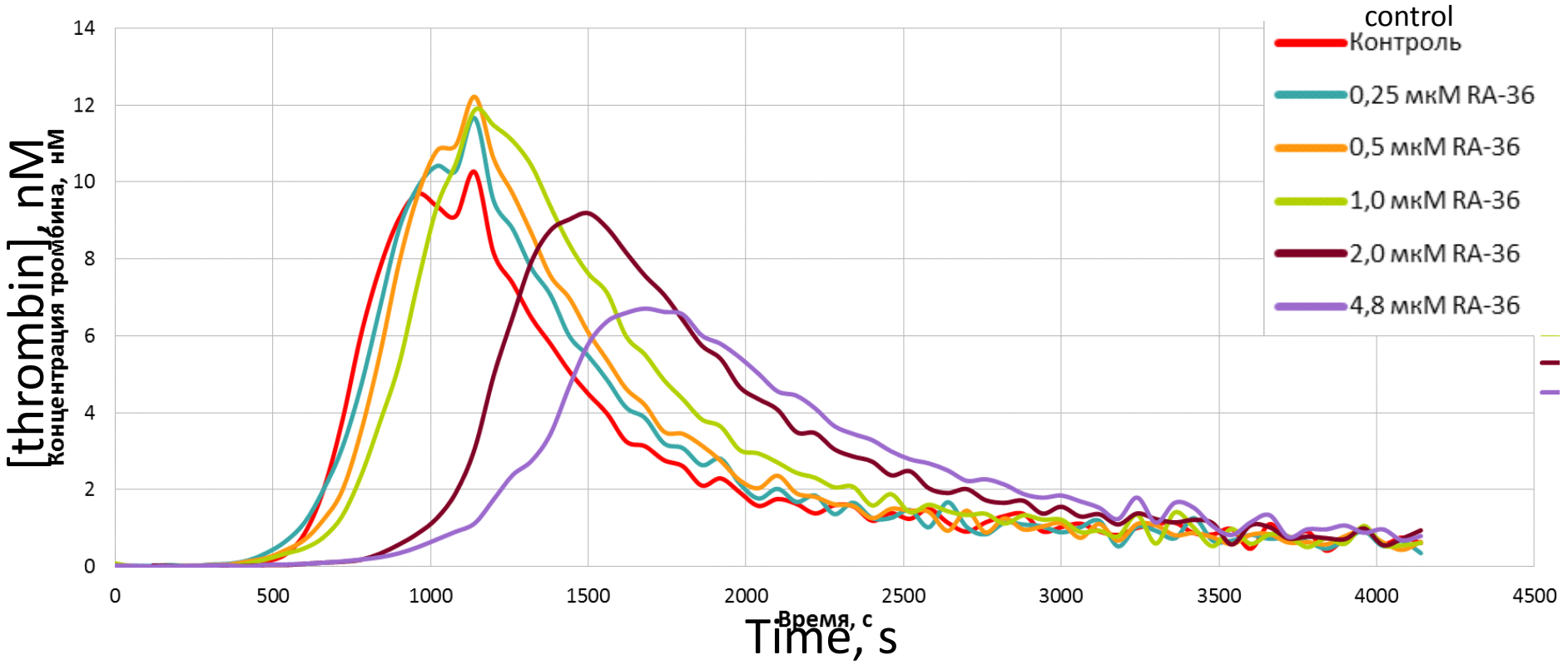
PCT WO 2011/075004 A1, Dec 13, 2010

Blood plasma tests for RA-36: species specificity



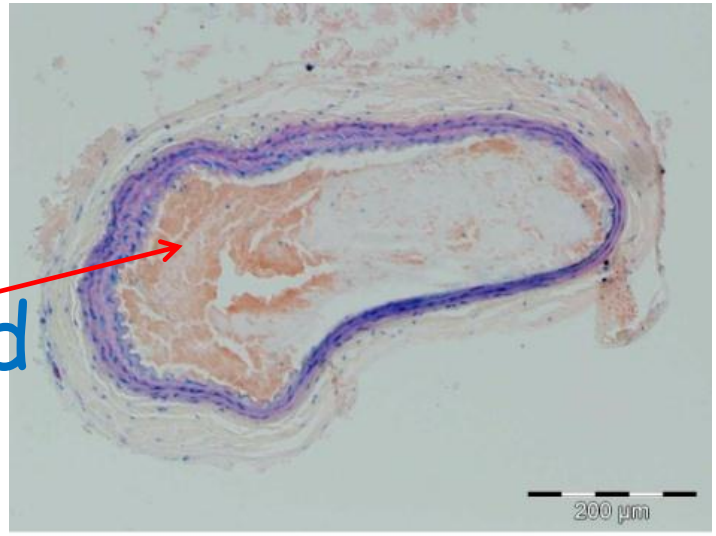
INR = 3 (like for coumarin)

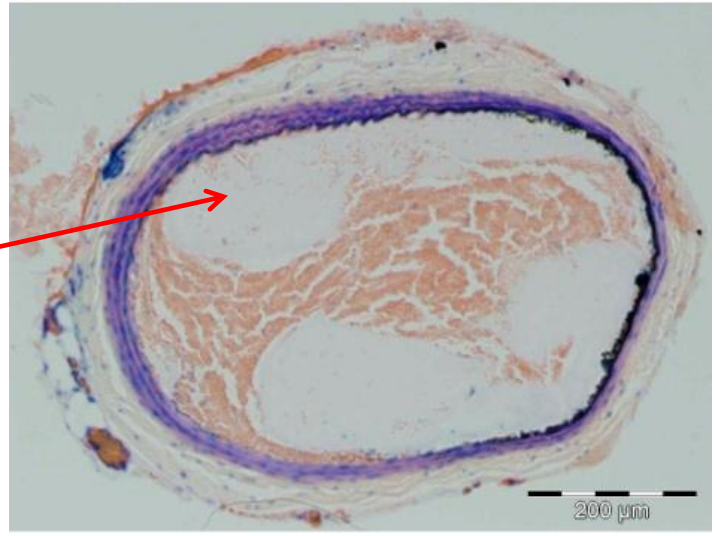
Thrombin Generation Assay



Mouse model for venous thrombosis

RA-36 inhibits clot formation: vessel cross-sections





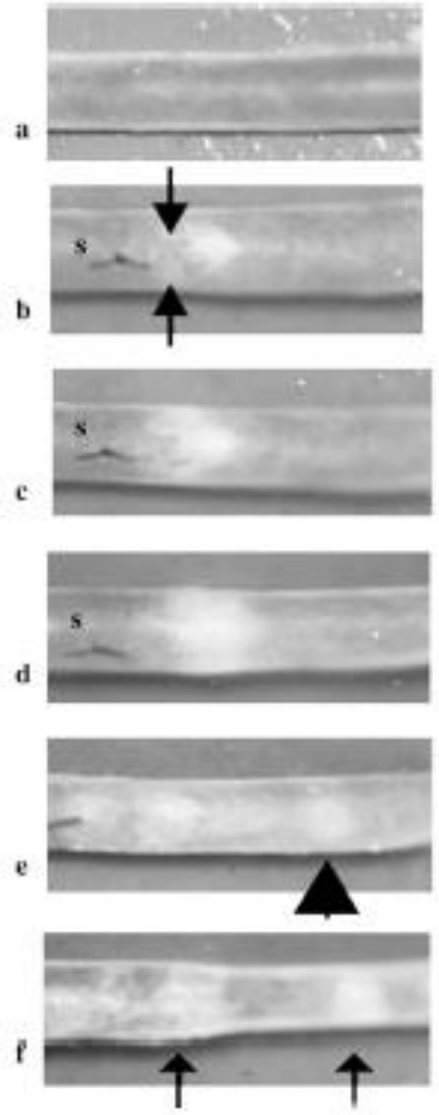
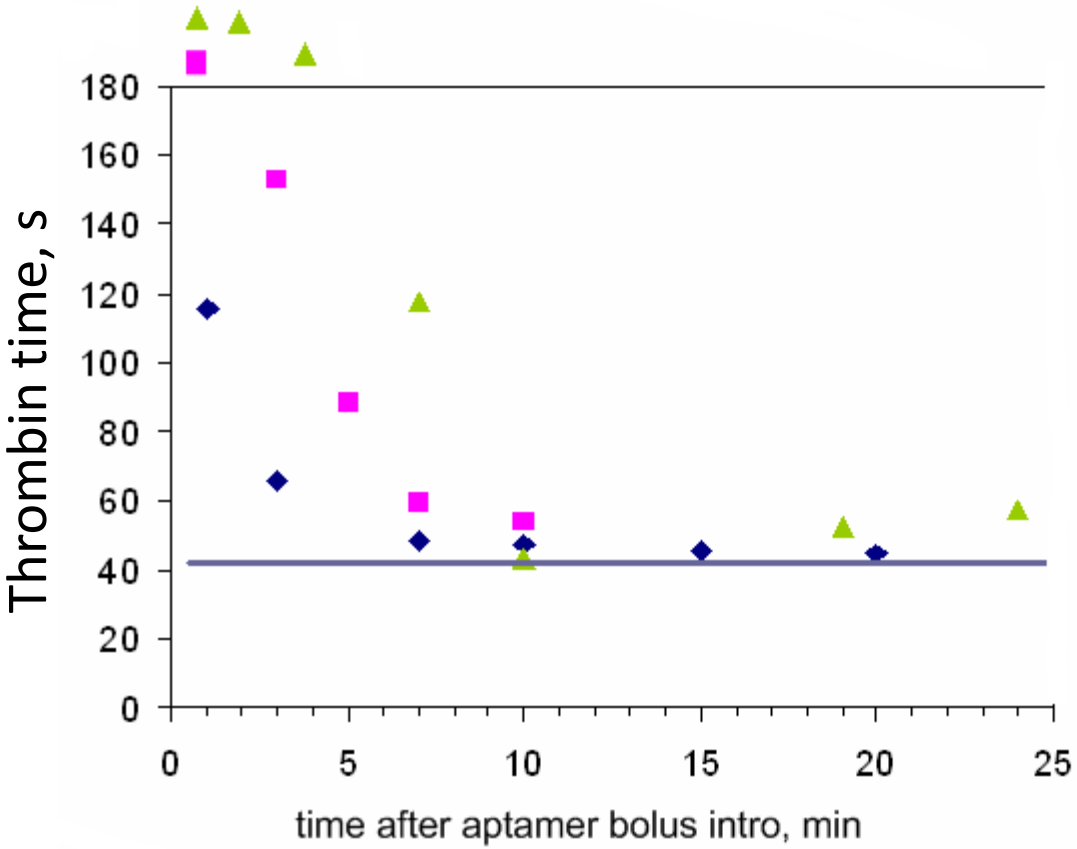


Figure 1 Micro-photographs (taken through an operating microscope) of the electric injury site and developing thrombus. (a) Normal carotid artery; (b) discolored/blanched site of electric injury (between arrows) 3 min after injury; developing thrombus, seen at (c) 8 min and (d) 15 min; (e) embolized thrombus (arrowhead) breaking off; f) occlusive thrombus with evident thrombi blocking flow (arrows) and with decreased vessel diameter on right. Flow is from left to right in all images; s=marking suture proximal to electric injury site.

Animal tests:
Short duration time – several min

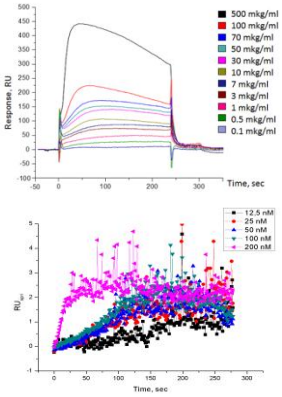


CURRENT STATUS: formal pre-clinical trials

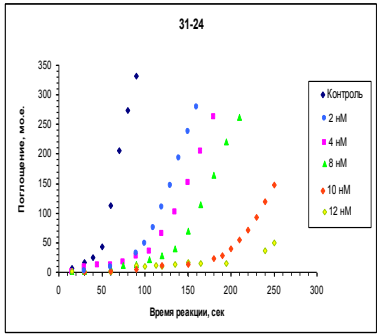
1st Moscow State Medical University

Modelling

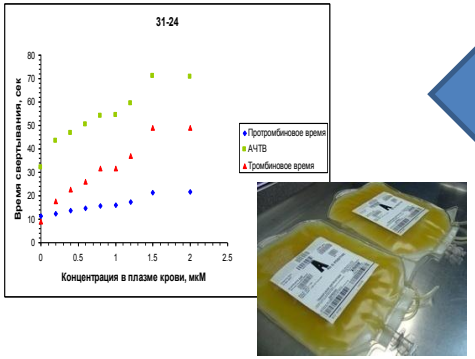
Synthesis



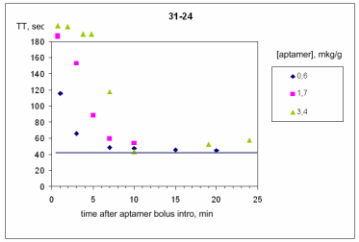
Affinity



Inhibition



Coagulation



Animal trials

PCT WO 2011/075004 A1, Dec 13, 2010

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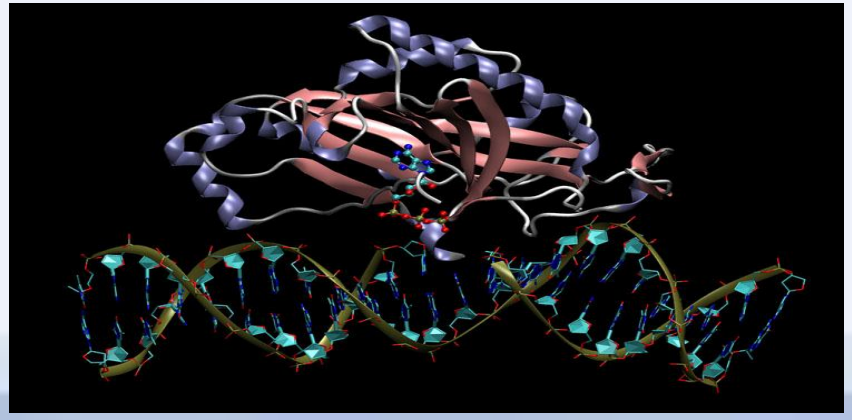

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