The Aptamer Technology: Powerful Tools in Basic Science, Diagnostics and Therapy

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SELEX
(Systematic Evolution of Ligands by Exponential Enrichment)
to find an aptamer (optimal fitting ligand or inhibitor)
or aptazyme (RNA enzyme)
with a desired action
on the target molecule
What is an Aptamer?

aptos: “to fit”

eres: “smallest unit of repeating structure”

Aptamers are single stranded folded oligonucleotides that bind to molecular (protein) targets with high affinity and specificity.

Mechanism of induced fit and complementarity to target-binding site.
Aptamers are antibody-like capture agents evolved *in vitro* to bind specific targets.
Isolation of Short Oligonucleotides (Aptamers)

An Aptamer Library = A Vast Shape Library

An aptamer’s shape is dictated by its sequence

An Aptamer Library = A Vast Shape Library

4⁴⁰ possible sequences

GGGAGGACGAUGCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAGACGACUCGCCCGA

40 nucleotide random region

In vitro selection (SELEX)

SELEX = Systematic Evolution of Ligands by Exponential Enrichment
Aptamer Development

1. Screening of the combinatorial library for target binders.
2. Competition for a limited number of binding sites results in identification of high-affinity aptamers
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
In Vitro Selection of High-affinity RNA Ligands
The Original Experiment: Selection of RNA Molecules that Bind to T4 DNA-polymerase
(Türk and Gold, 1990, Science 249, 505)

Known wild type sequence binding the enzyme:

\[
\text{AAUAACUC}
\]

Selected mutant seq. (from a 8 nt. random sequence)

\[
\begin{array}{c}
\text{AU} \\
\text{GC}
\end{array}
\]

Sequences found:

\[
\begin{align*}
\text{AAUAACUC} & : 9/20 \text{ clones (wild type)} \\
\text{AAUGACUC} & : 1/20 \text{ clones} \\
\text{AAUAACUU} & : 1/20 \text{ clones} \\
\text{AGCAACCU} & : 8/20 \text{ clones} \\
\text{AGCGGACCU} & : 1/20 \text{ clones}
\end{align*}
\]

Natural x *In vitro* Evolution

**NATURE**
- Variability in natural populations
- Natural selection
- Propagation of selected individuals

**IN A TUBE**
- Randomization of nucleic acids (DNA synthesizer) $10^{15}$ sequences and tertiary structures
- Binding to substrate / protein
- *In vitro* amplification (PCR, RT-PCR)
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)
Chemical Modification of the Ribose of Pyrimidines Results in Nuclease-resistant Aptamers

Ulrich et al. Cytometry A, 2004
Aptamers are easily developed for transcription factors and other soluble proteins.

However, can aptamers be developed for cell surface proteins or entire cell surfaces or organisms?
Example 1: **Aptamers Binding to the Cocaine-site of the Nicotinic Acetylcholine Receptor**

Class I aptamer 14  Displaces cocaine and inhibits receptor function

Class II aptamer 3  Displaces cocaine and protects the receptor against inhibition

Ulrich et al., PNAS, 1998
Hess, Ulrich et al., PNAS, 2000
Abused Drugs, Toxins and Compounds of Therapeutic Importance that Inhibit the Nicotinic Acetylcholine receptor (nAChR)

HOW DO THEY ACT ON THE nAChR?

CAN WE REVERT RECEPTOR INHIBITION?
The muscle-type nAChR ($\alpha_2\beta_2\gamma\delta$).

Model for studying receptor:

- Electric organ from Torpedo
- BC$_3$H1 cells expressing the fetal muscle nAChR

Adapted from N. Unwin
SELEX for Aptamers Binding to Membrane Proteins

Method 1: Gel Shift Preselection Procedure

Spot incubation mix in 5% glycerol loading buffer

Run Gel @ 10 V/cm

RNA-Receptor Complex

3% Nondenaturing Polyacrylamide Gel

Unbound RNA

Purification of eluted RNA

Incubation with receptor

Immobilize receptor-aptamer complex on nitrocellulose

Cut nitrocellulose strip containing Receptor-bound RNA and incubate in cocaine-solution

Amplify eluted cocaine-displaceable RNA and proceed with gel-shift selection
## Gel-Shift Comparison of SELEX Pools

<table>
<thead>
<tr>
<th>Membrane nAChR:</th>
<th>SELEX 0</th>
<th>SELEX 4</th>
<th>SELEX 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Bound RNA**

**Unbound RNA**

Ulrich et al., PNAS 95, 14051, 1998
Reiterative SELEX Cycles Result in Nanomolar Binding Affinity of selected RNA molecules
Aptamers Containing Consensus Sequences

Class I cocaine-displacing aptamers

Class II cocaine-displacing aptamers

Ulrich et al., PNAS 95, 14051, 1998
Displacement of Class I and II Aptamers by Cocaine

Class I aptamers 14
$K_D^{(Apt.)} = 2 \text{ nM}$

Class II aptamer 3
$K_D^{(Apt.)} = 12 \text{ nM}$

Ulrich et al., PNAS 95, 14051, 1998
PART 1: PROOF OF PRINCIPLE

Secondary Structures of Class I and Class II RNA Aptamers

Class I aptamer 14

Class II aptamer 3

Inhibitor binds to inactive receptor

Inhibitor binds equally to both active and inactive receptors

Ulrich et al., PNAS 95, 14051, 1998
Hess; Ulrich et al., PNAS 97, 13895, 2000
The Protector: **Class II Aptamer 3 Does not Affect nAChR Function, but Displaces Cocaine from the Receptor.**

Inhibition by 150 \( \mu \text{M} \) cocaine

-60 mV, pH 7.4, 22°C

Hess; Ulrich et al., PNAS 97, 13895, 2000

Ulrich et al., PNAS 95, 14051, 1998
Example 2: Development of Aptamers as Blockers of Host Cell Invasion by *Trypanosoma cruzi*
Chagas’ Disease in Brazil and Latin America

<table>
<thead>
<tr>
<th>Exposed population</th>
<th>Infected population</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At risk</strong></td>
<td><strong>% of total population</strong></td>
</tr>
<tr>
<td><strong>In Brazil</strong></td>
<td></td>
</tr>
<tr>
<td>41,000,000</td>
<td>32%</td>
</tr>
<tr>
<td><strong>In Latin America</strong></td>
<td></td>
</tr>
<tr>
<td>90,000,000</td>
<td></td>
</tr>
</tbody>
</table>
Receptor-ligand Interactions between *T. cruzi* and Host-cell Surfaces are Necessary Prerequisites for Host-cell Invasion by the Parasite

1. Midgut of revuidid-bug vector
2. Transmission
3. Invasion of mammalian host cells
4. Multiplication
5. Invasion of previously uninfected cells
Host Cell-matrix Molecules Bind with High Affinity to their Receptors on *T. cruzi* Trypomastigotes

**Laminin**

- $K_d = 9.4$ nM

**Thrombospondin**

- $K_d = 83$ nM

**Fibronectin**

- $K_d = 108$ nM
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
Displacement of the SELEX 7 RNA Pool from Trypomastigote Cell Surfaces by Host Cell-matrix Molecules

[Diagram showing the displacement of labeled RNA from cell surfaces in the presence of different competitors.]

- Absence of competitor
- Laminin (250 nM)
- Heparan-sulfate (5 μM)
- Fibronectin (250 nM)
- Thrombospondin (83 nM)

[32P-RNA] bound (%)
Consensus Sequences of Aptamers Binding to *T. cruzi* surfaces

**Family I: Fibronectin-displaceable aptamers**

01
5' GACUAGCAGGCUGACAAAGUUCCUGCCUGAAAUU 3'

04
5' GACCUUCCCGCAGCCAGGCAUGUAGGCAUCCACG 3'

09
5' ACAUGGAACUAACCAACGUGCAGUAGGCAUCCCGC 3'

10
5' CCGACACUCCUGCAUCCACCCUUCUACCCAGCA 3'

**Family II: Throbospondin-displaceable aptamers**

03
5' CAAAACGGUGGCGGGCAUUAUU 3'

05
5' CGCCAUAAACGGCGUGGCAGCGC 3'

06
5' UACUCAAGCGCGUUGCCGCAUGUUGCCG 3'

08
5' AAAGGCGACGCAGCCGCGGCAUUAUU 3'

09
5' CACACUCCUUUGGCAGCGCAGCGGCAUUAUU 3'

10
5' UCCGCACAGCUGCAGACGAGCCG 3'

18
3' CGCAUGCGAAGCGUGUCCGACGCG 5'

19
5' AAAGGCGACGCAGCCGCGGCAUUAUU 3'

**Family III: Heparan sulfate displaceable aptamers**

03
5' AUCAUCAACGGUCGGCAUCGGGCAUCCGUC 3'

06
5' GGGGCGUUGGCGGUUGCCGCAUUGGUACG 3'

07
5' CAAAAAGUCGCUGUCCCGAGCUUGGUG 3'

08
5' UCGCUAGCAGCGACGAGCCGCAUUAUU 3'

09
5' AGCCUCUGUCGCGGCAUUAUU 3'

13
5' UGGCCGUAAGCGGCAUUAUU 3'

15
5' UGUCGCAAGCGGCAUUAUU 3'

19
3' AGUCCUUAAGCGGCAUUAUU 3'

21
3' ACAGCGGCAAGCGGCAUUAUU 3'

22
3' GCCGCGCGGGGCAUUAUU 3'

24
5' ACCGCGGCAAGCGGCAUUAUU 3'

26
3' CACCCUCUAAACGGGCACGCAUUAUU 3'

27
5' AGUCCUUAAGCGGCAUUAUU 3'

**Family IV: Laminin-displaceable aptamers**

01
5' GGCCCAUAACGUGCAUAACUACGUAUCCCAUCCAGGUGUUCCCACG 3'

05
5' CGGAGGACAGCAGGCAUCCAAACGUGCAGUGAUUCUUCCCACG 3'

06
5' CGGAGGACAGCAGGCAUCCAAACGUGCAGUGAUUCUUCCCACG 3'

06
3' CGGAGGACAGCAGGCAUCCAAACGUGCAGUGAUUCUUCCCACG 5'

16
3' CGGAGGACAGCAGGCAUCCAAACGUGCAGUGAUUCUUCCCACG 3'

26
5' AAGGAGGACAGCAGGCAUCCAAACGUGCAGUGAUUCUUCCCACG 3'

27
5' CGGAGGACAGCAGGCAUCCAAACGUGCAGUGAUUCUUCCCACG 3'

28
3' CACCCUCUAAACGGGCACGCAUUAUU 3'

38
3' UCAUCCUUAACGUGGCAUCCAAACGUGCAGUGAUUCUUCCCACG 5'
Saturation Analysis of Consensus Aptamer Binding to T. cruzi Trypomastigotes

Heparan sulfate consensus

Fibronectin consensus

Thrombospondin consensus

Laminin consensus
The selected RNA Aptamers Partially Inhibit Invasion of LLC-MK₂ Monkey Kidney Cells by *T. cruzi*

* p < 0.05
** p < 0.005

Ulrich et. al. J. Biol. Chem. 277, 20576, 2002
Actual projects
Aptamers as

1. Specific high-affinity inhibitors for neurotransmitter receptors

2. Stem cell specific ligands

3. Aptamers for identification of parasite proteins in malaria-infected erythrocytes

4. Ligands for tumor cells (lung cancer and glioblastoma)
Aptamers as Specific Inhibitors of P2Y2 Receptors
Purinergic P2X and P2Y Receptor Activation

ATP

P2X1-7

Gq coupled

Ca2+

Cation influx

P2Y1,2,4,6,11

Ca2+

Calcium mobilization

P2Y12,13,14

Gi coupled

cAMP

Subtypes P2X1 – P2X7

P2Y1,2,4,6,11,12,13,14

Many receptor subtypes do not have specific agonists and antagonists
Aptamer Selection for P2Y₂ Receptors

Aptamer Selection against HEK Cells Expressing the Recombinant Human P2Y₂ Receptor

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

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

Subtype P2Y₂ Receptor Specific Aptamer
Identified anti-P2Y$_2$ Aptamers have Conserved Sequence Motifs

<table>
<thead>
<tr>
<th>Consensus</th>
<th>AGUUCACUUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>Base</td>
<td>A  G  U  U  C  A  C ou G  U  U  C</td>
</tr>
<tr>
<td>Occurrence</td>
<td>14 16 13 12 9 6 11 12 15 14</td>
</tr>
</tbody>
</table>

Patent application INPI 1106312-2
Selected aptamer B7 Preferentially Binds to P2Y2 Receptors

1321N1-P2Y2

$R^2: 0.94$

$K_d: 187.5 \pm 1.3 \text{ nM}$

Affinity to P2Y2 receptors is 20-50 times higher than to P2Y1 and P2Y4 receptors

1321N1-P2Y1

$R^2: 0.66$

$K_d: 3.3 \pm 2.6 \mu M$

1321N1-P2Y4

$R^2: 0.82$

$K_d: 9.3 \pm 2.6 \mu M$
Aptamers Abolish P2Y$_2$ R-mediated Protection of Embryonal Carcinoma Cells Against Apoptosis
Aptamers for Isolation of Adipose-derived Mesenchymal Stem Cells
Isolation of Stem Cells Based on Marker Expression Profiles

- Multipotent Stem Cell
- Neuronal
- Skin
- Smooth/Skeletal/Cardiac Muscle
- Bone/Cartilage/Fat tissue
- Blood
- Gastrointestinal tract
- Respiratory tract
- Endocrine glands

Usage of hMSC (ADSC or BMSC)

Cell Sorting

Cytometry (using specific antigens)

Basic Research

hMSC (ADSC)

CD29+ (97%)

CD90+ (86%)

Nery et al. Cytometry A 2013
Aptamer SELEX for human stem cell specific ligands

Cell-type specific aptamers
Three initial cycles of stem cell SELEX showed enrichment of stem-cell binders in the SELEX library.

Various cycles of SELEX and substraction of binders to both stem and non-stem cells.

Identification of stem-cell specific aptamers.
Biotin modification and separation of stem cells from cell mixture using magnetic bead –coupled streptavidin.
Aptamers for Recognition of Mesenchymal Stem Cells in Adipose Tissue
Aptamers can Substitute CD29+/CD90+ MSC Labeling

CD29

CD90

Apt+

R12

Br5

Cells

46% Apt+ and CD29+ and CD90+
26.6% Apt+ and CD29+
10.7% Apt+ and CD90+
16.7% Apt+

38.5% Apt+ and CD29+ and CD90+
18.5% Apt+ and CD29+
20.7% Apt+ and CD90+
22.3% Apt+
Aptamers Purify Human Adipose Stem Cells

Stem cell purification with aptamers coupled to magnetic beads

No Aptamer
Br5 / R12

Washing off “non-magnetic” cells
Differentiation of Purified Adipose-MSCs into Neural Phenotypes

- NSE
- β-3-tubulin
- MAP-2
Aptamers Label subpopulations within Stem Cells

Can aptamers be used for isolation of such lineages counting for distinguished differentiation potential?
Cloned Aptamers are Screened for Novel Specific Markers of Mesenchymal Stem Cells

ADSC (adipose stem cells)
- CD31
- CD29
- CD34
- CD90 (Thy-1)
- CD105
- CD144 (Ve-Cadherin)
- CD146
- CD45
- CD117 (c-Kit)
- Sca-1
- CD34

BMSC (bone marrow stem cells)
- CD45
- CD117 (c-Kit)
- Sca-1

Novos ligantes para alvos **conhecidos**

Novos ligantes para alvos **desconhecidos**
Aptamers Label Plasmodium falciparum-infected Erythrocytes in Live Cell Imaging
### In vitro Evolution of RNA Molecules for Therapeutic Applications

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

- Increased plasma stability
- Increased affinity
- Increased potency

• Proprietary processes
• Multiple chemistries employed

early lead

- single substitutions, nucleotide A
- composites

optimized lead

- single substitutions, nucleotide B, etc
- composites

+ polyethylene glycol or nanoparticle

Target binding (nM)

- single substitutions, nucleotide A
- composites

Binding affinity measurement

- beneficial
- tolerated

P=O \rightarrow P=S
2’-deoxy \rightarrow 2’-OMe
P=O \rightarrow P-Me
2’-OMe \rightarrow 2’-deoxy
Development of an anti-VEGF Aptamer to a Therapeutics for Treatment of Age-Related Macular Eye Disease

### Aptamers vs. other therapeutics

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

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