Development of Therapeutic siRNAs against TNF-α and Novel Peptide Delivery Agents for the Treatment of Rheumatoid Arthritis

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RNAi Therapeutics: A Solution to the Delivery Challenge

- RNAi holds immense promise, but delivery of siRNA is primary hurdle:
  - Passing through cell membrane barrier
  - Maintaining molecule stability
  - Delivery into cells in effective quantities
- Nastech’s novel carrier molecules enable intracellular delivery
- Nastech is first to demonstrate systemic *in vivo* delivery of an RNAi therapeutic, and clinical effect, in rheumatoid arthritis
Presentation Overview

- Challenges for RNAi therapeutics
- RA and TNF-α as a therapeutic target
- siRNA screening and characterization
- New peptide based delivery agents
- *In Vivo* Evaluation of peptide-siRNA formulations for systemic delivery
Challenges for RNAi Therapeutics

- **Target Validation**
- **siRNA Compound Selection**
  - Potency – sub nM IC$_{50}$
  - Selectivity – off-target effects
  - Chemical modification – stability
- **Delivery and Pharmacokinetics**
  - Plasma / tissue / cell stability
  - Circulation time
  - Tissue / cell type targeting
  - Interferon response
- **Toxicology**
- **Assay Development**
- **Process Development, Scale-up, Manufacturing**
Why TNF-α? Why siRNA?

- To establish clinical validation of RNAi as a therapeutic approach.
- TNF-α is a clinically validated target for RA.
- RNAi is NOT yet clinically validated.
- Existing therapies (Remicade, Humira, Enbrel) neutralize TNF-α activity by direct binding, leading to:
  - Stabilization of TNF-α protein in the bloodstream.
  - Increased TNF-α mRNA in circulating monocytes.
- siRNA can reduce mRNA and protein to basal level; will a different MOA be clinically advantageous?
- Established regulatory path for approval for TNF-alpha targeting RA treatments at FDA.
Rheumatoid Arthritis

Activated mφ

IL-1, TNF-α

Activated synovial fibroblast

GM-CSF

IL6/IL8

Recruit cells

PGE2/Protease

Erode bone/cartilage
Induction of TNF-α mRNA and Protein in LPS-activated Human Monocytes

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Activities for siRNAs were ranked by % knockdown at a defined concentration relative to a negative control siRNA (Qiagen).
Map Positions of siRNAs against hTNF-α
siRNA Knockdown of TNF-α mRNA and Protein in Activated Human Monocytes

TNF-α mRNA

TNF-α ELISA

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siRNA Potency Determination

- IC$_{50}$ determination
- Maximum knockdown activity (Max. KD)
- Dependence on multiple factors
  - Transfection efficiency
    - Lipofectamine vs peptide
  - Cell types
    - Cultured cells vs primary monocytes
    - Donor variation for human monocytes
  - Assay condition
    - Induction vs non-induction
  - Cellular localization of siRNA
Dose Response Curves for Selected siRNA Candidates

Human Monocytes

SiRNA activity on MTF

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀</th>
<th>Max KD (%)</th>
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<tbody>
<tr>
<td>LC13</td>
<td>0.116nM</td>
<td>67%</td>
</tr>
<tr>
<td>LC17</td>
<td>0.35nM</td>
<td>74%</td>
</tr>
<tr>
<td>LC19</td>
<td>0.19nM</td>
<td>76%</td>
</tr>
<tr>
<td>LC20</td>
<td>0.065nM</td>
<td>74%</td>
</tr>
<tr>
<td>YC12</td>
<td>0.102nM</td>
<td>83%</td>
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</table>
Screening Peptide Libraries for siRNA Delivery

- Existing CPP motifs
- Novel designs (NA binding proteins; cell surface binding ligands)
- Phage display
  - M13 system (7, C7C, 12)
  - T7 system (defined structural motifs)
- siRNA-peptide conjugates & complexes
Peptide Candidates for siRNA Delivery

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Percent Uptake</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>9L/lacZ Cell Line</td>
</tr>
<tr>
<td>PN27</td>
<td>86</td>
</tr>
<tr>
<td>PN28</td>
<td>79</td>
</tr>
<tr>
<td>PN29</td>
<td>87</td>
</tr>
<tr>
<td>PN58</td>
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<td>PN173</td>
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<td>PN182</td>
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<td>PN202</td>
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<td>PN204</td>
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<td>PN250</td>
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<tr>
<td>PN361</td>
<td>42</td>
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<tr>
<td>PN365</td>
<td>81</td>
</tr>
<tr>
<td>PN404</td>
<td>not tested</td>
</tr>
<tr>
<td>PN453</td>
<td>not tested</td>
</tr>
<tr>
<td>PN509</td>
<td>not tested</td>
</tr>
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</table>

Peptides were tested as complexes with fluorescently labeled siRNA in 9L/lacZ cell line and primary mouse tail fibroblast (MTF) cells. Most peptides had relatively comparable uptake values in both cell types (except PN58). All the values listed are the ones with cytotoxicity less than 20%.
FAM-conjugated siRNA Uptake Detected by Flow Cytometry.
Fluorescently labeled siRNA-peptide conjugates were tested for uptake in mouse tail fibroblast cells. In all cases, cytotoxicity was less than 10%.
siRNA Localization in Primary Mouse Tail Fibroblasts

Lipofectamine

PN73 (complex)
### Activities of Cholesterol siRNAs

<table>
<thead>
<tr>
<th></th>
<th>% knock down</th>
<th>% uptake</th>
<th></th>
<th>% knock down</th>
<th>% uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>89.7</td>
<td>44.4</td>
<td>I</td>
<td>70.1</td>
<td>42.7</td>
</tr>
<tr>
<td>B</td>
<td>88.0</td>
<td>43.6</td>
<td>J</td>
<td>68.0</td>
<td>35.2</td>
</tr>
<tr>
<td>C</td>
<td>87.9</td>
<td>35.4</td>
<td>K</td>
<td>66.0</td>
<td>53.0</td>
</tr>
<tr>
<td>D</td>
<td>83.8</td>
<td>47.9</td>
<td>L</td>
<td>53.6</td>
<td>37.1</td>
</tr>
<tr>
<td>E</td>
<td>83.2</td>
<td>55.5</td>
<td>M</td>
<td>34.6</td>
<td>15.0</td>
</tr>
<tr>
<td>F</td>
<td>81.0</td>
<td>43.4</td>
<td>N</td>
<td>22.9</td>
<td>42.6</td>
</tr>
<tr>
<td>G</td>
<td>76.9</td>
<td>17.2</td>
<td>O</td>
<td>22.2</td>
<td>42.5</td>
</tr>
<tr>
<td>H</td>
<td>72.4</td>
<td>44.8</td>
<td>P</td>
<td>1.2</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Effect of Serum on siRNA Uptake

Percent uptake in primary MTF cells in the presence of increasing fetal bovine serum concentration. Uptake of siRNA-peptide complexes is not compromised up to 20% FBS; however, uptake of cholesterol-siRNA dramatically drops in the presence 5% FBS.
Effect of PN73 on Cell Uptake of siRNA-Cholesterol in Serum

Inhibition of Uptake of cholesterol conjugated siRNA by 5% serum is rescued by PN073
hTNF-α Murine Expression Construct

- 2.8kb genomic fragment
- 0.77kb β-globin downstream regulation element

Microinjection

- Taconic hTNF-α Mice (1006-T)
- Hellenic Pasteur Institute hTNF-α Mice (Tg197)
## Mouse Models Comparison

<table>
<thead>
<tr>
<th>Taconic hTNF-α</th>
<th>Hellenic hTNF-α</th>
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<tbody>
<tr>
<td>SLOW Disease Progression</td>
<td>FAST Disease Progression</td>
</tr>
<tr>
<td>LOWER hTNF-a Plasma Level &lt; 10pg/ml</td>
<td>HIGHER hTNF-a Plasma Level &gt; 20pg/ml</td>
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</table>
Human TNF-α transgenic mice were treated with Remicade (5μg/kg) and siRNA (LC20) TNF-α/PN73 complex through tail vein injections; siRNA dose: X, twice a week. The binding of TNF-α by Remicade appears to increase serum concentrations, perhaps by stabilization against metabolism. Dissociation of TNF-α from Remicade could be a mechanism for disease escape from treatment.
Effect of siRNA-peptide Treatment of h-TNF-α Transgenic Mouse Model

Dose: X, siRNA, iv injection twice a week; Formulation: siRNA complexed with peptide PN73. RA scores determined using the following criteria: 0, normal; 1, edema or distortion of paw or ankle joints; 2 distortion of paw and ankle joints; 3, ankylosis of wrist or ankle joints.

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**Additional Work**

**Completed**
- siRNA stabilization (plasma) by chemical modification.
- Delivery peptide (PN073) analog development and optimization.
- Mouse TNF-\(\alpha\) siRNA candidate for pre-clinical toxicology.
- Demonstrated lack of interferon response.
- RA patient cytokine profiling; TNF-\(\alpha\) KD analysis.

**In Progress**
- Evaluation of off-target effects by global expression profiling.
- siRNA-delivery peptide formulations development.
- Dose optimization in Animal models.
- Process development for scale-up manufacturing.