Developing an RNAi Therapeutic for Liver Disease Associated With Alpha-1-Antitrypsin Deficiency


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Abstract

AIM:
- Transgenic human Z-AAT expressing mice develop tumors with age, ultimately compromising the hepatocyte and subsequently leading to liver disease. We propose that down regulating the mutant allele (Z-AAT) at the mRNA level can chronic dosing in aged mice with fibrotic livers decrease the tumor incidence?

RESULTS:
- ALN-AAT, a N-acetylgalactosamine (GalNAc) -conjugated siRNA, was developed. This allows for subcutaneous administration and is taken up by the hepatocytes via asialoglycoprotein receptor.
- Subcutaneous administration of the GalNAc-conjugated AAT siRNA led to dose-dependent inhibition of AAT mRNA on Day 21 (sac) in liver, serum hAAT by ELISA for up to 24 weeks.
- Repeat dosing of 0.5 mg/kg twice a week decreased AAT serum levels below 10% levels (90% suppression).
- 25–46-week-old Z-AAT transgenic mice (30% of control) showed decreased hAAT mRNA on Day 0 and serum human AAT was followed for 21 days post dose. Each point represents average of 3 mice and the error bars reflect the standard deviation.

CONCLUSION:
- GalNAc-conjugated AAT siRNA leads to dose-dependent, durable silencing of AAT.
- AAT siRNA is effective in decreasing Z-AAT levels in transgenic mice with fibrotic livers, leading to a reduction in tumor incidence with reduction in Z-AAT.
- Chronic dosing of the siRNA maintains low target levels, leading to less hepatocyte damage, decrease in fibrosis and hepatocyte proliferation, and a decrease in inflammation, fibrosis, and cirrhosis, HCC.

Figure 1. RNA interference (RNAi) is a highly evolutionarily conserved mechanism of gene regulation.

Figure 2. Cummings and McEver, 1997.

Figure 3. The top 10 compounds were screened at different concentration to separate them by their IC50 values were tested for efficacy. The siRNA were injected at 10 mg/kg in transgenic mice expressing human Z-AAT allele. These transgenic mice express the mouse AAT at the normal levels, so do not get the lung disease. The mice were dosed on day 0 and serum human AAT was followed for 21 days post dose. Each point represents average of 3 mice and the error bars reflect the standard deviation.

Figure 4. The efficacy curve showing maximum knock-down achieved at different doses tested in mice. Each point is an average of 3 animals and the error bar represent the standard deviation.

Figure 5. Decreased tumor incidence with reduction in Z-AAT.

Chronic dosing of si-AAT in aged diseased mice.

(A) depicts the study design. Animals treated with AAT siRNA.

(B–D) The siRNA was chemically-modified to enhance stability and conjugated at the 3'-end of the sense strand with a trivalent N-acetyl galactosamine (GalNAc) ligand to allow for efficient delivery to hepatocytes following subcutaneous administration.

(E) No macroscopic tumor

(F) PAS staining of two littermates treated with PBS or AAT siRNA. The pink dots represent the globules or Z-AAT aggregates.

(Z-AAT aggregates in liver lead to inflammation, fibrosis, cirrhosis, HCC.)
**Abstract**

AIM: Liver disease associated with Alpha-1-antitrypsin (AAT) deficiency is a common cause of both morbidity and mortality in patients. In >95% of these patients, expression of a mutant AAT allele called Z-AAT results in AAT protein misfolding and aggregation. Intracellular accumulation of Z-AAT protein aggregates ultimately compromises the hepatocyte and subsequently leads to liver disease. We propose that down regulating the mutant allele (Z-AAT) at the mRNA level will prevent the progression and development of liver disease.

METHODS: A set of chemically modified siRNAs designed using bioinformatic algorithms was synthesized and screened by transfection in Hep3B cells for activity leading to selection of a highly potent siRNA for further studies. The siRNA was tested in transgenic mice expressing human Z-AAT. These mice develop liver disease and show protein accumulation similar to the human patients.

RESULTS: We have developed a N-acetylgalactosamine (GalNAc) -conjugated siRNA (ALN-AAT). This allows for subcutaneous administration and is taken up by the hepatocytes via asialoglycoprotein receptor. Subcutaneous administration of the GalNAc-conjugated AAT siRNA, led to dose-dependent inhibition of serum human Z-AAT, with maximum inhibition of >95% observed at a dose of 3 mg/kg. A single dose of 1 mg/kg maintained 40% levels of AAT for at least 15 days. Repeat dosing of 0.5 mg/kg twice a week decreased AAT serum levels below 10% levels (90% suppression). Finally, 25–46-week-old Z-AAT transgenic mice administered GalNAc-AAT for 4 months exhibited decreased liver tumor incidence compared to untreated control animals.

CONCLUSION: The robust suppression of AAT and subsequent phenotypic amelioration achieved by administration of GalNAc-AAT in a well-established mouse model of AAT-deficiency associated liver disease supports further development of this novel therapeutic strategy.

**Figure 1. RNA Interference (RNAi)**

RNA Interference (RNAi) is a highly evolutionarily conserved mechanism of gene regulation. RNAi occurs at the post-transcriptional level and is triggered by short double-stranded RNA (dsRNA), known as short interfering RNA (siRNA), which is endogenously processed from long dsRNA by the RNAse III enzyme Dicer or introduced into the cell exogenously as synthetic siRNAs. After being loaded into the RNA-inducing silencing complex (RISC) in the cytoplasm, the siRNA causes sequence-specific degradation of its homologous mRNA sequences which in turn reduces the protein encoded by the mRNA. By the introduction of synthetic therapeutic siRNA, this natural, endogenous mechanism may be utilized to down-modulate any protein of interest.

**Figure 2. ALN-AAT**

**A** GalNAc-siRNA conjugates

ALN-AAT
- siRNA conjugated to N-acetylgalactosamine (GalNAc) ligand targeting AAT mRNA
- Efficient delivery to hepatocytes following subcutaneous administration

ASGPR
- Highly expressed in hepatocytes
- 0.5-1 million copies/cell
- Clears serum glycoproteins via clathrin-mediated endocytosis
- High rate of uptake
- Recycling time ~15 minutes
- Conserved across species

**B** Therapeutic hypothesis ALN-AAT

2-AAT aggregates in liver lead to fibrosis, cirrhosis, HCC
Reduction in mRNA will reduce mutant protein
Decrease in 2-AAT polymers, aggregate deposition in liver, less hepatocyte damage
Decrease in fibrosis and hepatocyte proliferation

Figure 2. ALN-AAT. (A) An siRNA conjugate was developed against alpha 1 antitrypsin to allow for targeted delivery to hepatocytes in vivo. The siRNA was chemically-modified to enhance stability and conjugated at the 3’-end of the sense strand with a trivalent N-acetyl galactosamine (GalNAc) ligand to allow for targeting to the asialoglycoprotein receptor (ASGPR) on hepatocytes. The GalNAc-AAT siRNA is administered via subcutaneous injection. (B) The GalNAc-AAT siRNA will degrade the AAT mRNA in the liver and thereby prevent the synthesis of mutant protein 2-AAT. Reduced levels of 2-AAT will translate into less polymer accumulation in the hepatocytes and a healthier liver.
An siRNA conjugate was developed against alpha 1 antitrypsin to allow for targeted delivery to hepatocytes. It is chemically modified to enhance stability and conjugated at the 3'-end of the sense strand with a trivalent N-acetyl galactosamine (GalNAc) ligand to allow for uptake into hepatocytes via the asialoglycoprotein receptor (ASGPR).

Figure 2. ALN-AAT.

**METHODS:**
A set of chemically modified siRNAs designed using bioinformatic algorithms was synthesized and screened by transfection in Hep3B cells for activity. The most potent siRNAs were selected based on their ability to reduce the protein expression of mutant AAT allele. These siRNA sequences were then chemically modified and conjugated with GalNAc ligand.

**RESULTS:**
In vitro screening of siRNA for efficacy showed that the GalNAc AAT-siRNA has a high rate of uptake and is taken up by hepatocytes. The siRNA causes sequence-specific degradation of its homologous mRNA sequences which in turn reduces the protein encoded by the mRNA. By reducing the expression of mutant AAT allele, the siRNA can ultimately compromise the hepatocyte and subsequently leads to liver disease.

**CONCLUSION:**
The results of this study support the development of an RNAi therapeutic strategy for treating alpha-1 antitrypsin deficiency liver disease. The GalNAc AAT-siRNA shows promising efficacy in transgenic mice expressing human Z-AAT allele. These transgenic mice express the mouse AAT at the normal levels, so do not get the lung disease. The mice were dosed on day 0 and serum human AAT was followed for 21 days post dose. Each point represents average of 3 mice and the error bars reflect the standard deviation. The mice were sacrificed on Day 21 and their livers were processed to measure mRNA levels. The graph on right shows hAAT normalized to GAPDH for each group. The bars reflect the average and error bars depict the standard deviation.

**Figure 3. In vitro screening of siRNA for efficacy.** (A) Hep3B cells were transfected with a set of siRNA sequences at 10 nM and 0.1 nM final concentration. The top 10 compounds were screened at different concentration to separate them by their IC50 values. The IC50 curve for the lead molecule is shown in the right-hand side graph. (B) Five molecules with lowest IC50 values were tested in vivo for efficacy. The siRNA were injected at 10 mg/kg in transgenic mice expressing human Z-AAT allele. The transgenic mice express the mouse AAT at the normal levels, do not get the lung disease. The mice were dosed on day 0 and serum human AAT was followed for 21 days post dose. Each point represents average of 3 mice and the error bars reflect the standard deviation. The mice were sacrificed on Day 21 and their livers were processed to measure mRNA levels. The graph on right shows hAAT normalized to GAPDH for each group.

**Figure 4. Efficacy of GalNAc AAT-siRNA in transgenic animals.**
(A) ED50 curve in transgenic mice. The dose response curve showing maximum knock-down achieved at different doses tested in mice. Each point is an average of 3 animals and the error bar represents the standard deviation. (B) Durable AAT suppression in dose responsive manner. The mice were dosed at different time intervals to measure the serum hAAT protein levels using human AAT specific ELISA. (C) Repeat dosing at 0.5 mg/kg leads to >90% protein suppression. The study is still ongoing. Each data point is average of 4 animals and the error bars reflect the standard deviation.

**ED50 curve in transgenic mice**

**Durable AAT suppression in dose responsive manner**

**Repeat dosing at 0.5 mg/kg leads to >90% protein suppression**
Figure 5. Decreased tumor incidence with reduction in Z-AAT

Experiment hypothesis
• Transgenic human Z-AAT expressing mice develop tumors with age
• Can chronic dosing in aged mice with fibrotic livers decrease the tumor incidence?

<table>
<thead>
<tr>
<th>Tumor incidence</th>
<th>PBS male</th>
<th>AAT female</th>
<th>AAT-male</th>
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<tbody>
<tr>
<td>A No macroscopic tumor</td>
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<tr>
<td>B Large tumor in left lateral lobe, 5 mm diameter</td>
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<tr>
<td>C 4 mm tumor in caudate lobe, many lesions in 2nd aux lobe</td>
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<tr>
<td>D 1.5 mm tumor in caudate lobe, 1 mm lesion in right medial lobe, multiple 1 mm lesions in 1st aux lobe</td>
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<tr>
<td>E 3 mm tumor in left lateral lobe</td>
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<tr>
<td>F No macroscopic tumor</td>
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Conclusions
• GalNAc-conjugated AAT siRNA leads to dose-dependent, durable silencing of AAT
• AAT siRNA is effective in decreasing Z-AAT levels in transgenic mice with fibrotic livers
• Chronic dosing of the siRNA maintains low target levels
• The decreased Z-AAT levels show a physiological benefit in form of healthier livers
  - Treated animals show less fibrosis
  - There is reduction in the mis-folded protein aggregates and globules
  - The decrease in mis-folded protein translates into less liver tumor incidence

References