An RNAi Therapeutic Targeting Antithrombin Increases Thrombin Generation in Nonhuman Primates

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Introduction

The hemostatic system balances the need to control blood loss with the need to prevent thrombosis. In hemophilia, the loss of certain procoagulant factors (Factor VIII (FVIII) and Factor IX (FIX), in the case of hemophilia A and B, respectively) results in an imbalance of the hemostatic system toward a bleeding phenotype. Interestingly, there have been reports suggesting that coinheritance of prothrombotic mutations (eg, Factor V Leiden, protein C deficiency, protein S deficiency, antithrombin deficiency, prothrombin G20210A) may ameliorate the clinical phenotype in hemophilia.\textsuperscript{1,4} We are currently investigating the use of RNA interference (RNAi) to target the natural anticoagulant antithrombin (AT) as strategy to rebalance the hemostatic system and improve thrombin generation in hemophilia.

Previously, a short interfering RNA (siRNA), ALN-AT3, employing a hepatocyte targeting ligand was developed against AT and demonstrated potent activity in both wild-type and hemophilia mice after single subcutaneous (SC) administration (ED\textsubscript{50} ~ 1 mg/kg).\textsuperscript{5} Further, treatment of hemophilia mice with ALN-AT3 has resulted in normalization of thrombin generation.\textsuperscript{6} In this work, we investigate the ability of ALN-AT3 to silence AT, and consequently, increase thrombin generation in nonhuman primates using a Calibrated Automated Thrombinoscope (CAT). CAT assay results demonstrated that AT reduction was correlated with increased thrombin generation, with up to 4-fold increases in peak thrombin noted.

These data suggest that the use of a novel RNAi therapeutic targeting AT is a promising approach for the treatment of hemophilia, and potentially, other bleeding disorders. Further, the SC route of administration, long duration of action, and applicability to persons with hemophilia who have inhibitors, make this a particularly encouraging potential therapy.
Results

Figure 1. Background

A  Therapeutic hypothesis: rebalancing the hemostatic system

(A) Coagulation model depicting two separate initiations, intrinsic (contact) and extrinsic pathways, which ultimately merge at the level of Factor Xa (common pathway). In hemophilia A and B the lack of FVIII and FIX, respectively, leads to a decrease in thrombin potential, resulting in a bleeding phenotype. Inhibiting antithrombin, a powerful natural anticoagulant in the pathway, has the potential to increase thrombin generation in hemophilia A or B and correct the bleeding phenotype. Using RNAi, we explored the impact of reducing antithrombin as a means to rebalance the hemostatic system in hemophilia.

B  RNA interference (RNAi)

(B) 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.
An siRNA conjugate was developed against antithrombin 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-AT siRNA (ALN-AT3) is administered via SC injection. Figure adapted from Cummings and McEver.
Figure 3. ALN-AT3 pharmacology in mouse

A Dose response and duration

- 30 mg/kg
- 10 mg/kg
- 3 mg/kg
- 1 mg/kg
- 0.3 mg/kg

Relative antithrombin level (PBS = 1)

Day

(B) Normalization of ETP in hemophilia B mice

ETP (nM mm

WT Control
HB Control
ALN-AT3 Treated

(A) Dose-dependent reduction of antithrombin after a single SC administration of ALN-AT3 in wild-type mice (C57BL6). At various time points post-administration, animals were bled and serum was collected. Serum antithrombin levels were analyzed by ELISA. Data points represent group mean, error bars represent standard deviation (N = 5). (B) Treatment of Hemophilia B mice (FIX <1 IU/dl) with 30 mg/kg ALN-AT3. At 72 hours post-administration, animals (N = 3) were sacrificed and plasma was collected. Thrombin generation assays were performed using tissue factor = 0.5 pM. Endogenous thrombin potential (ETP) (AUC) for WT mice treated with PBS (N = 2), HB mice treated with PBS (N = 3), and HB mice treated with ALN-AT3 (N = 3). Bars represent group mean, error bars represent standard deviation. HB mouse work courtesy of Y. Dargaud and C. Negrier.
<table>
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Figure 4. Single SC dose of ALN-AT3 mediates potent and durable suppression of AT and increases thrombin generation in NHPs

Relationship between relative AT level and fold change in peak thrombin value at (A) 1 mg/kg, (B) 3 mg/kg, (C) 10 mg/kg, and (D) 30 mg/kg. Cynomolgus monkeys were administered ALN-AT3 at the dose levels indicated via single SC injection. Serum was collected at various time points after administration and analyzed for AT protein level by ELISA. AT levels are represented relative to the average of three pre-dose measurements. Relative AT level is depicted on primary y-axis (dark blue). Thrombin generation curves were generated from plasma samples collected at various time points using a Calibrated Automated Throminoscope (CAT) (tissue factor = 1 pM). Fold change in peak thrombin was calculated relative to the average peak thrombin value for two pre-dose values of each animal. Fold change in peak thrombin is depicted on secondary y-axis (light blue). (E) Consolidated AT dose response and duration data. Dose dependent AT silencing was observed, with approximately 50, 70, 80 and >90% silencing at 1, 3, 10 and 30 mg/kg, respectively. (F) Consolidated scatterplot of fold change increase in peak thrombin as a function of relative AT silencing. Data points represent mean value and error bars represent standard deviation (N = 3).
Cynomolgus monkeys were administered ALN-AT3 at two different cumulative weekly dose levels (0.5 mg/kg and 1.5 mg/kg cumulative weekly dose) via SC injection at two different dose intervals (weekly, qw and every other weekly, q2w). Serum was collected at various time points and analyzed for antithrombin protein level by ELISA. AT levels are represented relative to the average of three pre-dose measurements. Dose dependent AT silencing was observed, with 0.5 mg/kg cumulative weekly dose (0.5 mg/kg qw and 1 mg/kg q2w) resulting in approximately 80% AT suppression and 1.5 mg/kg cumulative weekly dose (1.5 mg/kg qw and 3 mg/kg q2w) resulting in approximately >90% AT suppression. Steady-state levels of suppression are achieved by Day 25. Data points represent group mean, error bars represent standard deviation (N = 5).
Conclusions

- ALN-AT3 is a subcutaneously administered RNAi therapeutic targeting antithrombin (AT) for the treatment of hemophilia
- ALN-AT3 treatment results in potent and durable inhibition of AT in nonhuman primates, with a single 1 mg/kg dose leading to approximately 50% silencing and a weekly dose of 0.5 mg/kg resulting in approximately 80% suppression
- Given the long duration of action of ALN-AT3, once weekly or twice monthly SC dosing is envisioned
- RNAi mediated inhibition of AT results in up to 4-fold increase in thrombin generation in nonhuman primates
- A hemostatic rebalancing approach utilizing ALN-AT3 represents a potentially new prophylaxis therapy option in persons with hemophilia and other bleeding disorders

References


Disclosures

Akin Akin, Affica Sehgal, Julia Hettinger, Josh Brodsky, June Qin, Tim Racie, Klaus Charisse, Scott Barros are all employees of Alnylam Pharmaceuticals.