Toxicological Considerations For Oligonucleotide Therapeutics

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Northern CA SOT, May 2010
Oligonucleotide therapeutics

- What do I include?
  - Short strands of chemically linked, native or modified nucleotides, single or double stranded

- What is not included?
  - Nucleosides/nucleoside analogs
  - Long sequences of coding genetic material (e.g. gene therapy)
  - Non-nucleic acid transcriptional regulators (e.g. nuclear hormones, small molecule transcriptional regulators)
# The Major Classes of Oligonucleotide Therapeutics

<table>
<thead>
<tr>
<th>Class</th>
<th>Structure</th>
<th>Length</th>
<th>General MOA</th>
<th>Specific MOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense</td>
<td>ssDNA</td>
<td>13-25</td>
<td>Inhibit protein expression via complementary hybridization to mRNA</td>
<td>Cleave the target mRNA or inhibit translation by steric hindrance</td>
</tr>
<tr>
<td>Ribozyme / deoxyribozyme</td>
<td>ssRNA or ssDNA</td>
<td>&gt;20</td>
<td>Catalytically active; cause RNA cleavage</td>
<td></td>
</tr>
<tr>
<td>siRNA</td>
<td>Duplex RNA or ssRNA</td>
<td>19-21</td>
<td>Induce mRNA degradation via RNAi pathway</td>
<td></td>
</tr>
<tr>
<td>miRNA</td>
<td>ssRNA</td>
<td>20-25</td>
<td>Inhibit translation via binding to 3’UTR and/or promote mRNA degradation</td>
<td></td>
</tr>
<tr>
<td>Decoy</td>
<td>dsDNA</td>
<td>&gt;20</td>
<td>Inhibit protein function through high affinity binding</td>
<td>Binds to DNA binding site of transcription factor, inhibiting gene expression</td>
</tr>
<tr>
<td>Aptamer</td>
<td>ss or dsRNA, DNA</td>
<td>30-40</td>
<td></td>
<td>Binds to target protein</td>
</tr>
<tr>
<td>Immunostimulatory (CpGs)</td>
<td>dsDNA</td>
<td>&gt;20</td>
<td>Trigger immune response by activating Toll-like receptors (TLRs)</td>
<td></td>
</tr>
</tbody>
</table>

From: Scott Barros, Alnylam Pharmaceuticals
Mechanisms of the Four Major Classes of Oligonucleotide Therapeutics

**Antisense**

**siRNA**

**Immunostimulatory**

**Aptamer**

From: Antisense oligonucleotide-based therapeutics for cancer

From: adjunet.net/pubadjuvantdatabase/id_cpg.htm
DMPK Considerations and Optimization

- A historical limitation of oligonucleotide therapeutics is short half-life
  - Metabolic instability – nucleases degradation
  - Renal clearance – elimination by glomerular filtration
- The vast majority of OTs are chemically modified to block nuclease based metabolism and prolong duration of action
- Many OTs are chemically conjugated or encapsulated to facilitate delivery, block renal filtration, or otherwise modulate pharmaceutical properties
  - e.g. cholesterol, lipid nanoparticles, HDLs, PEG, another oligo, small molecule functional group, etc. (limited only by imagination)
- The final properties of modified oligonucleotides are highly “tunable” by site-directed chemical modifications, many of which are now well characterized
Metabolism of Oligonucleotides

**Nuclease Mediated Metabolic Pathway**

PS ODN
5’-gcacgtctgtctcgacct-3’

5’- PS-deoxy 3’

3’ exonuclease (plasma)

3’ and 5’ exonuclease (tissue)

2’-MOE gapmer
5’-GCATC agtctgcttc GCAC-3’

5’- 2’-MOE PS-deoxy 2’-MOE 3’

Endo-nuclease (tissue)

3’ and 5’ exonuclease

urine
Oligonucleotide-Based Therapeutics
Chemical Modifications

Sugar modifications: target binding affinity, nuclease stability, potency, duplex binding affinity (siRNA), immunostimulation

Base modifications: target binding affinity, potency

Phosphate linkage modifications: nuclease stability, potency, immunostimulation

Adapted from Watts et al. 2008, Drug Disc Today 13(19-20), 842-855.

From: Scott Barros, Alnylam Pharmaceuticals
# Bioanalytical Techniques for Oligonucleotide Therapeutics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bioanalytical Method</th>
<th>LLOQ (ng/mL)</th>
<th>Aptamer</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charge</td>
<td>CGE-UV</td>
<td>1000</td>
<td>Oligo and metabolite</td>
<td>++++</td>
</tr>
<tr>
<td>Charge</td>
<td>SAX (IEX)-HPLC-UV</td>
<td>100</td>
<td>Oligo. PEGoligo and metabolite</td>
<td>++++</td>
</tr>
<tr>
<td>Mass</td>
<td>RP-ESI-LC-MS</td>
<td>10</td>
<td>Oligo and metabolite</td>
<td>++++</td>
</tr>
<tr>
<td>Mass</td>
<td>MALDI-TOF-MS</td>
<td>10</td>
<td>Oligo and PEGoligo</td>
<td>++++</td>
</tr>
<tr>
<td>Binding</td>
<td>Oligreen</td>
<td>520</td>
<td>Oligo, PEGoligo and metabolite</td>
<td>-</td>
</tr>
<tr>
<td>Binding</td>
<td>Hybridization ELISA</td>
<td>1</td>
<td>Oligo, PEGoligo, and metabolite</td>
<td>++</td>
</tr>
<tr>
<td>Radiolabeled</td>
<td>LSC</td>
<td>&lt;0.1</td>
<td>Oligo, PEGoligo, and metabolite</td>
<td>++</td>
</tr>
<tr>
<td>Radiolabeled</td>
<td>Autoradiography (QWBA or MARG)</td>
<td>&lt;0.1</td>
<td>Oligo, PEGoligo, and metabolite</td>
<td>++</td>
</tr>
</tbody>
</table>
Typical Biodistribution of Oligonucleotides at the Whole Body Level

5 min post dose

8 hour post dose

336 hour post dose
Biodistribution at the Tissue Level

Liver: Anti-PEG antibody 60x
Liver: anti-aptamer ISH 60x
Oligonucleotide “class effects” were initially established with ASOs

- **Polaronion effects** (non-specific protein interactions)
  - Activation of C’ alternative pathway
  - Inhibition of coagulation cascade

- **Immune stimulation**
  - Somewhat dependent on sequence, independent of target
  - CpG motif/TLR-9 interaction

- **Tissue accumulation**
  - kidney – proximal tubule
  - tissue macrophages – liver, spleen, lymph nodes, others
  - There is a threshold [ ] of oligo for cytotoxicity and tissue injury
Regulatory Considerations for Development Oligonucleotide Therapeutics

- There are no specific guidance for oligos, therefore standard NME guidance’s need to be adapted.

- Oligonucleotides are at the intersection of small molecules and biotherapeutics when considering program design:
  - Chemically synthesized and derived from solid phase synthesis
  - Metabolized by endogenous metabolic processes and metabolites can be bioanalytically defined
  - Composed of endogenous (or modified endogenous) building blocks
  - ~5-50 kDa molecular weight
  - Pharmacological species specificity is typical
  - Tend to behave in “platform” specific patterns in terms of general pharmaceutical properties
Specific Considerations for Antisense Oligonucleotides (ASOs)

• General Properties
  - ssDNA, 2’modified, P=S backbone, ~20 mer (classical design)
  - Generally administered by intermittent SC or IV routes
  - High degree of protein binding (mostly albumin)
  - Highly stable to nucleases
  - Rapid immediate distribution from the plasma into certain tissues and cells and long persistence in those sites

• Key considerations in preclinical testing
  - Pharmacological species restriction – Use of murine surrogate of the same composition and target mRNA for pharmacology and toxicology is typical

• Key challenges for the technology
  - Effective intracellular delivery to desired target cells
  - Potency (on a delivered dose basis)
Immune Stimulation in Rodents with ASOs

- **Design**
  - C57BL/6 Mice
  - 100 mg/kg 104838 by SC
  - Q2d Load/q1w Maintain
  - 3 and 6 weeks of Tx

- **Results**
  - MCP-1, MIP-1a, and IP-10 levels increased 4 hr after dose
  - No increase in IL-6 or IL-12
  - Increase sustained throughout dose interval
  - No difference in MCP-1 between 3 and 6 weeks of Tx
  - Variable incidence of mild mononuclear cell infiltrates in liver

From: Scott Henry, ISIS Pharmaceuticals
Basophilic Granules in Renal Proximal Tubules in the Cynomolgus Monkey

ISIS 353512 administered at 40mg/kg/wk

From: Scott Henry, ISIS Pharmaceuticals
doses of 20 mg/kg/wk produce up to 3,500 to 4,500 μg/g cortex conc.

Specific Considerations for Immunostimulatory Oligonucleotides (ISOs)

• General properties
  - ss or dsDNA, usually partially 2’ modified, ~ ≥20 mer
  - Endosomal → intracellular site of action
  - Agonist MOA inducing a cascade of biological response
  - Potent (μg/kg doses)
  - Clinical applications: vaccine adjuvant, cancer immunotherapy, chronic viral disease, and immune modulation in allergic diseases

• Key considerations for preclinical testing
  - Nature of immune response differs between species
  - Optimal molecule design differs between species
  - Use of surrogates for development?

• Key challenges
  - Harnessing the positive effect without intolerable side effects
Key Considerations – Immune effects

- The immune system evolved Toll-like receptors and other pathways to detect RNA (TLR3,7,8) and DNA (TLR9) from pathogens, and to trigger protective responses
  - These pathways are likely activated many times during life
    - Mice with TLR deficiencies are more susceptible to many types of infections
  - There are many checks and balances to prevent excessive or inappropriate responses
  - TLR responses differ between primates and rodents
    - no functional TLR8 in rodents
    - Differential expression of TLR9 between rodents and primates

- These pathways are readily activated by administration of exogenous RNA or DNA (intentionally or unintentionally)
  - Oligos are concentrated in endosomes: not cytoplasm/nucleus!
  - Cause dose-dependent systemic cytokine/chemokine expression
  - Transient broad spectrum immune activation
    - Protects against infectious challenge
    - Rejects tumors

From: Art Krieg, Pfizer
Immune Recognition of Nucleic Acids (PRRs)

Toll-Like Receptors (TLRs)

- Bacteria
- Viruses
- Dying host cells

- ssDNA
- ssRNA
- dsRNA

- RIG-I
- MDA-5

- Tri-P ssRNA
- dsDNA

- IFNs
- Cytokines

- NUCLEUS
- CYTOSOL
- ENDOSONME

From: Art Krieg, Pfizer
# Safety Findings From TLR9 Activation Differ In Rodents And Primates

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rodents</th>
<th>NHP</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>extramedullary hematopoiesis, splenomegaly</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>multi-organ lymphoid infiltrates</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Lethal SIRS</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Lymphoid hyperplasia</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Injection site reactions</td>
<td>Rare</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hypersensitivity reactions</td>
<td>No</td>
<td>No</td>
<td>Rare</td>
</tr>
<tr>
<td>Complement activation acute DLT</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Transient leukopenia</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

From: Art Krieg, Pfizer
Stimulation of human TLR7, TLR8 or TLR9 leads to cell type specific cytokine secretion

- RNA oligos are less stable, require formulation for delivery

From: Art Krieg, Pfizer
Therapeutic effects of RNA resulting from immune activation

- TLR-induced immune activation provides:
  - a transient but broad spectrum defense against many pathogen challenges
  - Anti-tumor activity
  - Strong vaccine adjuvant

- An RNAi could have the same effects from immune stimulation, not through RNAi MOA

From: Art Krieg, Pfizer
Specific Considerations for Aptamers

• General properties
  – Aptamers are single stranded structured oligonucleotides that bind to molecular (protein) targets with high affinity and specificity
  – Most therapeutic aptamers target extracellular proteins and disrupt protein:protein interactions
  – 15-40 mer, variety of 2’ modifications, often PEG conjugated
  – Site of action in the plasma and interstitial fluid, outside of cells
  – Dose regimens vary widely depending on the aptamer compositions and the intended use (IV bolus, infusion, repeated bolus, SC bolus, etc…)

• Key considerations in preclinical testing
  – Species restriction is often observed; similar to mAbs
  – Two species toxicology testing, typically rat (off-target) and monkey (on-target)
    – Use of surrogates for development?

• Key challenges
  - Finding applications where properties of aptamers are uniquely advantageous
  - Limits to the systemic T1/2 without active recirculating mechanisms
Typical Findings in General Toxicology Studies with Aptamers

- **Exaggerated pharmacology**
  - Expected based on target biology

- **Anticoagulation**
  - Generally a modest effect with good safety margins

- **C’ activation**
  - Rarely seen and only at very high concentrations with aptamers tested to date

- **Hematopoietic bone marrow suppression**
  - Seen in repeated-dose toxicity studies, modest effect with good safety margins

- **Hemodilution (PEGylated oligos only)**
  - Osmotic properties of PEG at high plasma concentrations

- **Basophilic granulation and/or vacuolization**
  - Mononuclear phagocytes and kidney tubule epithelial cells
  - Presence of drug-related material in these specific cells
Off-target Anti-coagulation

Concentration-dependent prolongation of aPTT
Vacuolization, and/or Basophilic Granules Mostly in Mononuclear Phagocytes

- Presence of test article-related material in cells has not been associated with apparent adverse effects on those cells or tissues
- Therefore, this finding alone is not considered to be an adverse effect (thus far)
Specific Considerations for siRNAs

• General Properties
  - dsRNA, 2’modified, ~2x21 mer (classical design)
  - Intracellular site of action
  - Generally administered by intermittent local or IV routes
  - Variable stability to nucleases depending on degree of 2’ modification
  - Generally require carrier to access site of action (cytoplasm of target cells)

• Key considerations in preclinical testing
  - Pharmacological species restriction – Use of surrogates?
  - Poorly understood mechanisms of immunostimulation
  - Delivery systems often dominate the toxicity profile of the combined product candidate

• Key challenges for the technology
  - Delivery, delivery, delivery!
  - Specificity
Potential siRNA Safety Issues to Consider

- **Off-target RNA effects**
  - immunostimulation (TLR activation & IFN response)
  - “aptamer” effects (C’ activation, anticoagulation, other)
  - non-specific knock down of mRNA
  - perturbation of endogenous RNAi machinery

- **Carrier system effects**
  - Nanoparticles (various liposomes and others)
  - mAb, aptamer, or small molecule targeted nanoparticles
  - Lipid conjugates
  - Folate receptor-mediated uptake
Oligonucleotide Therapeutics are a Prominent Class of Products in Development; Be Ready!

- Oligo Therapeutics IND Submissions (n = 38)

Only INDs of new molecular entities are shown

- Miscellaneous: siRNA, aptamer, LNA, decoy, DNAi, non-PS-ASO (one each)

- Clinical development: Phase 1 through 3

- New chemically modified oligonucleotides emerged after 2000

- Withdrawn: 4 (PS-ASO, 3 before 2000)

Data from S-L Lee, FDA CDER, presentation at AAPS November 2008
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