Use of DNA Aptamers as analytical tools for anti-doping peptide (r-hGH) analysis

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WORLD ANTI-DOPING AGENCY
SYMPOSIUM ON DEVELOPMENTS & CHALLENGES IN THE DETECTION OF DOPING WITH PEPTIDE HORMONES & RELATED SUBSTANCES
Rome, Italy
15/16 June 2011
Aptamers

- DNA/RNA molecules that mimic antibodies
- Made entirely in vitro, obviate host animals
- Can produce higher affinity & specificity
- Pioneered by Gold & Ellington, early 1990's

Oligonucleotide
Position: 1 2 3 4 5... n

Sequence Diversity: 4 16 64 256 1,024 $4^n$

Random DNA Library (10^{15} Sequences)

Affinity Selection on Magnetic Beads
Displaying Immobilized Target Molecules

Heat Elute Bound DNA
PCR Amplify Rare DNA Binders (Aptamers)
Repeat Selection & PCR Amplification 5-10 Times
Clone & Sequence

Wash & Discard Unbound DNA

Ref: Bruno JG and Kiel JL
BioTechniques, 2002
Potentially Higher Affinity vs. Antibodies

Crimean-Congo Viral Envelope DNA Aptamers

Kinetic Analysis of CCHF 3 Peptide with Immobilized C3 – 6R Aptamer

\[ k_a \ (1/\text{Ms}) = 2.65 \times 10^5 \]

\[ k_d \ (1/\text{s}) = 6.27 \times 10^{-7} \]

\[ K_A \ (\text{M}^{-1}) = 4.22 \times 10^{11} \]

\[ K_D \ (\text{M}) = 2.37 \times 10^{-12} \]
Side-by-Side Comparison w/ Antibody

Vivekananda & Kiel
Lab Invest. 2006
Aptamer specificity

- Aptamers were selected against ACh immobilized in middle of molecule
- Aptamers bind acetyl & choline moieties
- Aptamers cross-react with other ester moieties
- Aptamers did not cross-react with unrelated small molecules such as Pinacolyl-MPA (GD acid) Or Diazinon


C-telopeptide from Bone Collagen (CTx)
Aptamers for NASA
Rationale for WADA Pilot Study

• Hepner et al., had shown modifications (deamidations, amino acid substitutions, etc.) in about 2% of Genotropin r-hGH by mass spectral analysis
• Research grade r-hGH from Genway Biotech (San Diego, CA) had N-terminal methionine (signal sequence) to distinguish it (proof-of-concept)
• Aptamers might be found that bind tighter to these modified regions of r-hGH and discriminate r-hGH from natural hGH
• Used a negative selection or “adsorption” approach (anti-r-hGH aptamer pool exposed to natural hGH)
• These could be exploited in various assay formats from serum and urine samples for ultrasensitive (sub-picogram) r-hGH detection
Consistent Differences in ELASAs Between hGH and r-hGH Aptamers

DNA Aptamers

vs. 1 µg Natural hGH vs. 1 µg Recombinant-hGH
Tabulated Mean Δ Abs 405nm ≥ 0.5 (N = 3)

### ELASA Plate Scheme / Layout

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>9</th>
<th>10</th>
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<th>12</th>
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<tbody>
<tr>
<td>A</td>
<td>rH-31F</td>
<td>rH-31R</td>
<td>rH-34F</td>
<td>rH-34R</td>
<td>rH-35F</td>
<td>rH-35Fa</td>
<td>rH-35Rb</td>
<td>rH-36F</td>
<td>rH-36Ra</td>
<td>rH-36Fb</td>
<td>rH-36Rb</td>
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</tr>
<tr>
<td>D</td>
<td>Ar-31F</td>
<td>Ar-31R</td>
<td>Ar-32F</td>
<td>Ar-32R</td>
<td>Ar-33F</td>
<td>Ar-33R</td>
<td>Ar-34F</td>
<td>Ar-34R</td>
<td>Ar-36F</td>
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<td>Ar-49R</td>
<td>Ar-50F</td>
<td>Ar-50R</td>
<td>Ar-51F</td>
<td>Ar-51R</td>
<td>Ar-54F</td>
<td>Ar-54R</td>
<td>Ar-55F</td>
<td>Ar-55R</td>
<td>Ar-56F</td>
<td>Ar-56R</td>
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</table>

### WADA Delta Plate Result Averages (rhGH minus hGH Values)

#### PLATE 1

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<tr>
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<th>12</th>
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<tbody>
<tr>
<td>A</td>
<td>0.278</td>
<td>0.392</td>
<td>-0.103</td>
<td>-0.078</td>
<td>-0.403</td>
<td>-0.012</td>
<td>0.269</td>
<td>-0.339</td>
<td>-0.036</td>
<td>0.131</td>
<td>0.164</td>
<td>-0.282</td>
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<tr>
<td>B</td>
<td>-0.008</td>
<td>-0.093</td>
<td>0.132</td>
<td>0.037</td>
<td>-0.180</td>
<td>0.030</td>
<td>0.152</td>
<td>-0.016</td>
<td>-0.008</td>
<td>-0.150</td>
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<td>-0.141</td>
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<tr>
<td>C</td>
<td>0.520</td>
<td>0.125</td>
<td>-0.033</td>
<td>-0.033</td>
<td>0.467</td>
<td>-0.039</td>
<td>-0.333</td>
<td>0.033</td>
<td>0.428</td>
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<td>0.647</td>
<td>0.061</td>
<td>0.080</td>
<td>0.244</td>
<td>0.025</td>
<td>0.052</td>
<td>0.078</td>
<td>0.425</td>
<td>-0.192</td>
<td>0.186</td>
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<td>E</td>
<td>0.462</td>
<td>-0.025</td>
<td>0.026</td>
<td>0.145</td>
<td>-0.213</td>
<td>-0.033</td>
<td>0.087</td>
<td>-0.020</td>
<td>-0.037</td>
<td>0.019</td>
<td>-0.142</td>
<td>-0.256</td>
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<tr>
<td>F</td>
<td>0.217</td>
<td>-0.372</td>
<td>-0.191</td>
<td>0.167</td>
<td>0.409</td>
<td>0.232</td>
<td>0.004</td>
<td>0.091</td>
<td>-0.103</td>
<td>0.002</td>
<td>0.056</td>
<td>-0.037</td>
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<tr>
<td>G</td>
<td>0.076</td>
<td>-0.106</td>
<td>-0.330</td>
<td>-0.024</td>
<td>0.049</td>
<td>-0.039</td>
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#### PLATE 2

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<tbody>
<tr>
<td>A</td>
<td>0.281</td>
<td>0.271</td>
<td>0.127</td>
<td>0.196</td>
<td>0.027</td>
<td>0.223</td>
<td>0.418</td>
<td>0.437</td>
<td>0.322</td>
<td>0.343</td>
<td>0.337</td>
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<tr>
<td>B</td>
<td>0.278</td>
<td>0.482</td>
<td>0.343</td>
<td>0.227</td>
<td>0.382</td>
<td>0.356</td>
<td>0.486</td>
<td>0.360</td>
<td>0.513</td>
<td>0.462</td>
<td>0.467</td>
<td>0.501</td>
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<tr>
<td>C</td>
<td>0.273</td>
<td>0.282</td>
<td>0.161</td>
<td>0.259</td>
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<td></td>
<td></td>
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</table>
ELASA Titrations: Part 1

ELASA Titer H43R

ELASA Titer H49R

ELASA Titer H55F
ELASA Titrations: Part 2
Cross-Reactivity: Means of Top 8 Aptamers

- rhGH
- hGH
- BSA
- NTx
- CTx
- Myoglobin
- Gn611
- Blank
- ABTS Only

Absorbance at 405nm
Recombinant and Natural hGH (GenWay Biotech)

MFPTIPLSRLFDNAMLRAHRLHQFDTYQEFEEAYPEKEQKYSFLQNPQTSLCFS
ESIPTPSNREETQQKSNLELLLRLSLLLIQSWLEPVQFRLSVFANSLVYGASDSNVYD
LLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDALLKYGLLYC
FRKDMDKVETFLRIVQCRSVEGSCGF

Myoglobin

MGLSDGEWQLVLNVWGKVVEADIPGHGQEVLIRLFGHPETLEKFDFKFKHLKSE
DEMKAEDLKKHATVLTALGGILKKKGHHEAEIJKLMNOPSHATKHK
PVKYLEFI
SECIIQVLQSKHPGDFGADAOQGAMNKA
LELFRKDMASNYKELGFQG

Bovine Serum Albumin

MKWVTISLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHKGLVLIAFSQYL
QQCPFDEHVKVLVNELEFAKTCSVADESHAGCEKSLHTLFGDELCKVASLRETYG
DMADCCCEKQEPERNECFLISHKDDSPDLPKLKPDNTLCDEFKADEKKFWGKYL
YEIARRHPYFYAPELLLYANKNMGVFQECQAEDKGCALPKIETMEREKVLTTSS
ARQRLRCAIQKFGERALKAWSVARSQKFPKAEFVEVTKLVTDLTKVHKECCCH
GDLLECADDRADLAKYICDNQDTISSKLKECCDKPLEKSHCIAVEKDA
TPLENLPPLTADFAEDKDVCKNYQEAKDFAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATL
EECCA

TRKVPQVSTPTLVEVSRSLKGVGRCTCKPESERMPCTEDYLSSLILNRLCVLHEK
TPVSEKVTKCCTEMLVNRRPCFSALTDPETYVPKAFDEKLTFTFHADICTLPDTEKQ
IKKQTALVELLEKHKPKATEEQLKTMENFVAFVDKCCAADDKEACFAVEGPKL
VVSTQTALA
Non-Reducing 4-20% Gradient Mini-SDS-PAGE

Coomassie Blue

Silver Stained

Discontinuous or “bridge” epitope?

< 20 kD and a doublet?

Dimer

Monomer (< 20 kD)
Summary of Pilot Study & Future?

- Cloned and sequenced 106 candidate hGH, r-hGH or absorbed DNA aptamers

- 8/106 aptamers gave Δ > 0.5 Å 405nm or bound consistently better to r-hGH (i.e., discriminated recombinant-hGH)

- The top 8 discriminatory aptamers shared some short sequence segments in loops

- Aptamers were made to research grade r-hGH which has an N-terminal methionine, but no 6x His tail or other affinity capture tail

- Aptamers could be detecting a discontinuous or “bridge” epitope on the dimer of r-hGH?

- Wish to target pharmaceutical r-hGH (Genotropin & Norditropin, etc.) using same r-hGH aptamer adsorption methods and known modified epitopes from Hepner et al. 2005 & 2006 in future work

- The ultimate aptamer-based solution would require specific epitope mapping to develop one or more highly r-hGH-specific aptamer assays.
WADA’s Critique of Pilot Study & Solutions

• ELASA not sensitive enough – **Solution:** ECL with sub-picogram/mL LODs

• R-hGH aptamers cross-reacted with BSA & myoglobin – **Solution:** Adsorb r-hGH aptamers with various human serum proteins including albumins, Hb & Mb to increase selectivity

• Many pharmaceutical r-hGH products would require vast amount of work to restart aptamer development for each – **Solution:** Find common host cell modifications from Hepner et al. in Genotropin and Norditropin and target those “epitopes” as initial aptamer development targets (Next slide)
Hepner et al., 2005/06 – Common *E. coli* modifications to Target?

Table 2: Results of electrospray LC-MS/MS and MALDI-TOF MS/MS measurements

<table>
<thead>
<tr>
<th>Sequence</th>
<th>M calculated (Da)</th>
<th>M observed (Da)</th>
<th>delta M (Da)</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>EETQQKSNLELLR</td>
<td>1586.83</td>
<td>1614.74</td>
<td>27.91</td>
<td>K&lt;sup&gt;70&lt;/sup&gt;: di-methylation or → R *</td>
</tr>
<tr>
<td>FDTNHSVDDALLK</td>
<td>1488.68</td>
<td>1489.60</td>
<td>0.92</td>
<td>N&lt;sub&gt;149&lt;/sub&gt; / N&lt;sub&gt;152&lt;/sub&gt; deamidation</td>
</tr>
<tr>
<td>RLEDGSPR</td>
<td>928.47</td>
<td>900.46</td>
<td>-28.01</td>
<td>R&lt;sub&gt;127&lt;/sub&gt; → Q or K *</td>
</tr>
<tr>
<td>LFDNAMLRL</td>
<td>978.50</td>
<td>994.40</td>
<td>15.90</td>
<td>M&lt;sub&gt;14&lt;/sub&gt;: oxidation</td>
</tr>
<tr>
<td>DMKVETFCLR</td>
<td>1252.61</td>
<td>1268.40</td>
<td>15.79</td>
<td>M&lt;sub&gt;170&lt;/sub&gt;: oxidation</td>
</tr>
<tr>
<td>DLEEGTLMGKR</td>
<td>1360.61</td>
<td>1376.66</td>
<td>16.05</td>
<td>M&lt;sub&gt;125&lt;/sub&gt;: oxidation</td>
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<tr>
<td>LFDNAMLRL</td>
<td>978.50</td>
<td>960.40</td>
<td>-18.10</td>
<td>M&lt;sub&gt;14&lt;/sub&gt; → I</td>
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<tr>
<td>DLEEGTLMGKR</td>
<td>1360.61</td>
<td>1342.60</td>
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<td>DMKVETFCLR</td>
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<td>1234.60</td>
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<td>978.50</td>
<td>1035.40</td>
<td>56.90</td>
<td>Carbamidomethyl – N terminus</td>
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</table>

* Nearly isobaric mass differences

Sequences and mass differences of modified peptides detected in the Genotropin sample. Column 1: sequence of modified peptides following tryptic digestion of Genotropin<sup>®</sup>; column 2: calculated monoisotopic masses of the unmodified tryptic peptides; column 3: observed monoisotopic masses; column 4: differences of calculated and observed masses; column 5: possible explanations of the mass differences.

Modifications to approx. 2% of Genotropin made in *E. coli* K12

Hepner et al., 2006: Norditropin M14 → V14
Ultrasensitive Electrochemiluminescence (ECL)

IGEN / Bioveris – Defunct Research ECL Analyzer

Origen® Analyzer

Roche ELECSys Clinical ECL Analysis Systems

Roche Cobas e 411
ECL Sandwich Screening Results

Capture Aptamer

MB

Reporter Aptamer

Mean ECL Intensity

Sandwich Assay Combination Number
Sub-pg hGH detection by Aptamer-Magnetic Bead ECL Sandwich Assay

r-hGH Assay Combination 18 Titration in 50% Human Serum

![Graph showing the relationship between concentration of hGH (pg/mL) and ECL intensity. The graph includes two lines with R² values of 0.9561 and 0.8895.](image)
Presumptive Lateral Flow Aptamer Strips for On Site r-hGH Testing?

- Traditional colloidal gold
- Enhanced sensitivity w/ fluorescent NPs or Q-dots and a UV penlight
Rapid sensitive on site FRET-aptamer assays for r-hGH?

**Software CDs**

**D-Salt Column Kit (for CTx)**

**Handheld Picofluor Fluorometer**

**Serial to USB Adapter**

**Lyophilized Cuvette Assays**

**Handheld Bone Peptide Sensor Delivered to NASA Phase 2 SBIR**

$r^2 = 0.9982$
Key References


