CHEMICALLY MODIFIED SHORT INTERFERING RNA (siRNA)
FOR
TARGETED GENE SILENCING

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Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States.

Estimated new cases and deaths from pancreatic cancer in the United States in 2013:

New cases: 45,220  
Deaths: 38,460
Genes and Disorders
Any technique or mechanism in which the expression of a gene is prevented.
Strategies for Gene Silencing

- Regulation of transcription
- Inhibition of translation
- Degradation of the mRNA
- Protein inhibition

- siRNA
- AONs
Gene Silencing

- Food and agriculture
- Gene therapy
- Cancer
- Cardiovascular and Cerebrovascular Diseases
- Neurodegenerative Disorders (Huntington's disease)
- Viral infections

Antisense Oligonucleotides (AONs)

Short Interfering RNA (siRNA)

The siRNA is combined with RISC proteins.

One of the two RNA strands is degraded.

siRISC binds to mRNA.

mRNA

Exactly complementary

siRNA

Degradation of mRNA

Inhibition of mRNA translation

siRISC

Seed Region

Guide

Passenger

2012 Pearson education, Inc
si-RNA

Importance of siRNA

Andrew Fire (1998)
“double-stranded RNA was substantially more effective at producing interference than was either strand individually.”

Thomas Tuschl (2001)
First report of using synthetic siRNA in mammalian cell line

- siRNA: More resistant to nuclease degradation
### siRNAs in Clinical Pipeline

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Disease</th>
<th>Target</th>
<th>Carrier</th>
<th>Phase</th>
<th>Company</th>
<th>Status (Clinicaltrials.gov identifier)</th>
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</thead>
<tbody>
<tr>
<td>Bevasiranb</td>
<td>AMD</td>
<td>VEG</td>
<td>Naked siRNA</td>
<td>II</td>
<td>Opko Health Inc.</td>
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<td>AMD</td>
<td>VEGF</td>
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<td>OSI-888</td>
<td>Wet AMD and DME</td>
<td>RTP801</td>
<td>Naked siRNA</td>
<td>II</td>
<td>Quark Pharmaceuticals</td>
<td>Ongoing for DME (NCT01445899); Completed for AMD (NCT00713518)</td>
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<td>QPI1007</td>
<td>Non-arteritic ischemic optic neuropathy</td>
<td>Caspase 2</td>
<td>Naked siRNA</td>
<td>II</td>
<td>Ongoing (NCT01064505)</td>
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<td>TD101</td>
<td>Pachyonychia congenitus</td>
<td>Keratin (K6a, K6b)</td>
<td>Naked siRNA</td>
<td>Ib</td>
<td>TransDerm</td>
<td>Completed (NCT00716014)</td>
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<tr>
<td>RXI109</td>
<td>Dermal scarring</td>
<td>Connective tissue growth factor</td>
<td>Self-delivering RNAi compound (si-RXRNA®)</td>
<td>I</td>
<td>RXi Pharmaceuticals</td>
<td>Initiate in 2012</td>
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<td>SYL040012</td>
<td>Ocular Hypertension</td>
<td>ADRB2</td>
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<td>Sylentis</td>
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<td>Evolin</td>
<td>Asthma</td>
<td>Syk kinase</td>
<td>Naked siRNA</td>
<td>II</td>
<td>ZnBeCor</td>
<td>Ongoing, ongoing</td>
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<td>ALN-RSV01</td>
<td>RSV infection</td>
<td>RSV Nucleocapsid 'N' gene</td>
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<td>siRNA 1D-LODER</td>
<td>Pancreatic cancer</td>
<td>KRASG12D</td>
<td>LODER polymer</td>
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<td>Liquid nanoparticles, MC3 lipid</td>
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<td>KM-Ebola</td>
<td>Zaire Ebola or other hemorrhagic fever viruses infection</td>
<td>RNA polymerase L protein</td>
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<td>OCTN2</td>
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<td>QPL-1002 (ISNP)</td>
<td>Delayed Graft Function and Acute Kidney Injury</td>
<td>p53</td>
<td>AtnRNAi chemically modified siRNA</td>
<td>II for Delayed Graft Function 1 for kidney injury</td>
<td>Silence Therapeutics, Geron, Mirati Pharmaceuticals</td>
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<td>CALAA-01</td>
<td>Solid tumors</td>
<td>RRM2</td>
<td>Cyclohextrin, PEG and Transform</td>
<td>I</td>
<td>Calando Pharmaceuticals</td>
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Nucleotides Structure
Natural Nucleotides

Adenine

Guanine

Cytosine

Thymine

Uracil
## Double Stranded Structures

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>A-DNA</th>
<th>B-DNA</th>
<th>Z-DNA</th>
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</thead>
<tbody>
<tr>
<td>Helix Direction</td>
<td>Right-handed</td>
<td>Right-handed</td>
<td>Left-handed</td>
</tr>
<tr>
<td>Average base pairs per turn</td>
<td>11</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Rotation per base pair</td>
<td>32.7</td>
<td>36</td>
<td>-30</td>
</tr>
<tr>
<td>Distance between adjacent bases</td>
<td>0.26 nm</td>
<td>0.34 nm</td>
<td>0.37 nm</td>
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<tr>
<td>Diameter</td>
<td>2.3 nm</td>
<td>1.9 nm</td>
<td>1.8 nm</td>
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<tr>
<td>Overall shape</td>
<td>Short and wide</td>
<td>Long and narrow</td>
<td>Elongated and narrow</td>
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Synthesis of siRNA

- Enzymatically Generated siRNAs
- Chemically Synthesized siRNAs
Enzymatically generated siRNA

1. **In Vitro Transcription**
   - 30 minutes at 37°C.

2. **DNase Treatment**
   - 30 minutes at 37°C.

3. **Annealing to Form siRNA**
   - 10 minutes at 75°C
   - 20 minutes at room temperature.

4. **Alcohol Precipitation**
   - 5 minutes on ice.
   - 10 minute spin in microcentrifuge.

5. **Resuspend, Quantitate, Analyze siRNA.**

**Advantages:**
- Quick
- Cost effective

**Disadvantage:**
- Limited to natural nucleotides
Chemically Synthesized siRNAs

1. Deprotection

2. Coupling

3. Capping

4. Oxidation

General Problems for siRNA as a Drug Candidate

- Stability
- Targeting
- Side effect

- Endonuclease
- Target delivery
- Off target effects
Modification of siRNA

- A/U at the 5’end antisense strand (guide strand)
- Avoiding immunostimulators (GU rich sequences, blunt structure at 3´ end etc.)
- siRNA conjugation and delivery systems
Frequently Utilized Modifications

Chemical Modification of Nucleotides

- Internucleotide linkage Modifications
- Sugar Modifications
- Nucleobase Modifications

Internucleotide Linkage Modifications

X=H, OH
Phosphodiester

X=H, OH
Phosphorothioate (PS)

X=H, OH
Boranophosphate

X=H, OH
Phosphonoacetate (PACE)

Decreasing Charge Density

Increasing Lipophilicity

Increasing Stability

Sugar Modification

\[
\text{X} = \text{H, OH}
\]

N3' Phosphoramide (NP)

Sugar Conformations

S-type

N-type

0.34 nm

0.26 nm
Commercial Available Products

- 2’-O-Me modification
  - Pegaptanib (FDA approved 2004)

- 2’-O-MOE modification
  - Mipomersen (FDA approved 2013)
Nucleobases Modification

Nucleobases modification is less common than sugar and backbone modifications.

Affecting Hydrogen bonding in order to control:

- Duplex Stability
- OTEs

Crystal Structure of *Thermus thermophilus* Argonaute

siRNA instability

- RNA-dependent Protein Kinase (PKR)
- Adenosine Deaminases (ADAR-1)
Minor Groove & Major Groove
Minor Groove Modification: a New Strategy for Increasing the Stability of siRNA

Development of a Method for Switching Steric Groups away from the Minor Groove when the Guide Strand Enters RISC
Hoogsteen Base Pairing

8-Oxopurines as a potential frameworks

N²-alkyl-8-oxo-7,8-dihydroguanosine

minor groove
OG (anti) : C (anti)
Watson-Crick

major groove
OG (syn) : A (anti)
Hoogsteen

Preparation of N²-Alkyl-8-oxo-7,8-dihydro-2′-deoxyguanosine Phosphoramidites

### Design of siRNAs

<table>
<thead>
<tr>
<th></th>
<th>passenger: 5'-GGAAUGCAAGAGAAACUGdTdT-3'</th>
<th>guide: 3'-dTdTCCUUUACGUUCUUCUGAC-5'</th>
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<tbody>
<tr>
<td>UA (native)</td>
<td>16</td>
<td>11</td>
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<tr>
<td>X4</td>
<td>16</td>
<td>11</td>
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<tr>
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<td>R</td>
<td>R</td>
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<td>R</td>
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<td>X4,11</td>
<td>R</td>
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X = O (R = H), X = P (R = n-propyl), X = B (R = benzyl)

The temperature at which under specific condition double stranded structure is changed (50%) to single stranded nucleic acid.
Thermal Stability Tm

R = H (O) > R = propyl (P) > R = benzyl
The presence of a single OG:A Hoogsteen pair in the RISC does not diminish the activity. The location of addition of single alkyl group (propyl or benzyl) is important for siRNA function.
Knockdown Studies with 2 or 3 Modification

Substitution of a U in two or three positions of the guide strand with an OG was detrimental to knockdown activity.

Multiple substitutions with the benzyl substituent compromise the activity of siRNA more severely!

PKR Binding Assay

PKR binding assay

So far ....

**Hoogsteen** base pair formed by 8-oxoguanine in the **syn** conformation pairing with a target adenosine is tolerated in the seed region.

**Single Site Modifications** of this type led to knockdown of protein expression that was more efficient than the unmodified siRNA. (only in specific locations)

**PKR binding assay** supported the model.
Improvement of Model

Alternative Purine Modifications

8-alkoxyadenosine phosphoramidites

Base Switch

8-AlkoxyA

Delivery as Hoogsteen pair

Watson-Crick pairing in the RISC

Synthesis of 8-AlkoxyA

Synthesis of 8-AlkoxyA

3 equiv. HF, pyridine
pyr. CH₂Cl₂
-10 °C, 2-2.5 hr

siRNA design

Thermal analysis

Alkoxy group appears to introduce instability in the RNA duplexes.

In Watson–Crick Model hydrogen bonding is still preferred.

Thermal analysis

Knockdown Ability of the modified nucleotides is sensitive to position.
Knockdown Studies (2 sites modification)  

Multiple 8-ROA substitutions at the guide strands showed significantly reduced silencing efficacy.
Importance of Switching the Steric Blockade from the Minor to the Major Groove in the RISC

PKR binding assay

Chemical modification is a promising strategy to increase the potency and specificity of siRNAs.

A purine ribonucleoside can exist in both syn and anti conformations around the glycosidic bond depending on the base-pairing partner.

Singly modified siRNAs were capable of inhibiting PKR–siRNA interactions.
Acknowledgement

- Dr Huang

- Group members: Hovig, Herbert, Isaac, Steven Qian, Berm, Peng, Jicheng, Zeren, Weizhun, Zhao-Jun, Claire, Joe

- My Friends

- And you for your attention
THE END