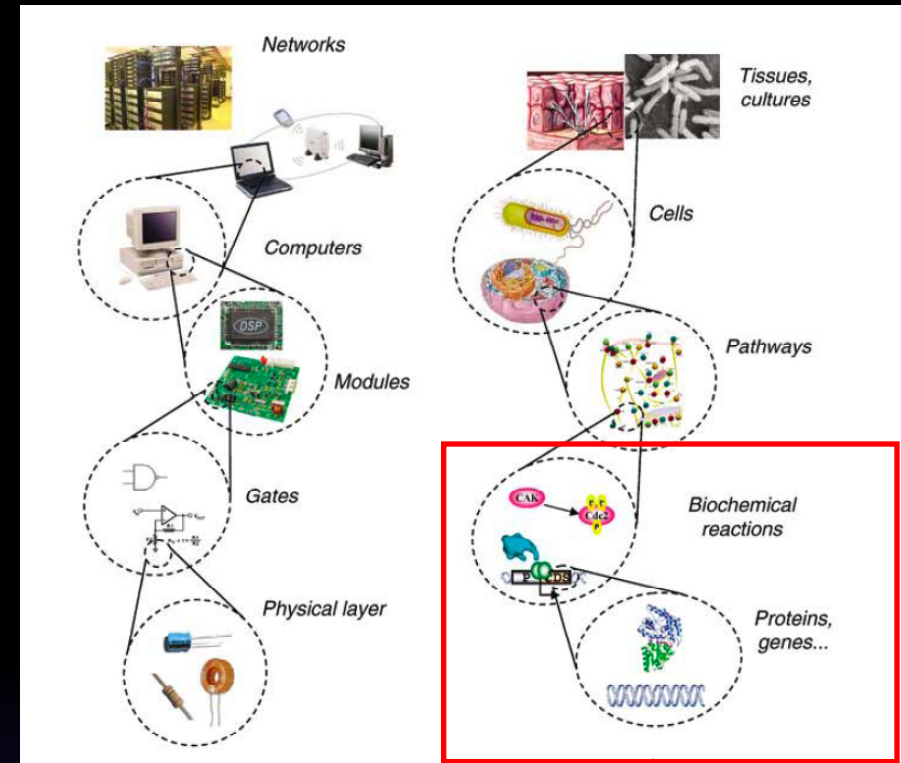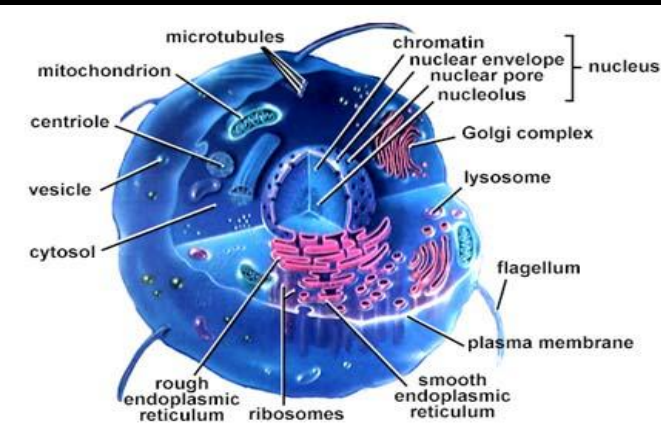# The Aptamer Technology: Promises of Synthetic and Robotic Biology

*Henning Ulrich*

**Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil**

# Synthetic Biology



**Substitute bioactive natural compounds (enzymes, receptor ligands by synthetic ones**

**The final goal is to be able to design biological systems in the same way engineers design electronic or mechanical systems.**

# Structure-Based Drug Design Versus Isolation of Biological Active Compounds from Combinatorial Libraries

**Structure-based**

**Knowledge on location and tertiary structure of the binding site on the target molecule**

**Combinatorial library**

**No structural knowledge on target-binding site required**

**Effective selection and screening procedure required**

# Drug Discovery:

## Synthetic versus natural compounds

## (Paradigma Shift for Drug Development)

'70 : **Natural Products**

'80 : **Rational Design**

'90 : **Combi. Chem. & High-throughput screening (HTS)**

'00 : **Information Guided Design**

´Since then : **Combi. Chem. & HTS & Automatization (Robotic Assays)**

# Combinatorial Chemistry Based Drug Discovery

A synthetic  strategy which leads to a large set of  compounds.

- Synthesis of many structures  (diversity)

- Product  of  matrix  chemistry (systematic synthesis)

- High-throughput Screening (HTS)

- Automation of synthesis (speed) and Screening

- Identification of lead compounds and drug development

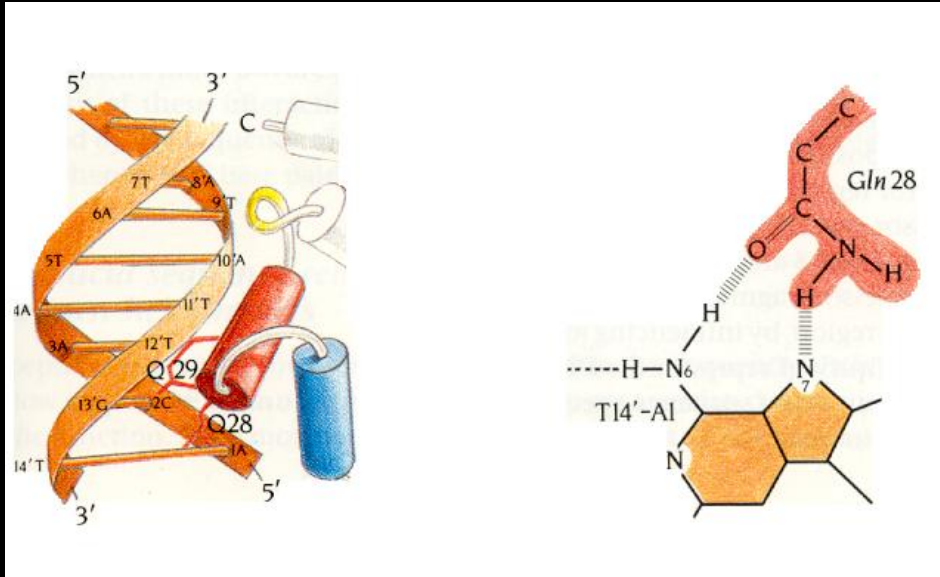# Compounds Used in Combinatorial Libraries for Drug discovery

| Compounds | Targets |
|---|---|
| - Oligonucleotides | Enzymes / receptors / ligands |
| - Nucleotide analogs (linked binding elements) | Tyrosine kinases |
| - Peptides | Enzymes / receptors / ligands |
| - Peptoids | Cholecystokinin and opioid recptors |
| - Benzodiazepenes | Peptide-, Neurotransmitter receptor antagonist |
| - Beta-Turn-Blocker | receptor-, peptides-, hormone receptors, enzymes |
| - Prostaglandins | Thrombin receptor |
| - Sialyl-Lewis X | Anti-tumor agents, carbohydrate receptors |
| - Polyamines | Neurotransmitter receptors / ion channels |
| - Triterpenes | Dimerization inhibitors of HIV protease |
| -Oligocarbamates | Replication  of HIV, thrombin |
| - Cocaine /scolopamine analogues | Neurotransmitter receptors, Reuptake systems |

## Static versus Dynamic Combinatorial Libraries

# Oligonucleotide Combinatorial Libraries



**DNA synthesizer**

**$10^{16}$ sequences**

GGGAGGACGATGCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAGACGACTCGCCCGA

**Aptamer**



```
GGGAGGACGAGCGGGGGAGCACAACCAGGAAUAUAUUUGAACCUUCAUUAUAUGCAGACGACCGCCCGA
GGGAGGACGAGCGGUUAUGGUGCCGCUUUUAGAACCCUCUAGGUUUUUCUUAGCCAGACGACCGCCCGA
GGGAGGACGAGCGGGUGAGGGAGGCCGGAUUGACUCUAGAUUCUCACGCUCCUGCAGACGACCGCCCGA
GGGAGGACGAGCGGUGAUUGCUGCCACAAGAGCUCUCCUCACACUAACUAGCAGCAGACGACCGCCCGA
GGGAGGACGAGCGGUAACUCCGACCUGUGUGUACCCUCUUCUGUAUUGCCGCCACAGACGACCGCCCGA
GGGAGGACGAGCGGGGUGUUCCCCAGUGAGCGCUUUGUAUAUAAACUUGAAAACCAGACGACCGCCCGA
GGGAGGACGAGCGGAAAGCCUGCAUCGUAAGCCUCCACAGUAAAUAGCAAAAGUCAGACGACCGCCCGA
GGGAGGACGAGCGGGAUGGAAUCUGGAAUAUGCCUUCGUAGAGUCUCCUUGUCUCAGACGACCGCCCGA
GGGAGGACGAGCGGAUCUGUCAAGCUUAUAUCCUCUCUAACAUCAUGUUUAGUACAGACGACCGCCCGA
GGGAGGACGAGCGGAAGUGGGAUCGGGGGUGAACUAGUCUGCAAUGUCACAUGACAGACGACCGCCCGA
GGGAGGACGAGCGGGUGGGAUCCCGACCAGGAUAAACAUAUGCGCGGUAGCAAGCAGACGACCGCCCGA
GGGAGGACGAGCGGUUGUCCACAGUAUAUAGAUUUCCAUAUGUUUCCGCGCGGUCAGACGACCGCCCGA
GGGAGGACGAGCGGACGCUCUGCUGUAAAGGAAUACGAUCCCUUGGCAAGCUCGCAGACGACCGCCCGA
GGGAGGACGAGCGGUGUGUCCGGGAGAUAGUCCACGUAUGAUGCUUUAACAGACAGACGACCGCCCGA
GGGAGGACGGCGGCGUGAUCACAUUGUAGCAUCCUUAAUUGCGCCUUAACGUCAGACGACCGCCCGA
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GGGAGGACGAGCGGCCAUAUUAGGGCAGUAAAGACGCCCCAAGCAAUUUCGUAUCAGACGACCGCCCGA
GGGAGGACGAGCGGUUCGGCCUAGACUCGUAAGCACAUUCAAUAGAACCGAUCACAGACGACCGCCCGA
GGGAGGACGAGCGGCGCUAGGGUACCACCGUUGAGCUCCGUUCGAGUUAUUCCCCAGACGACCGCCCGA
GGGAGGACGAGCGGUAGUAGGCGCCACCGUUAUGCCACUCAAUAAUUAUCUGCACAGACGACCGCCCGA
GGGAGGACGGGCUGUGGCCCCUGAUAGGCCCACUUGCCUAGAUAUCAGAGCAGACGACCGCCCGA
GGGAGGACGAGCGGACGAAUCCGCCAACUGAUGCCCUUCAUUAAGAUCUGUCAACCAGACGACCGCCCGA
GGGAGGACGAGCGGCGUGUUUUUAUCCGAGAGGCAACUCUUUAUAUAUUCCCAAUUCAGACGACCGCCCGA
GGGAGGACGAGCGGCUUACUCAAUAGCCUAAUCAUCCCAGGAAUUAAGGAGGGCAGACGGCCCGA
```

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.
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# Direct / Indirect Recognition of Protein Sites by Nucleic Acids



Sequence-specific because amino acid side chains H-bond with DNA base pairs in major groove.

Structural basis well understood.
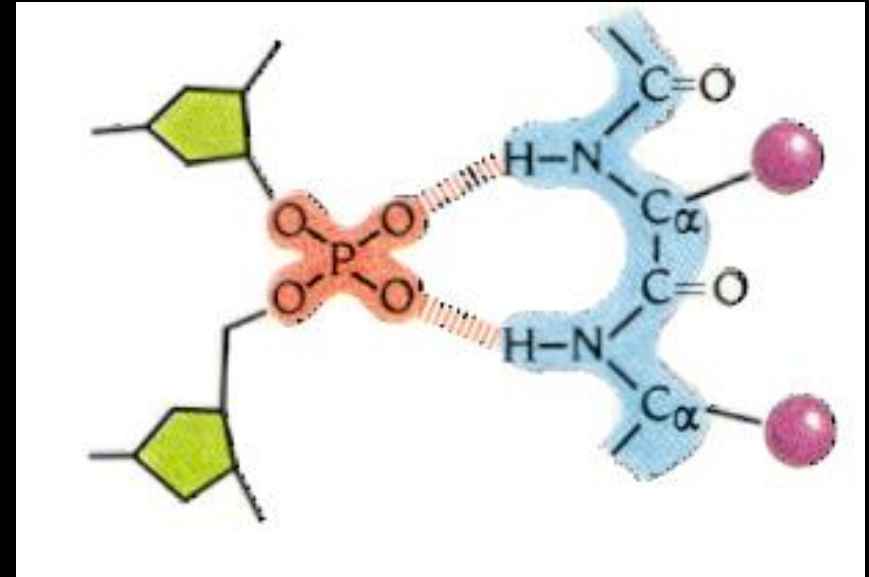
Protein recognizes DNA / RNA structure
May be sequence specific

# Why to Use Nucleic Acids?

**Nucleic acids form** complex secondary and tertiary structures **and bind with high affinity to their target proteins.**

**They can be** easily amplified **using PCR techniques.**
**DNA can be converted to RNA and RNA to DNA by in vitro transcription and reverse transcription procedures.**

**Oligonucleotide polymers are excellent for in vivo studies as they can be** chemically protected against enzymatically degradation.

**Oligonucleotides have a** low immunogenic **potential.**

Example for a biological active
RNA molecule (aptamer)
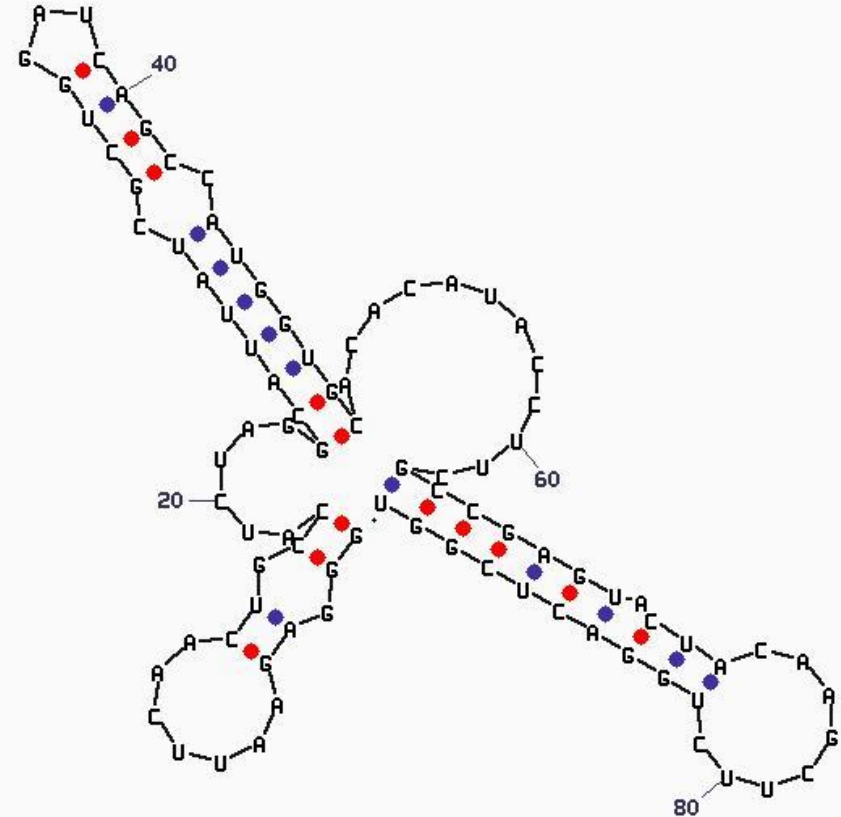


SELEX...

# SELEX

(**S**ystematic **E**volution of **L**igands by **Ex**ponential **E**nrichment)

to find an **aptamer** (optimal fitting ligand or inhibitor)

with a desired action on the target molecule

# The Original Experiment: Selection of RNA Molecules that Bind to T4 DNA-polymerase

**Known wild type sequence binding the enzyme: AAUAACUC**

**Selected sequence**   A $\begin{smallmatrix}AU\\GC\end{smallmatrix}$ AAC $\begin{smallmatrix}UC\\CU\end{smallmatrix}$   **(from a 8 nt. random sequence)**

**Sequences found:**

AAUAACUC    9/20 clones (wild type)
AGCAACCU    8/20 clones (variation)

*The binding constants of these RNAs to T4 DNA-polymerase are equivalent*

# Natural x *In vitro* Evolution
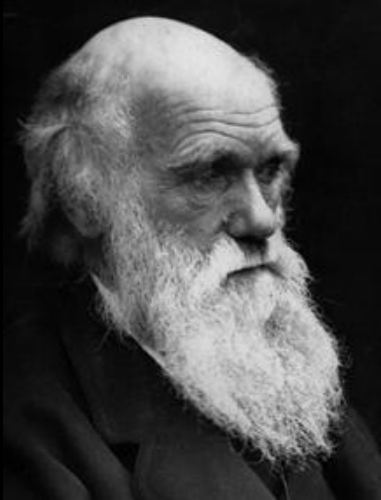

Charles R. Darwin


Albert R. Wallace

## NATURE

- ✓ Variability in natural populations
- ✓ Natural selection
- ✓ Propagation of selected individuals
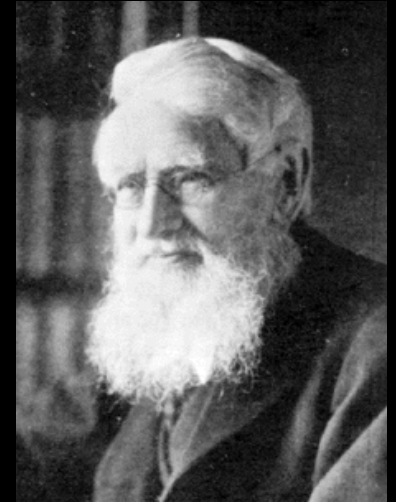
## IN A TUBE


Jack Szostak


Larry Gold

- ✓ Randomization of nucleic acids (DNA synthesizer)
  $10^{15}$ sequences and tertiary structures
- ✓ Binding to substrate / protein
- ✓ *In vitro* amplification (PCR, RT-PCR)

# Isolation of Short Oligonucleotides (Aptamers)

## *In vitro* selection (SELEX)

### Aptamer Library

$4^{40}$ possible sequences

GGGAGGACGAUGCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAGACGACUCGCCCGA

40 nucleotide random region

**An aptamer's shape is dictated by its sequence**

**An Aptamer Library = A Vast Shape Library**



Library

Repeat Process

Protein Target of Interest

Incubate with specific target

Partition Bound from Unbound RNA

Amplify Selected RNA's (RT/PCR)

**SELEX = Systematic Evolution of Ligands by Exponential Enrichment**

# *In Vitro* Selection of High-affinity DNA and RNA Ligands
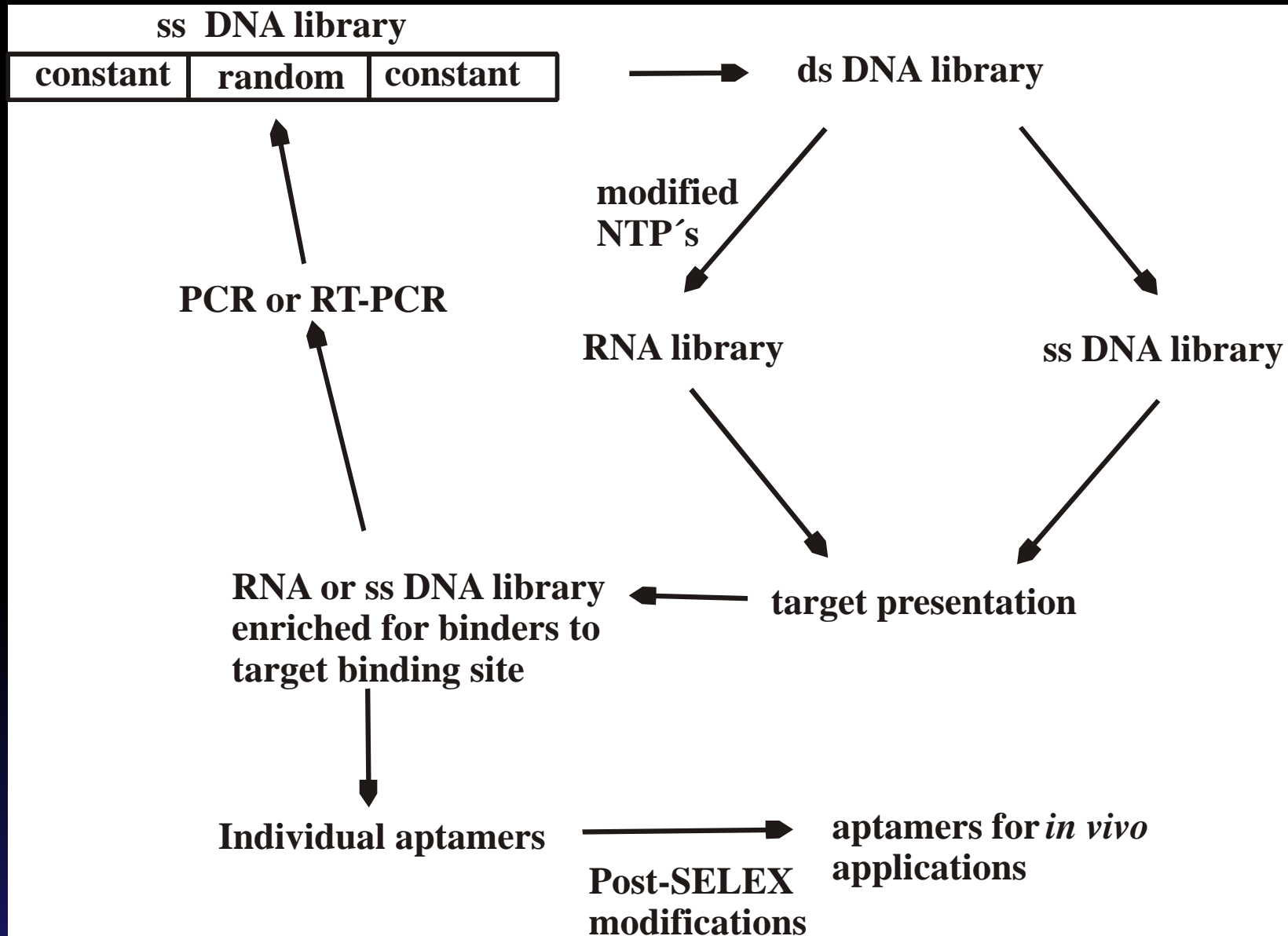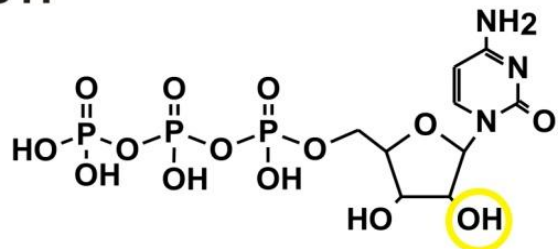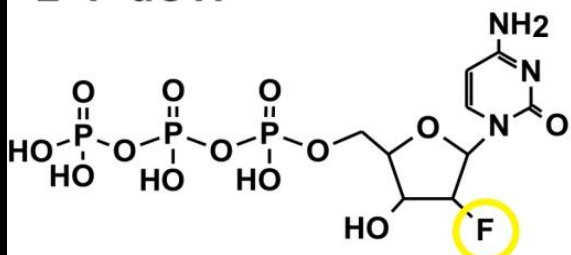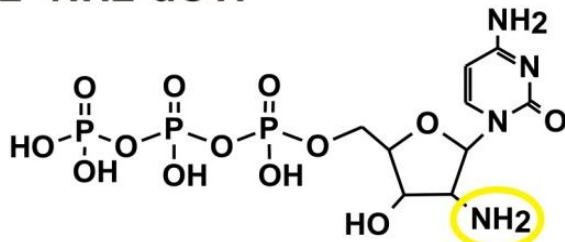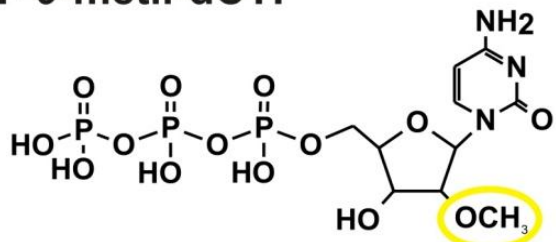
**Chemical Modification of the 2OH Position of the Ribose of pyrimidines Results in Nuclease-Resistance of the Transcripts**
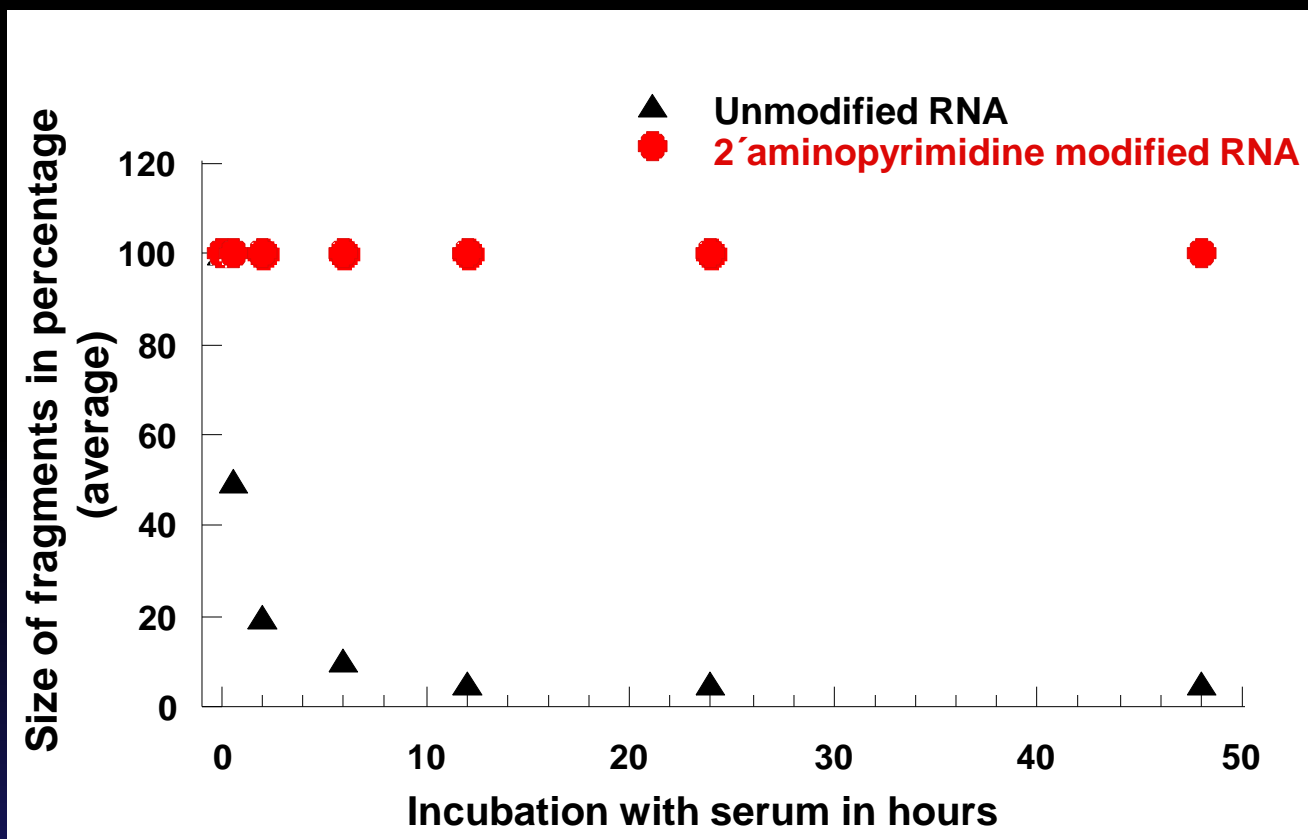
# History and Applications of SELEX

*In vitro* evolution of RNA aptamers (ligands) targeting:

Proteins naturally in contact with nucleic acids
Small molecules, organic dyes and short peptide motif

Soluble proteins that do not naturally bind nucleic acids
Substance P, VEGF, NGF, FGF, neuropeptide Y,.....

Cell surface antigens
CD4, selectins, ...

Whole organisms, cancer and stem cells, membranes and membrane-bound receptors
Membrane-bound acetylcholine receptors, erythrocyte ghosts, virus particles, African and American trypanosomes, tumor/normal vasculature, stem/differentiated cells

Modulation of intracellular function (intramers)
Inhibition of cell division during *Drosophila eye* development

Mapping of ligand-binding sites on proteins

Allosteric aptamer activation and inactivation (aptamer antidotes)

# Towards a RNA World



- •A RNA replicase made out of RNA

  - Library of $10^{15}$ different RNAs

  - Selection with substrate specific

  -  oligo affinity column



Bartel & Szostak, Science 1993

# Evolution becomes continous…

- Most in vitro experiments with ribozymes in a stepwise fashion but Continous Evolution seems to be possible!
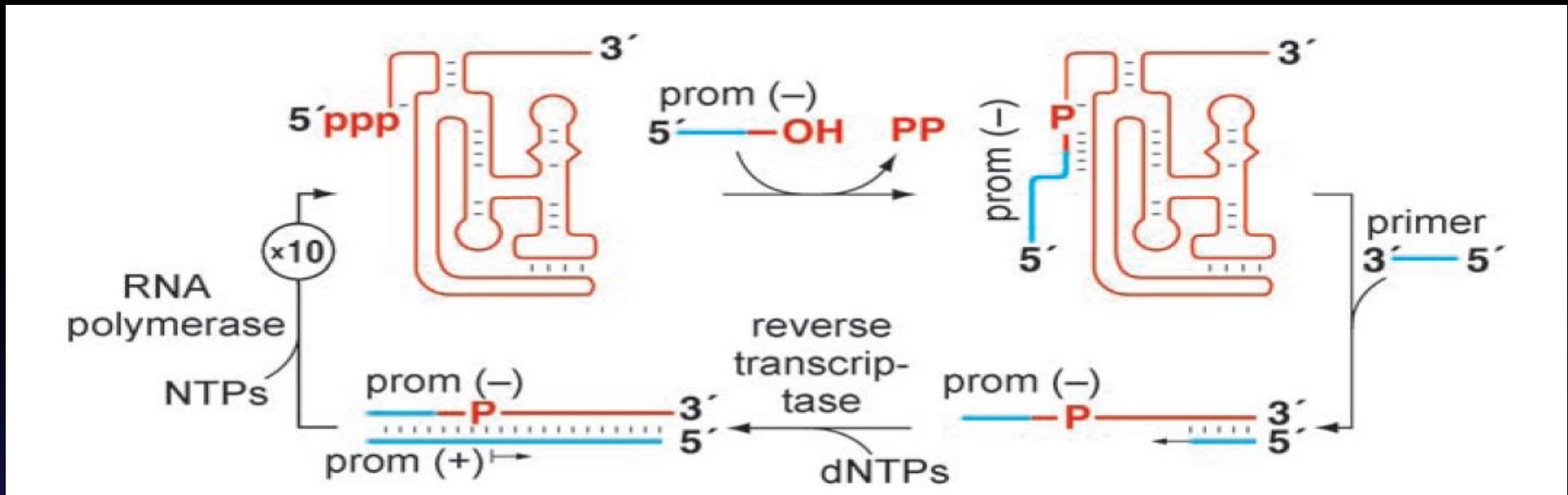
# Aptamers Towards Automatization: Robotic Selection
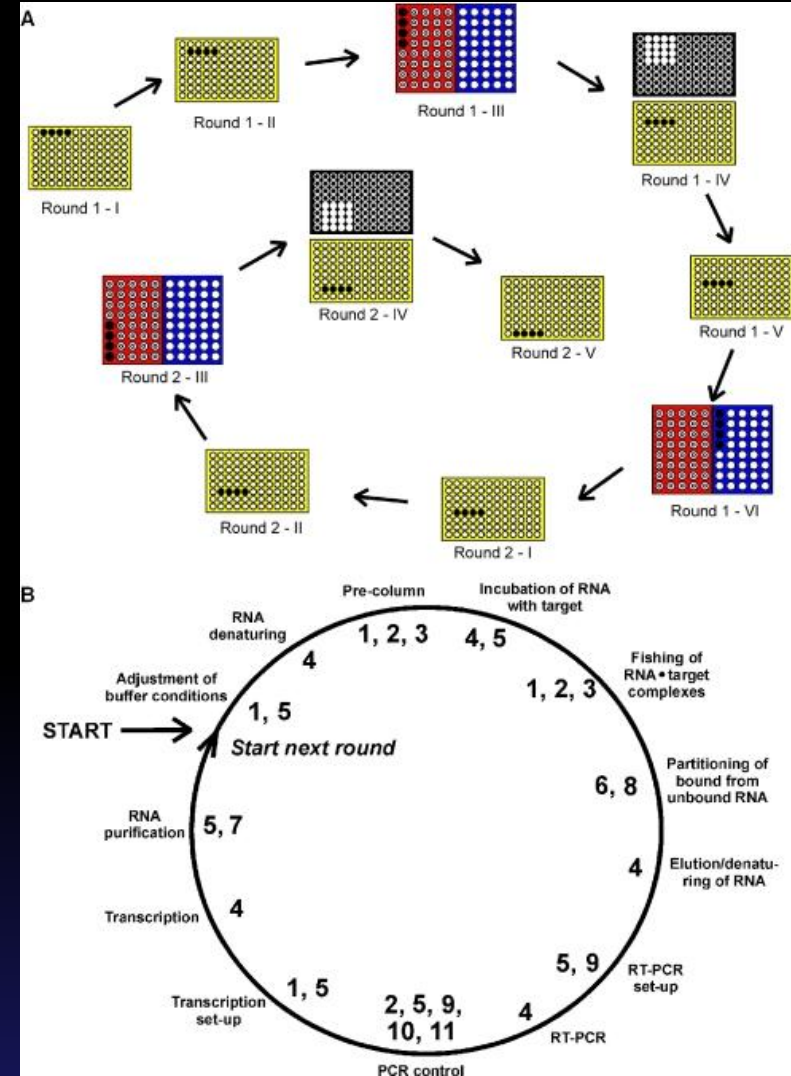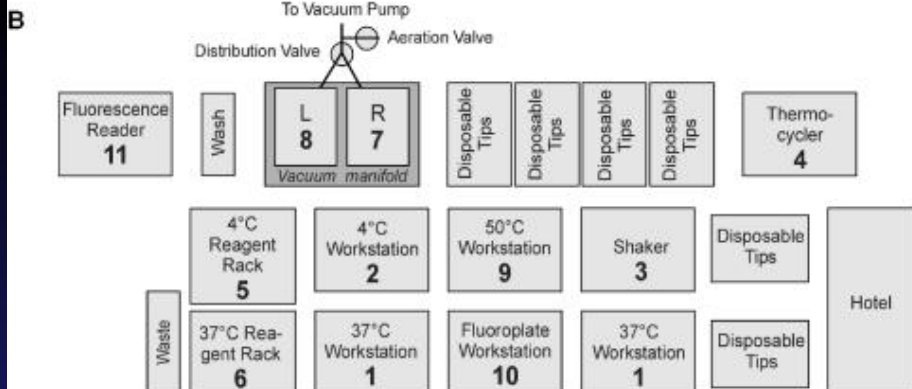
# Aptamers Binding to the Nicotinic Acetylcholine Receptor: Proof of Mechanism

**Hypothesis:**

**Cocaine does not sterically block the channel pore**

**Cocaine binds to a regulatory site of the inactive receptor**

**Proof:**

**The development of RNA aptamers that bind to an allosteric site of the acetylcholine receptor  and alleviate cocaine inhibition**

**The muscle-type nAChR (α2βγδ).**

**Model for studying receptor:**

- Electric organ from Torpedo

- BC$_3$H1 cells expressing the fetal muscle nAChR

Development of an aptamer against a
membrane–bound receptor, for the first time!
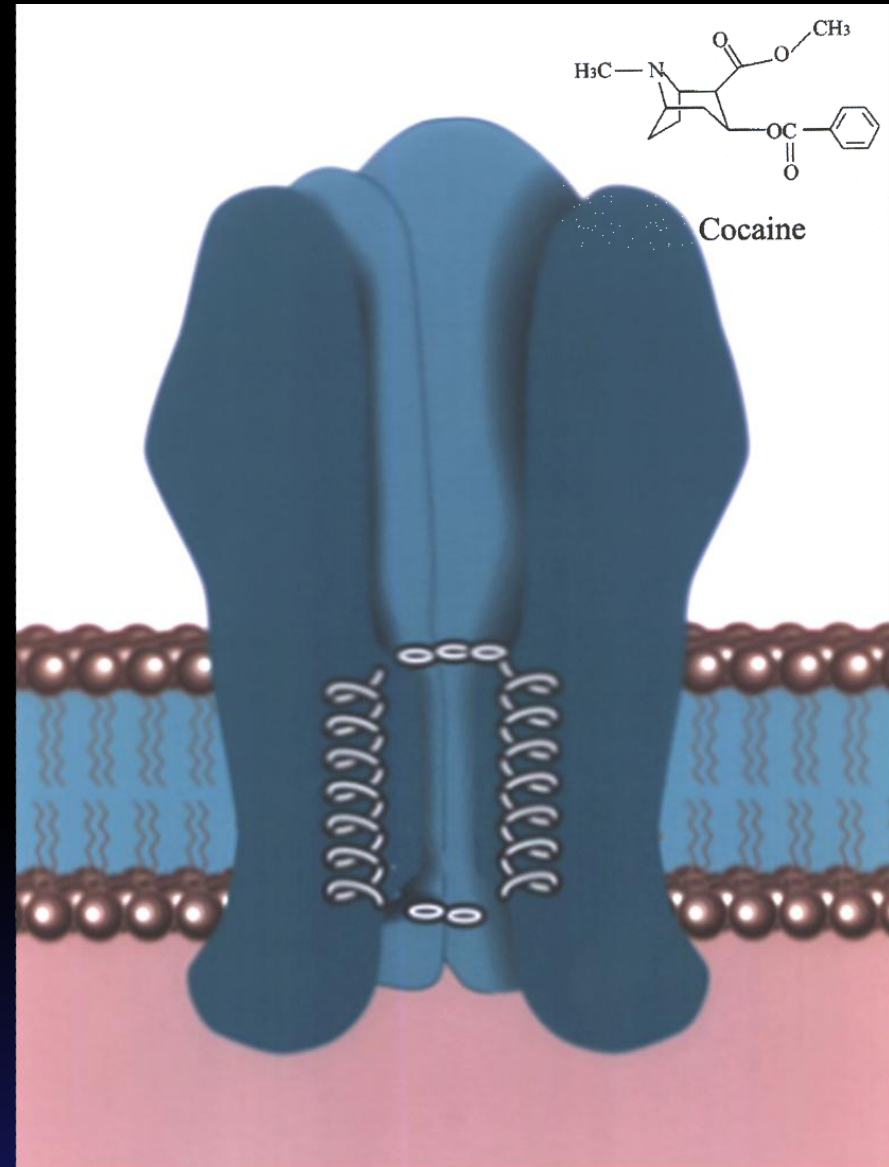


How to do it?...
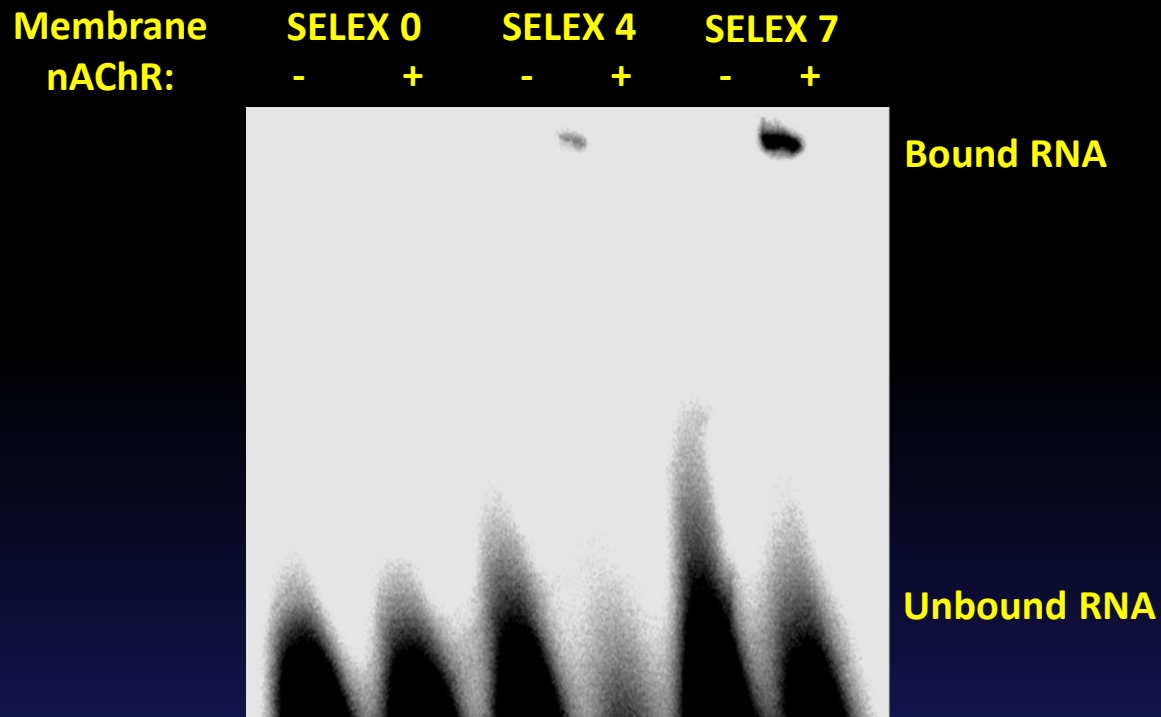
# Aptamers Containing Consensus Sequences

#01   ...ACCCCG<u>UUCACGG</u>UAGCCC...
#05   ...GUGGAA<u>UACACCG</u>ACAAG...
#06   <u>UCCACCG</u>AUCUAGA...
#07   ...CAACCA<u>GUCACCG</u>UUGCCC...
#09   ...AGUCCU<u>GUGUCCG</u>UUGAAU...
#11   ...UUGCCG<u>GCGACCG</u>CGUUCU...
#13   ...CUGGCG<u>UAGACCG</u>CGCAGA...
#14   ...CAUAGU<u>UUCGCCG</u>CUAUGC...
#16   ...GGAGCG<u>UUGACCG</u>GACCUC...
#18   ...GACUAC<u>GCACCCG</u>CUAGUC...
#19   ...UGAAUA<u>GUCACCG</u>UGAUGA...
#20, #21   ...GCAUUC<u>UUCACCG</u>GAAGUA...
#22   <u>UUCGCCG</u>CUGCAC...

Class I cocaine-displacing aptamers

#03   ...CGCUA<u>GGCUGAA</u>CAC
#08   ...GAGAUU<u>GCAGAA</u>AAACGC...
#23   ...UCCCUA<u>GCUGAC</u>GAUGGA...
#24   ...GCCGAC<u>GGUGGA</u>CCGUAC...
#26   ...ACGCCA<u>GGUGAA</u>CCCCUC...
#30   AAC<u>GCUGAA</u>UCCCCG...
#31   UA<u>CUGAA</u>UGAUCU...
#38   ...CCACCG<u>CCUGAA</u>GCUUUG
#39   ...CACCUU<u>CUUGAA</u>ACAUUU
#42   ...UGAUUG<u>CUUGAA</u>CCUCUA...

Class II cocaine-displacing aptamers

Ulrich et al., PNAS, 1998

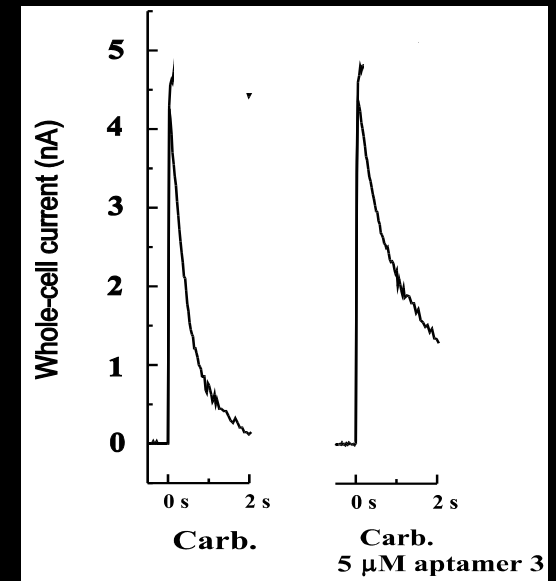# Secondary Structures of Class I and Class II RNA Aptamers
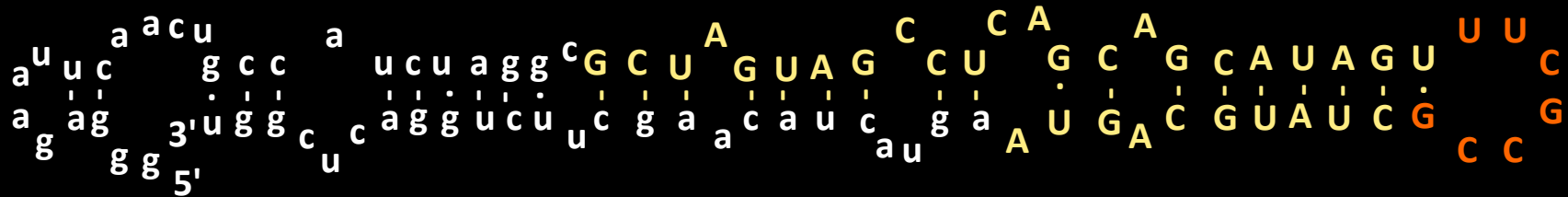
**Class I aptamer 14**    **Inhibitor binds to closed channel-form**
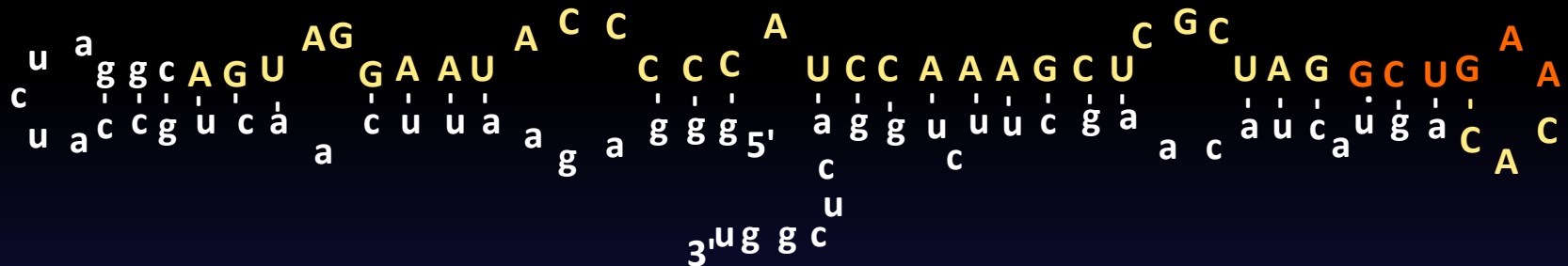
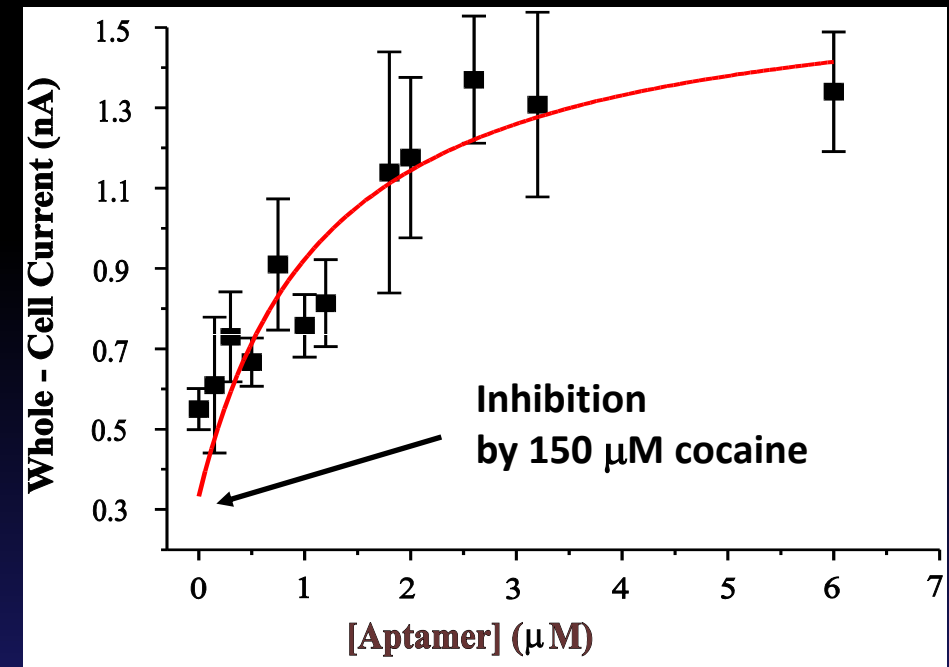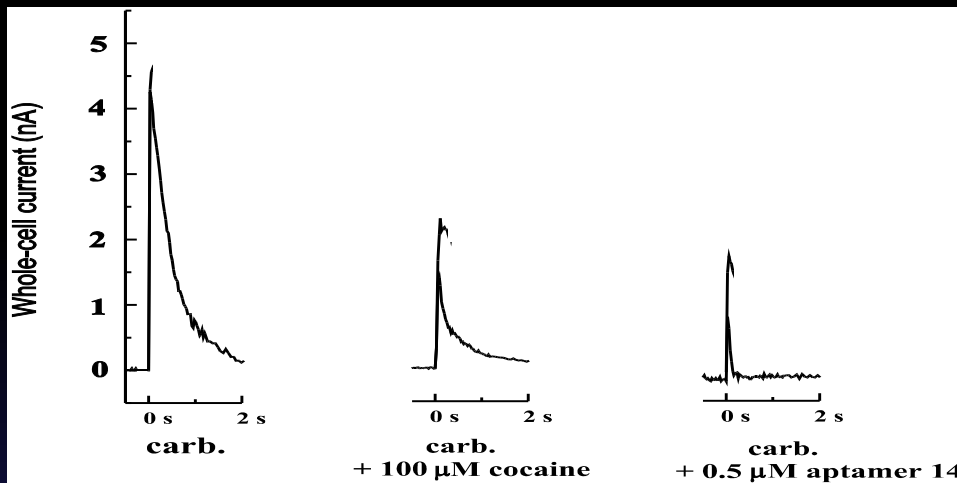**Class II aptamer 3**    **Inhibitor binds equally to open and closed channel-form**

**Ulrich et al., PNAS, 1998**
**Hess and Ulrich et al., PNAS, 2000**

# The Protector: Class II Aptamer 3 Does not Affect nAChR Function, but Displaces Cocaine from the Receptor.

## Class II Aptamer

## Class I Aptamer

# Example 2

## Development of Aptamers as Blockers of

## Host Cell Invasion by *Trypanosoma cruzi*

# Receptor-ligand Interactions between *T. cruzi* and Host-cell Surfaces are Necessary Prerequisites for Host-cell Invasion by the Parasite

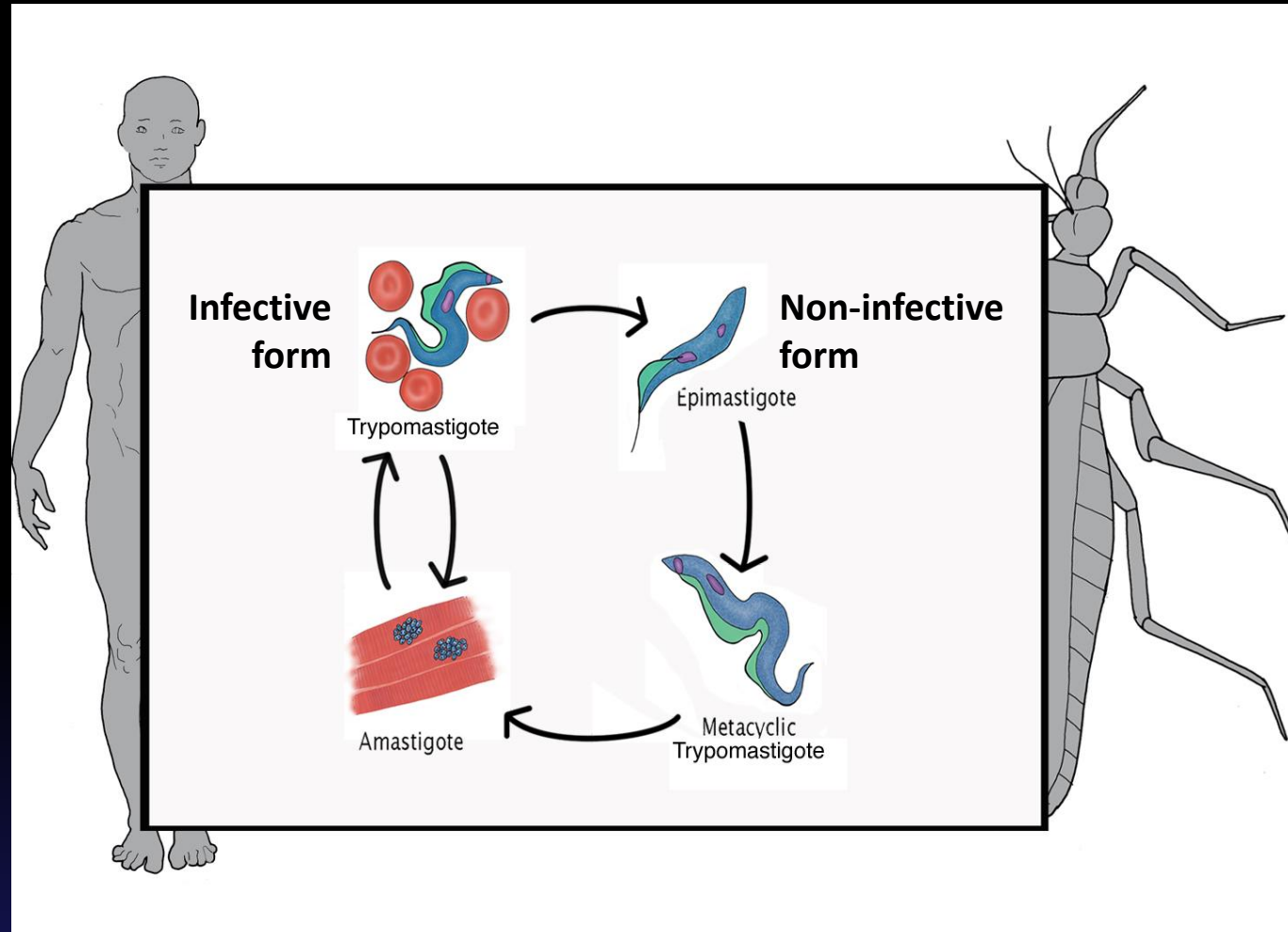**Revudid bug vector**

↓

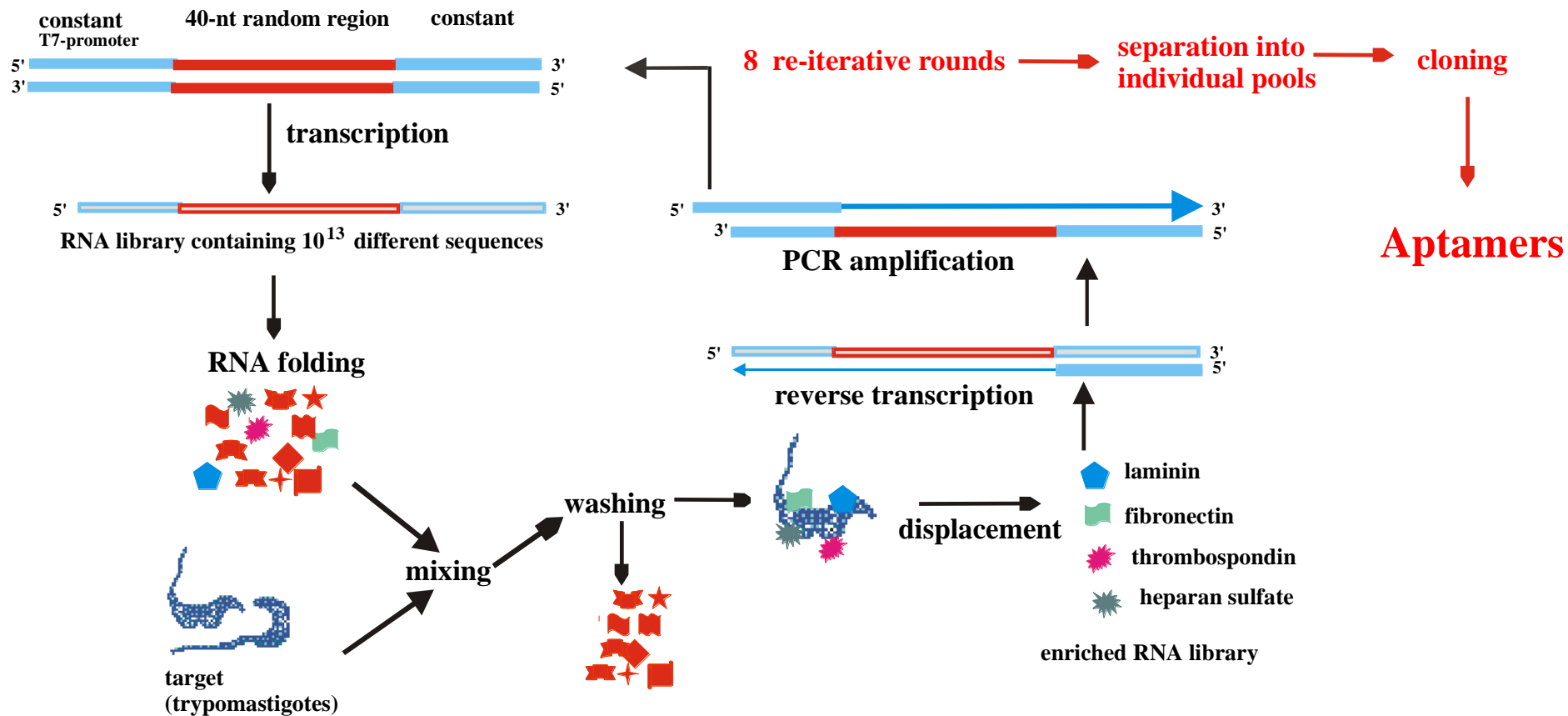**Transmission**

↓

**Invasion of mammalian host cells**

↓

**Multiplication**

↓

**Invasion of previously uninfected cells**



Infective form — Trypomastigote

Non-infective form — Épimastigote

Amastigote

Metacyclic Trypomastigote
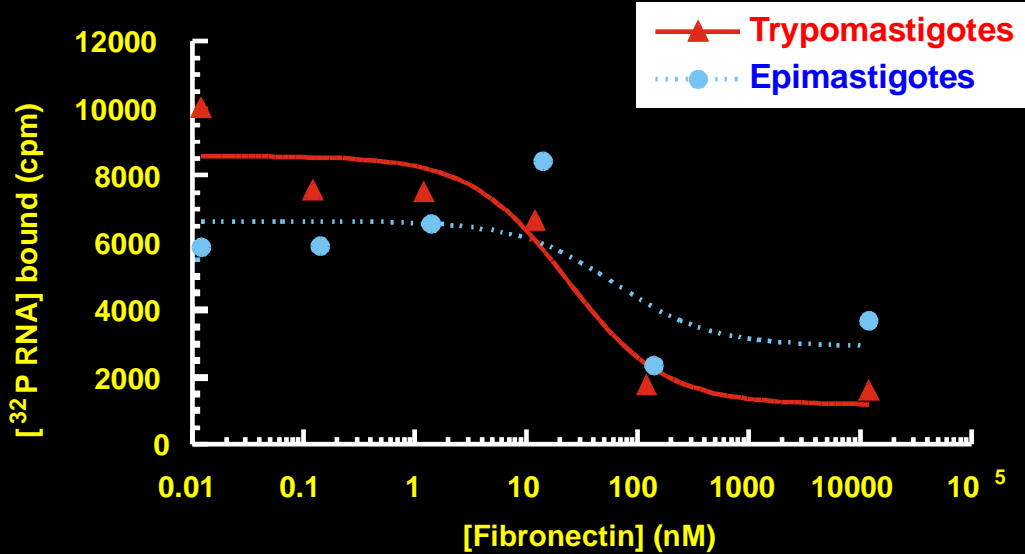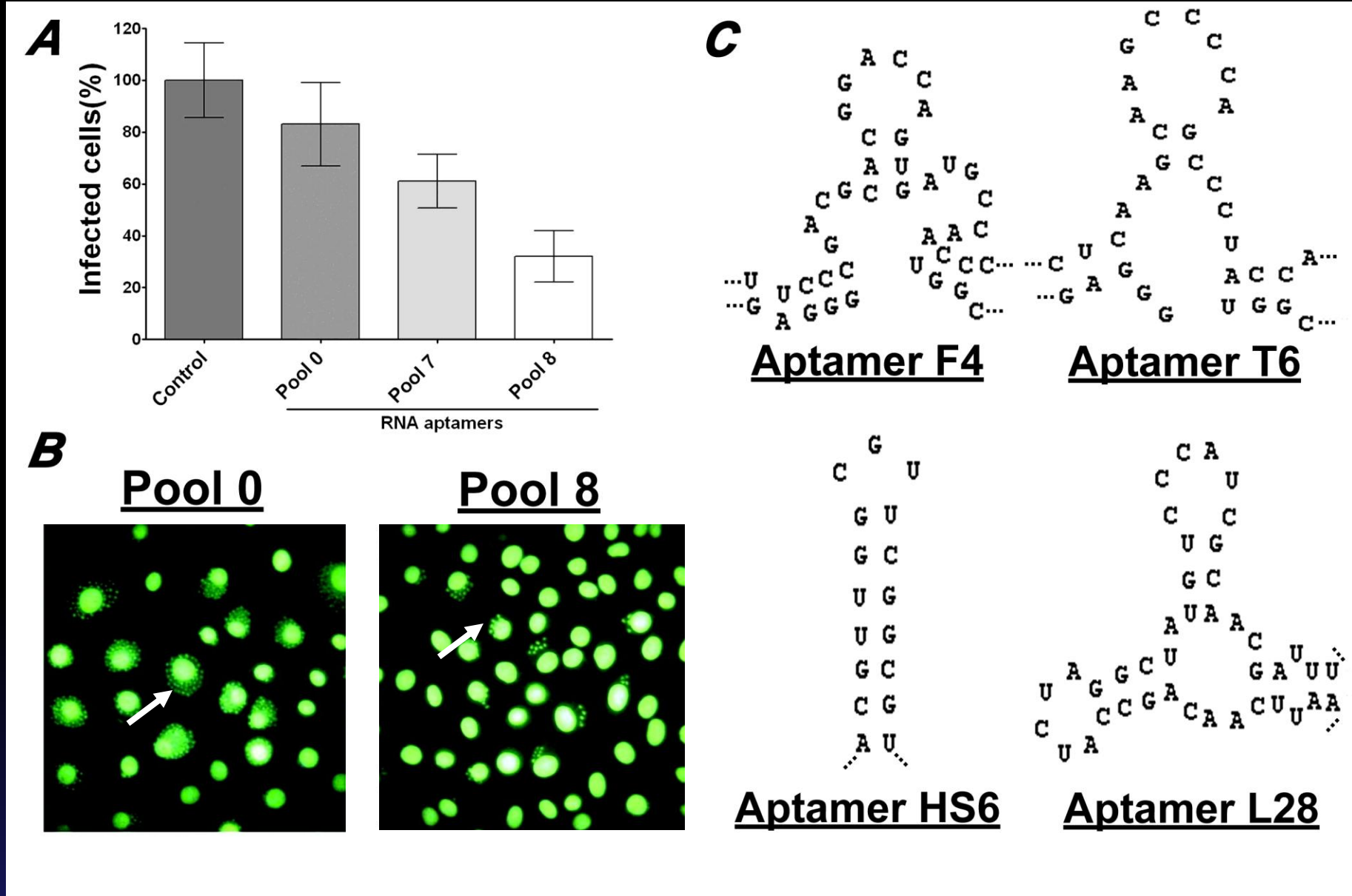
# SELEX Procedure for the Evolution of RNA Aptamers that Bind to the Receptors of Host-cell Matrix Molecules on *Trypanosoma cruzi*

# The Selected RNA Molecules Bind Mainly to their Targets on the Infective Trypomastigote Form as Shown by Displacement with Fibronectin
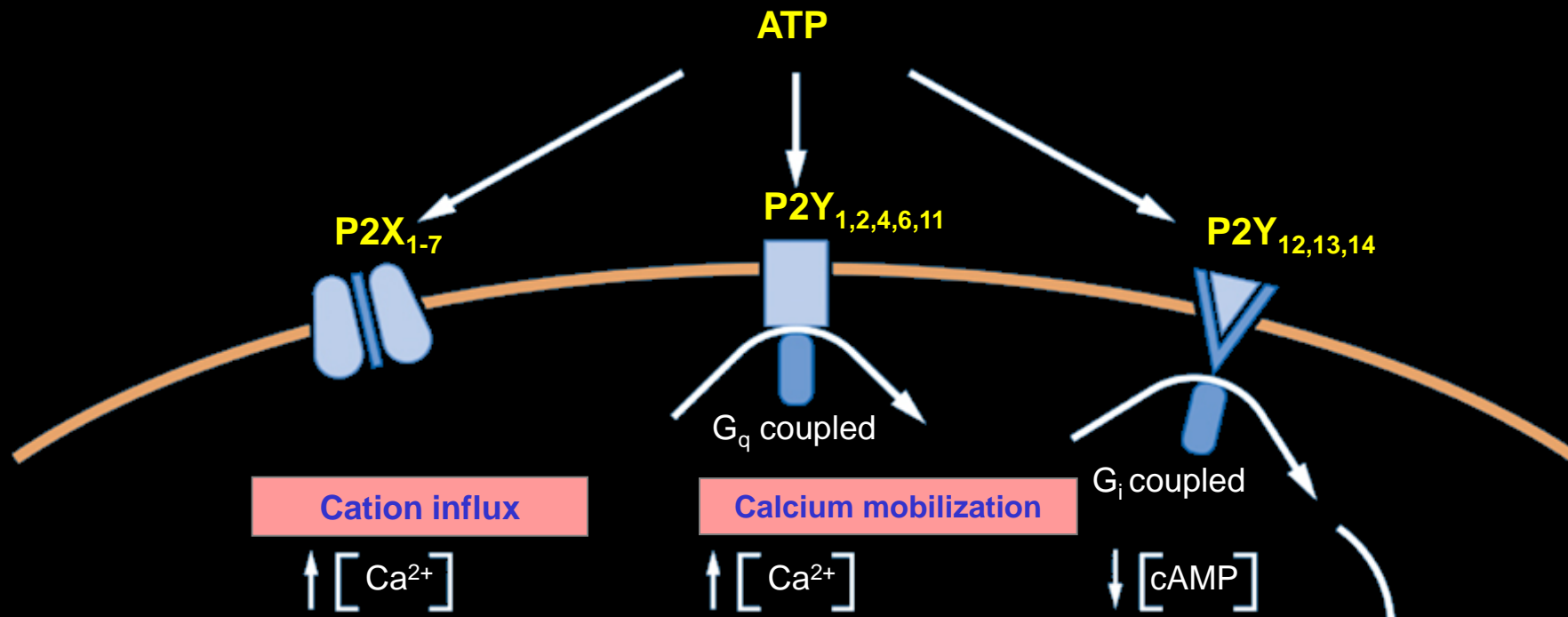


**SELEX-5 RNA**

**SELEX-7 RNA**
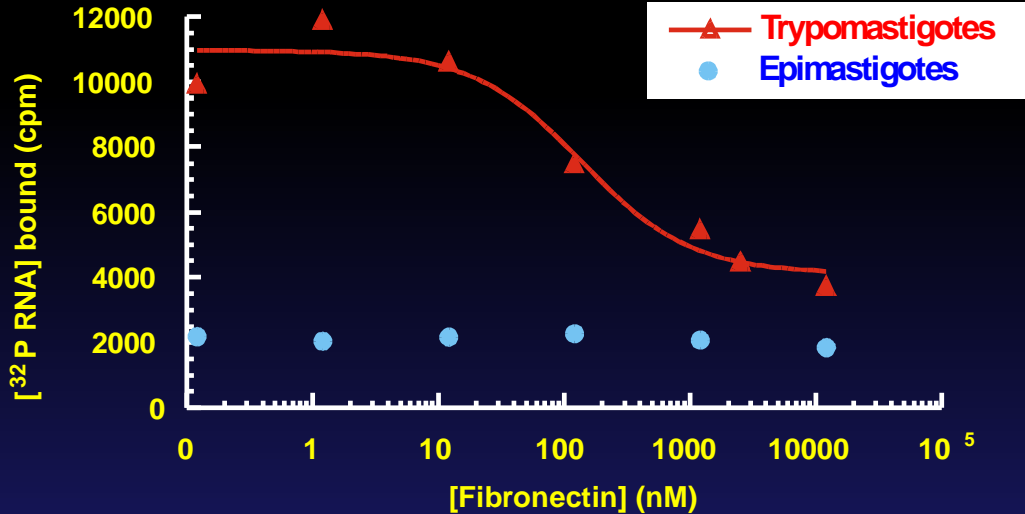
# Aptamers acting on *Trypanosoma cruzi* inhibting cell invasion

**Example 3**

**SELEX for Specific Inhibitors of the Purinergic Receptor Subtypes**

# Purinergic P2X and P2Y Receptor Activation

ATP

$P2X_{1-7}$

$P2Y_{1,2,4,6,11}$

$P2Y_{12,13,14}$

$G_q$ coupled

$G_i$ coupled

Cation influx

Calcium mobilization

$\uparrow [Ca^{2+}]$

$\uparrow [Ca^{2+}]$
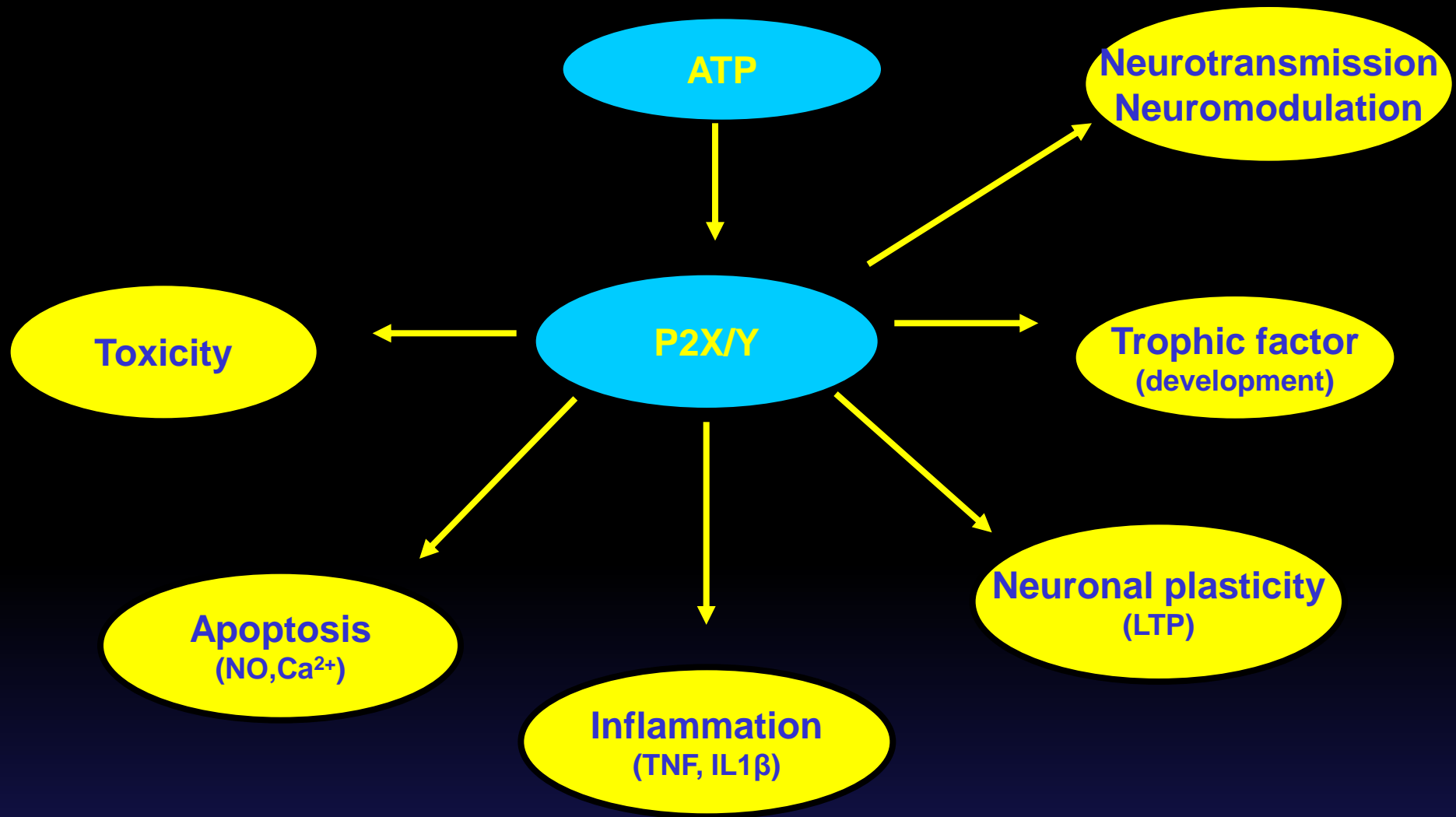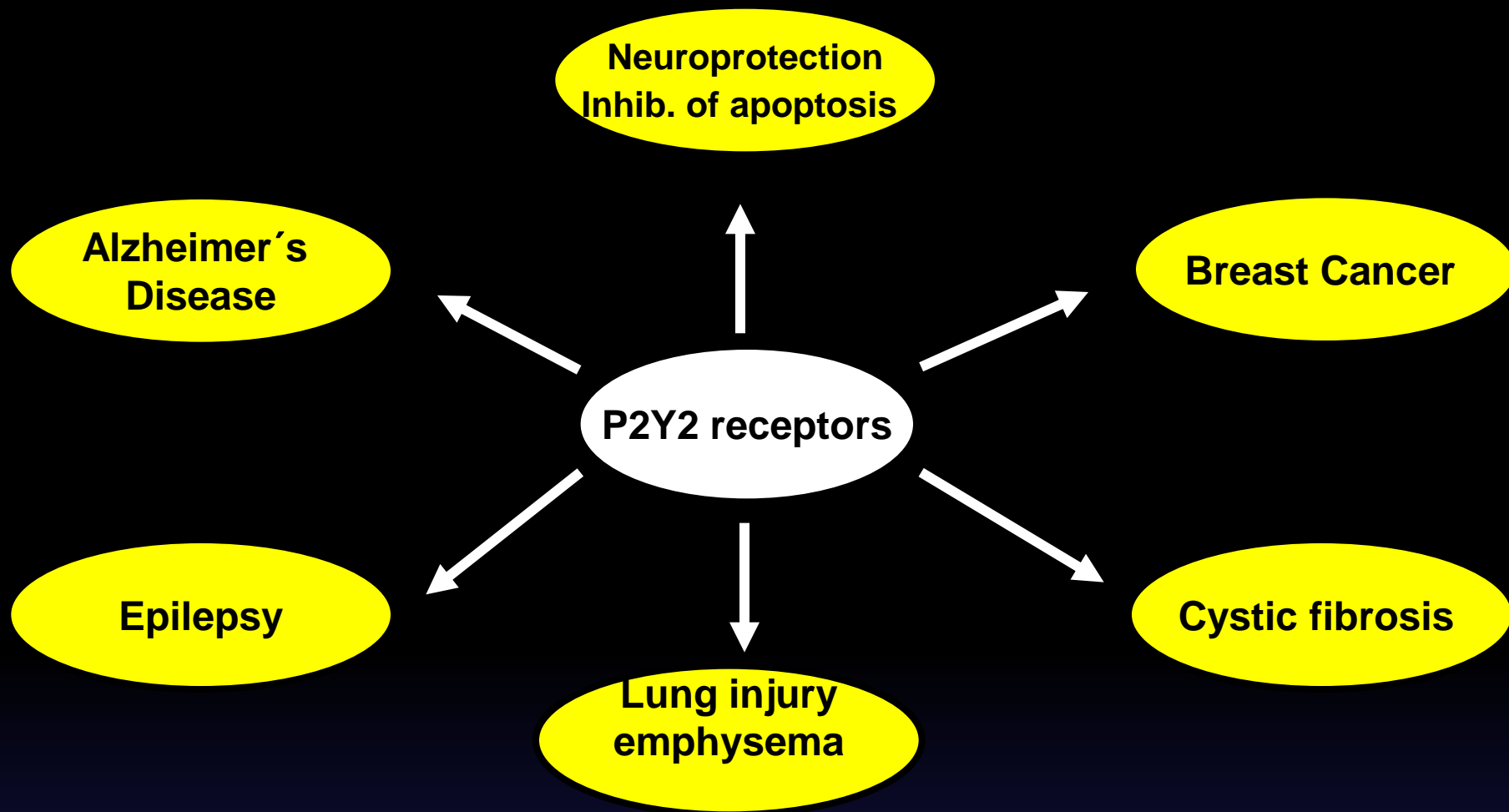
$\downarrow [cAMP]$

Subtypes $P2X_1 - P2X_7$

$P2Y_{1,2,4,6,11,12,13,14}$

Many receptor subtypes do not have specific agonists and antagonists

# Some of the ATP-induced Effects

# Aptamer Selection for P2Y$_2$ Receptors

**Aptamer Selection against HEK Cells Expressing the Recombinant Human P2Y$_2$ Receptor**
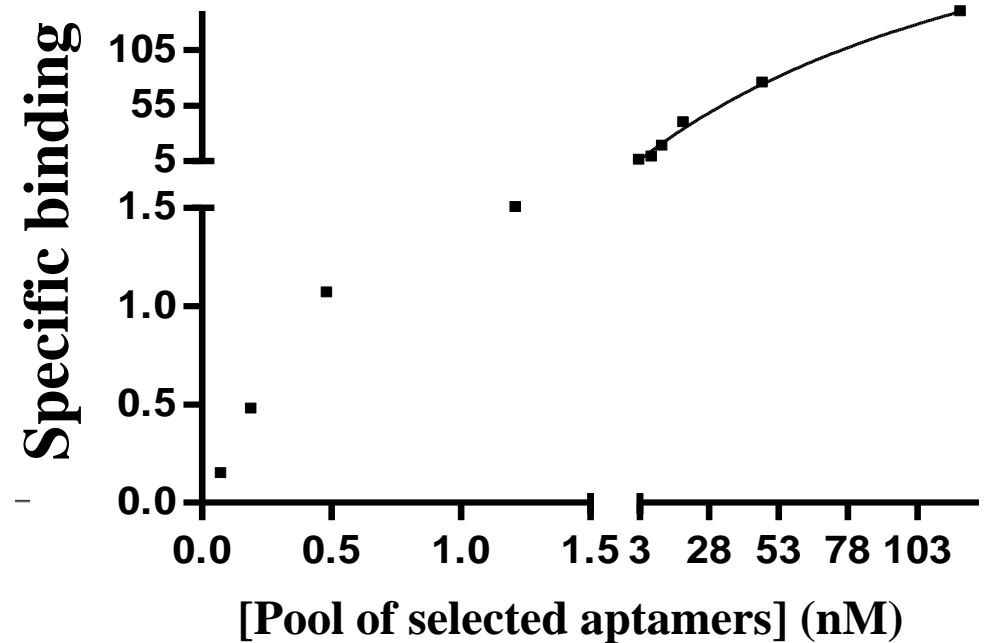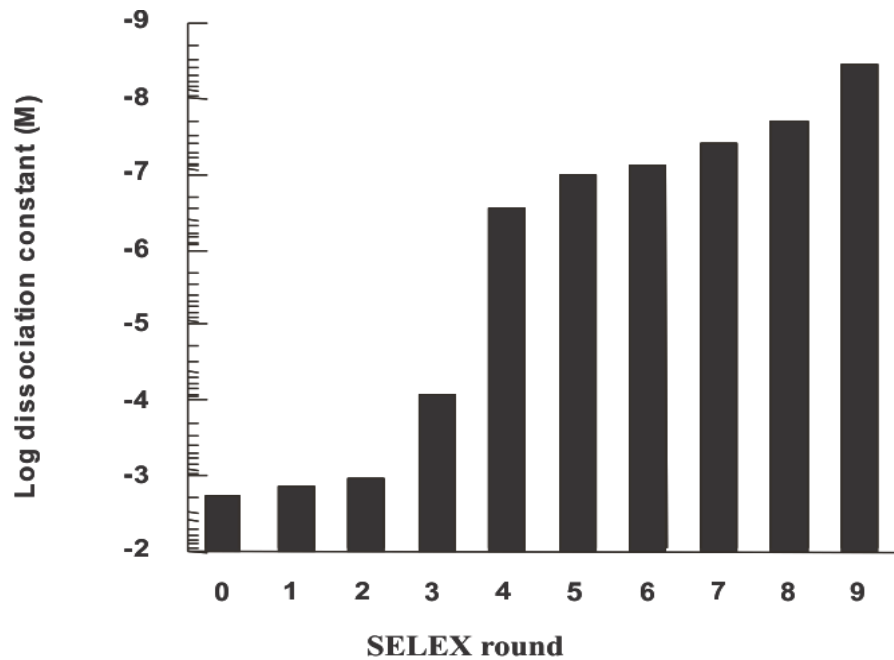
**9 x**

**Displacement of target-bound RNA molecules with γ [S]-ATP**

**Removal of Unspecific Binders by Negative Counter Selection against untransfected 1321N1 HEK Cells**

**Subtype P2Y$_2$ Receptor Specific Aptamer**

# Following Nine Cycles of SELEX, the Selected Pool Binds with Nanomolar Affinities to P2Y2 Receptors Expressed on HEK Cells

# Identified anti-P2Y₂ Aptamers have Conserved Sequence Motifs

| | |
|---|---|
| A5 | CACUGGCCUCGCUGGAACGCUCCGACUCUCGC |
| B1 | GCACUUAGUGUGCCUCGCUGGUCCACCCACAU |
| C5 | UUAAUGCCUCGCUGGAAGUCUCCACUAAUAGC |
| D7 | UGCUCGCACCUGGAUGUCCUGAUGUCUCUGGCC |
| D2 | GGCGUAACUCGCGCCUGAUGACUUGACCCUGA |
| B7,E6,G4 | GGUCGAUUGAUCCUCGCGCCUUACUCCAGGCU |
| C4 | AUUGACCUCGCCCACAACCGAUCCAUUAGGU |
| E3 | CACCUGCAAGGGCCUGGGUGUCAGUCGCUCCA |
| E4 | AGCUCUCGGUUCGCUCUCUAGCGAUUUAUUUG |
| C7 | AAGUCUGCCGGUGUUGUCUUUUCCCUAACUGA |
| A7 | GCGCUGCCUAGCGUGACAGCUUGCAUUGCGGU |
| B2 | AGUAGAUAUCGCCACGCCGCUGCUGGUCCAUC |
| D5 | AAGCCAGCUUGCUUAGACUCCUCCCUAUAUGC |
| F5,H3 | CACUCGGUGGUAGCUCGAUCCGCCCAAUUGUC |
| C2,H1 | AGUACGUCUCGAUGCACCAGUGAAUUGUCCCU |
| F4 | AAACCUAGAUCUCUGUGAGUUCUCUCCUCCUAG |
| H6 | ACCGCGGGAUGCUCACUGAGCAUCUUGUCCCA |
| C1 | CGUUAAGUUCUUACCACCCCCCUCCAUCGGUA |
| F1 | GCCCACUAAUUGGCACUGAUUGAACGCUCCGAC |
| A6 | GUUAACGCUAGUUUGGCGUUUCCCCAGUCGAA |
| G6 | AUCCUGCAGAGCCUGG UGGUAGUGUCACCUGA |

**Aptamer B7**
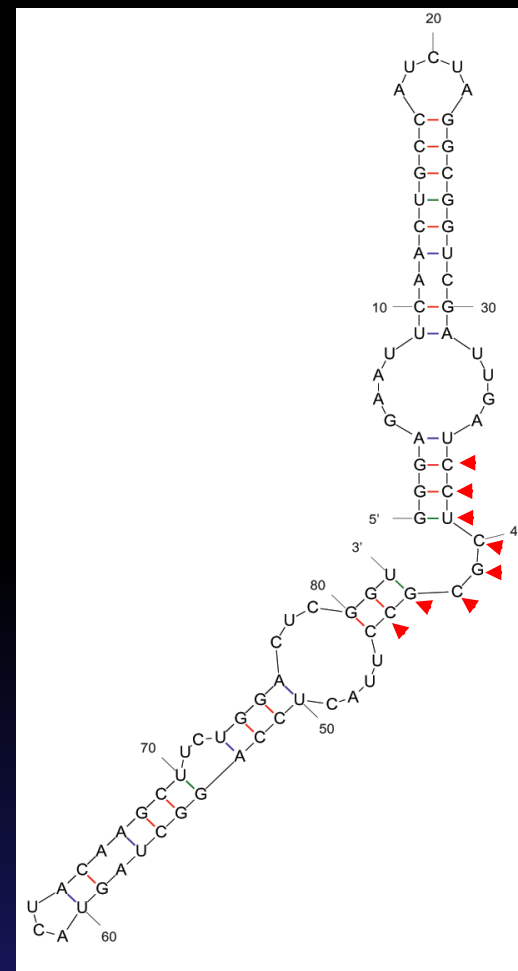


| Consensus | AGUUCACUUC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Base | A | G | U | U | C | A | C ou G | U | U | C |
| Occurrence | 14 | 16 | 13 | 12 | 9 | 6 | 11 | 12 | 15 | 14 |

# Selected aptamer B7 Preferentially Binds to P2Y2 Receptors

**1321N1-P2Y2**

$R^2$ : 0,94
$K_d$: 187,5± 1,3 nM

**1321N1-P2Y1**

$R^2$ : 0,66
$K_d$: 3,3± 2,6 µM

**1321N1-P2Y4**

$R^2$ : 0,82
$K_d$: 9,3 ± 2,6 µM
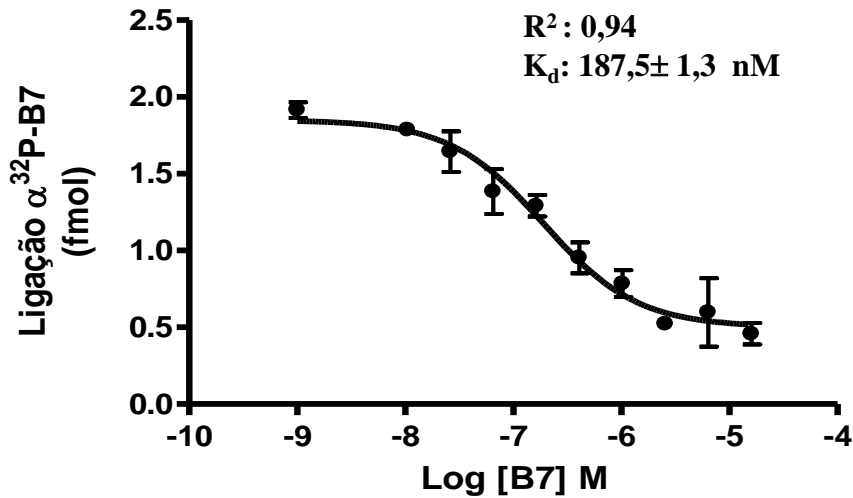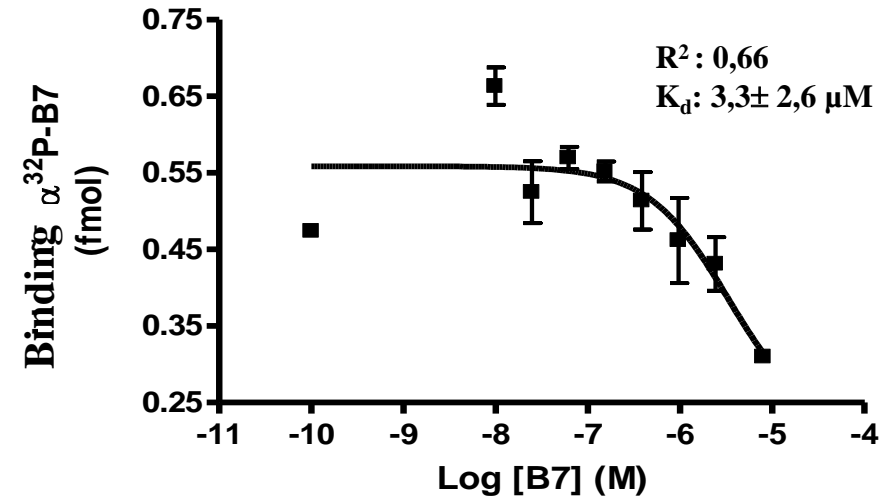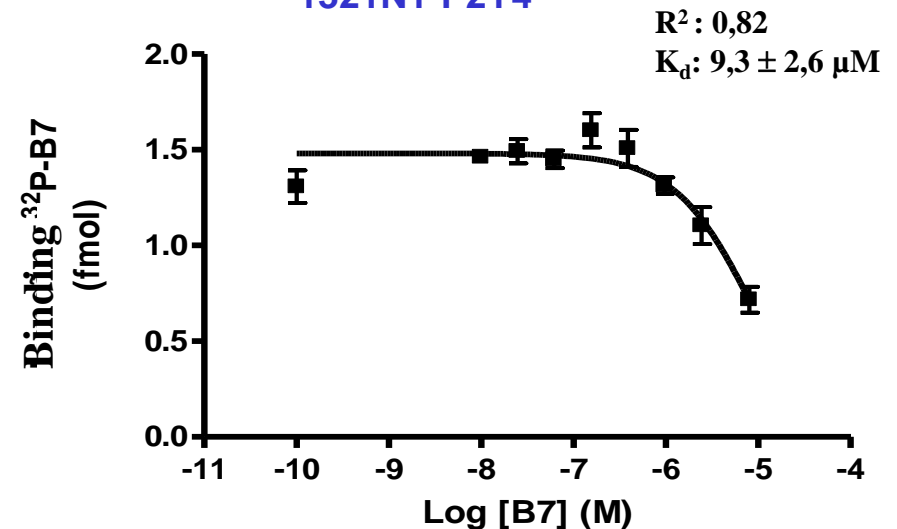
**Affinity to P2Y2 receptors is 20-50 times higher than to P2Y1 and P2Y4 receptors**

# Aptamers Abolish P2Y$_2$ R-mediated Protection of Embryonal Carcinoma Cells Against Apoptosis
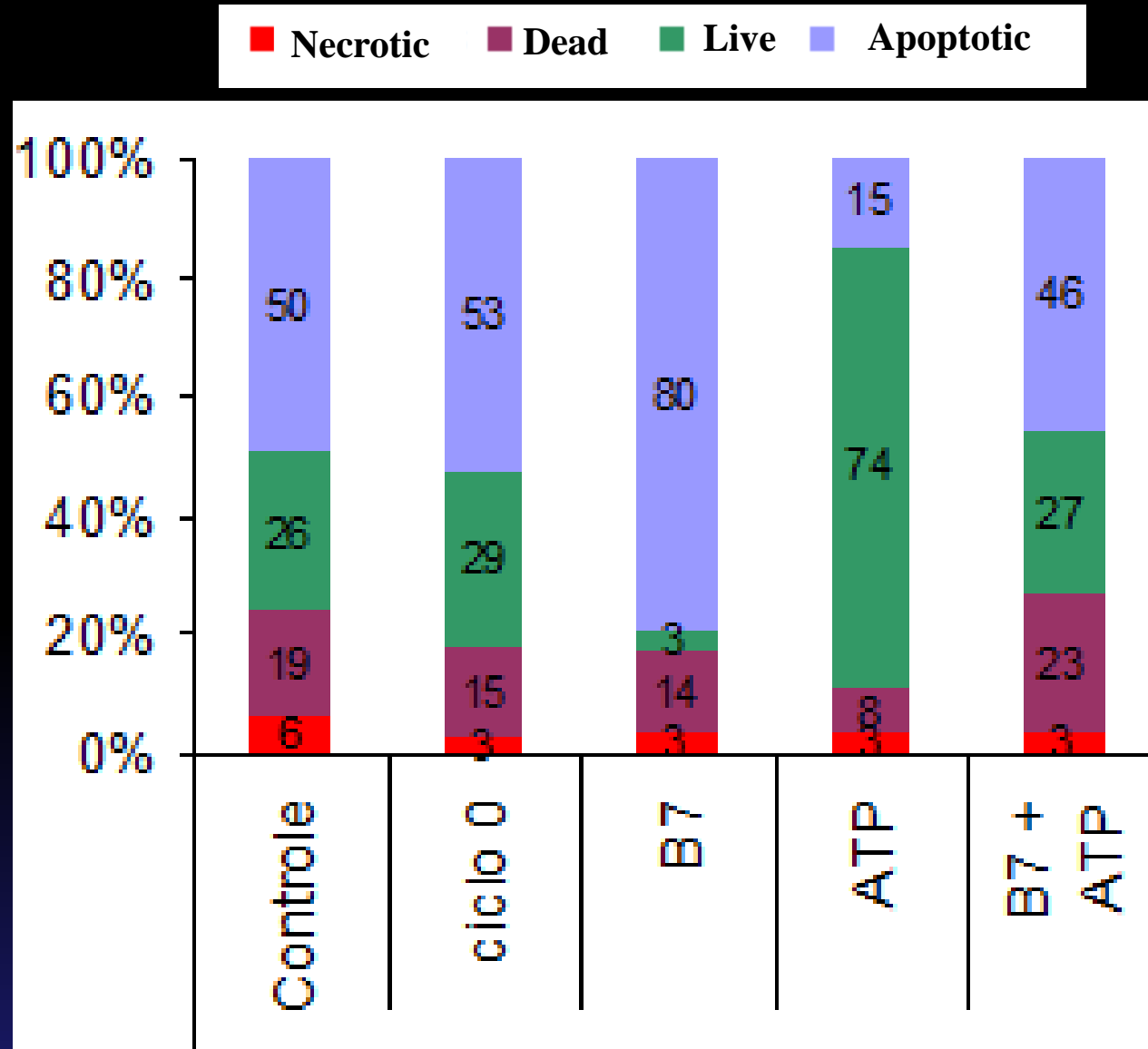
# Conclusions:

1. The SELEX technique has been used for the isolation of aptamers either inhibit nicotinic acetylcholine receptors or protect them against inhibition.

2. Aptamers were selected as inhibitors of cell invasion by *T. cruzi* trypomastigotes.

3. Aptamers are able to distinguish between ligand-binding sites of structurally related purinergic receptors.

Robotic, automated SELEX assays shorten the times necessary for aptamer identification and allow simultaneous selection against various target proteins. The identified aptamers came then by used in HTS.

# Whole-Cell Aptamer SELEX

**Aptamers binding to surface epitopes which are uniquely expressed by a cell-type (recognizing the molecular signature of a cell surface)**
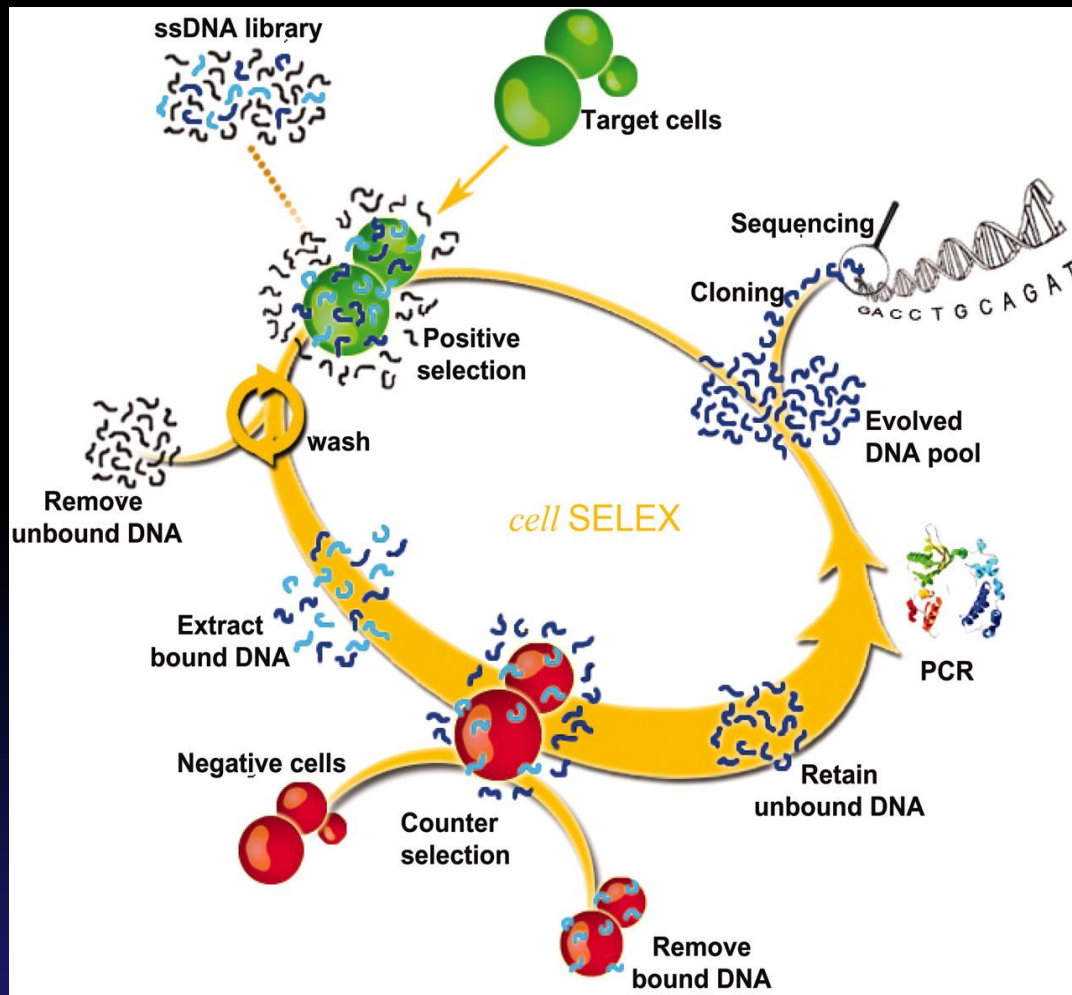


Aptamers differentiating between

- Stem cells and somatic cells

-Tissue stem cell-specific aptamers

-P. falciparum infected and healthy erythrocytes

-Aptamers as inhibitors of parasite-host cell invasion

| ALVO | $K_d$ [nM] |
|---|---|
| **HORMONES, GROWTH FACTORS** | |
| Substance P | 190 |
| Neuropeptíde Y | 30 |
| Nerve growth factor (NGF) | 500 |
| Fibroblast growth factor (b-FGF) | 1-3 |
| Platelet-derived growth factor (PDGF) | 0,05 |
| **Vascular endothelial growth factor** | **0,005-0,05** |
| Angiopoietin-2 | 3 |
| **COAGULATION-RELATED PROTEINS** | |
| Thrombin | 25 |
| Factor IXa | 0,6 |
| **CELL SURFACE ANTIGENS** | |
| L-selectin | 0,2 |
| P-selectin | 0,2-0,7 |
| Tenascin C | 150 |
| **VIRAL PROTEINS** | |
| HA1-influenza virus subunit | 0,2 |
| NS3 hepatitis C virus protein | 650 |
| REV-HIV virus protein | 76-180 |
| **RECEPTORS** | |
| Muscle nicotinic acetylcholine receptor | 3 |
| GABA Receptor | 0,4 |
| Neurotensin receptor | 0,4-19 |
| Purinergic P2Y2 receptor | 168 |
| **Entire organisms** | |
| *Trypanosoma cruzi* | 40-400 |
| *Trypanosoma brucei* | 60 |

**MACUGEN**

*Pfizer*

# *In vitro* Evolution of RNA Molecules for Therapeutic Applications

| Aptamer target molecule | Possible therapeutic application | Status |
|---|---|---|
| Nucleolin | Tumor growth | Clinical tests |
| IL-23 | Autoimmune disease | |
| IgE | Allergic disease | |
| Anti-AchR-autoantibodies | Myasthenia gravis | |
| Factor IXa + antidote | Anti-coagulation | Clinical tests |
| L-selectin | Inflammation | |
| VEGF | Age-related macular disease | Approved |
| PDGF | Age-related macular disease | Clinical tests |
| HIV-1 RT, HIV ver, HIV integrase | Virus replication | Clinical tests |
| α-thrombin, activated protein C | Thrombosis | Clinical tests |
| Phosphotyrosine phosphatase | Oncogenesis, viral and cell. regulation | |
| Phospholipase A2 | ARDS, septic shock | |
| Von Willebrandt factor | Platelet activation, thrombosis | Clinical tests |
| Aptazymes | | |
| Angiozyme | Inhibition of VEGF-gene expression | Clinical tests |
| Heptazyme | Ribozyme targeting of highly conserved Hepatitis C Virus sequences | Clinical tests |

# Aptamers vs. other therapeutics

| Property | Small molecule | Aptamer | Antibody |
|---|---|---|---|
| Targets | Active site inhibitors | Extracellular targets | Extracellular targets |
| Potency | pM–µM | pM–nM | pM–nM |
| Selectivity | Relatively poor | High, **easily tuned** | High, difficult to adjust |
| Time to discovery | 6-18 mos. | 4-6 mos. | 12-18 mos. |
| Manufacture | Chemical, few steps Scalable | Chemical, many steps **Scalable** | Biologics Limited scalability |
| Cost of goods | Low | **$100-1,000 / g** | $1,000-10,000 / g |
| Molecular weight | <500 Da | 10,000 Da 50,000 Da (PEG) | 180,000 Da |
| Elimination | hours | min → days | days |
| Toxicity / immunogenicity | Varies; toxicity is a major concern | **No evidence for toxicity or immunogenicity** | Varies; immunogenicity is a major concern |
| Administration | All routes | Intravenous, intramuscular, **subcutaneous** | Intravenous |
| Shelf-life | Generally stable | Generally stable | Limited; require refrigeration |

**LAB**

**Thank you!**

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Dr. Claudiana Lameu
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