

1204 RNAi-Mediated Inhibition of Activated Protein C – A New Approach for Hemophilia Treatment

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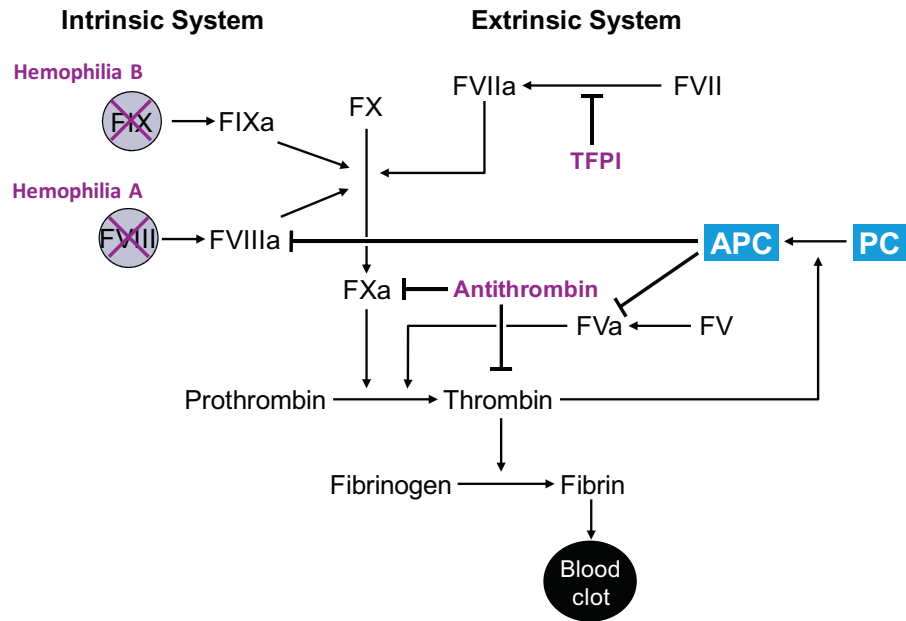
Abstract

Protein C is a major anticoagulation factor that serves as a key regulator of the clotting cascade. Activated protein C (APC), which is formed by limited proteolysis of the zymogen protein C by the thrombin–thrombomodulin complex, modulates thrombin generation. The anticoagulant effect of APC is due to subsequent inactivation of both activated factors V (FVa) and VIII (FVIIIa)¹.

The important role of APC in coagulation is highlighted in FV Leiden (FVL), the most common inherited form of thrombophilia. FVL is caused by a mutation in the FV gene at the initial APC cleavage site, which results in FVL being inactivated at approximately one tenth the rate of normal FVa, leading to high thrombin levels that create a procoagulant state². Several clinical studies have suggested that the severity or onset of bleeding phenotype in hemophilia patients is substantially reduced in association with impaired inactivation of FVa by APC in the presence of the FVL mutation^{3,4,5}. Co-inheritance of protein C deficiency and severe hemophilia B in a single patient demonstrates basal activation of the coagulation system and increased prothrombin 1.2 fragment and fewer bleeding episodes⁶. Transgenic animal studies also show that hemophilic mice, either heterozygous or homozygous for FVL, have improved clotting times with the ability to form clots at sites of laser-induced injury in a microvascular bed injury model, supporting the role of the FVL mutation in enhancing hemostasis⁷.

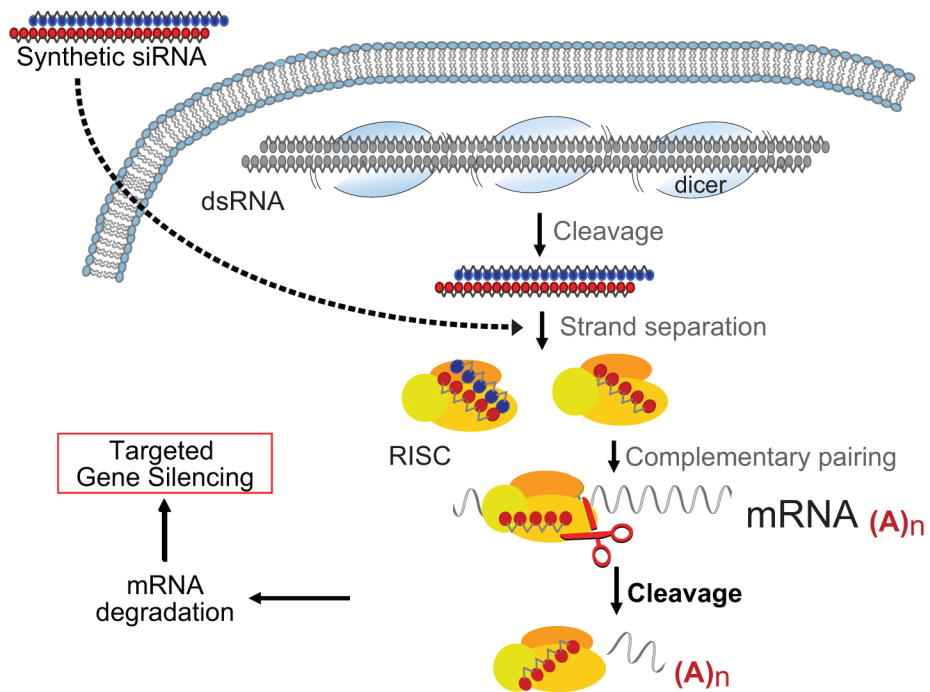
Therapeutic strategies aimed at reducing the level of protein C and activated protein C, thereby increasing levels of FVa and thrombin could thus prove efficacious in hemophilia A and B. Here we investigate the systemic administration of lipid nanoparticle (LNP) formulated siRNA directed against protein C (encoded by PROC). We have designed potent siRNA sequences against human, cynomolgus monkey, mouse and rat PROC mRNA. The lead human/cynomolgus candidate has IC₅₀ of 5 pM. The lead mouse/rat candidate has IC₅₀ of 30 pM and ED₅₀ of 20 ug/kg. We demonstrate robust and durable inhibition of PROC mRNA in normal mouse liver- a single injection of 0.3 mg/kg resulted in 90% silencing of PROC mRNA and reduction of more than 75% was sustained for more than two weeks. We also demonstrate significant reduction of circulating protein as measured by Western blot. Further testing using genetic models of hemophilia is being conducted to provide proof-of-concept for developing a PROC siRNA as a novel therapeutic agent in hemophilia management.

Figure 1. Waterfall/Cascade model of coagulation



Waterfall/Cascade model of coagulation consists of two separate initiations, intrinsic (contact) and extrinsic pathways, which ultimately merge at the level of Factor Xa (common pathway). Coagulation is triggered (initiation) by circulating trace amounts of FVIIa and locally exposed tissue factor (TF). Subsequent formations of FXa and thrombin are regulated by tissue factor pathway inhibitor (TFPI) and antithrombin (AT). Activated protein C (APC) is generated from the protein C zymogen by proteolytic activation by thrombin-thrombomodulin complex and down regulates formation of thrombin by inactivating FVa and FVIIIa. 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 protein C has the potential to increase thrombin generation in hemophilia A or B and correct the bleeding phenotype.

Figure 2. RNA interference



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.

Figure 3. Lead selection and development

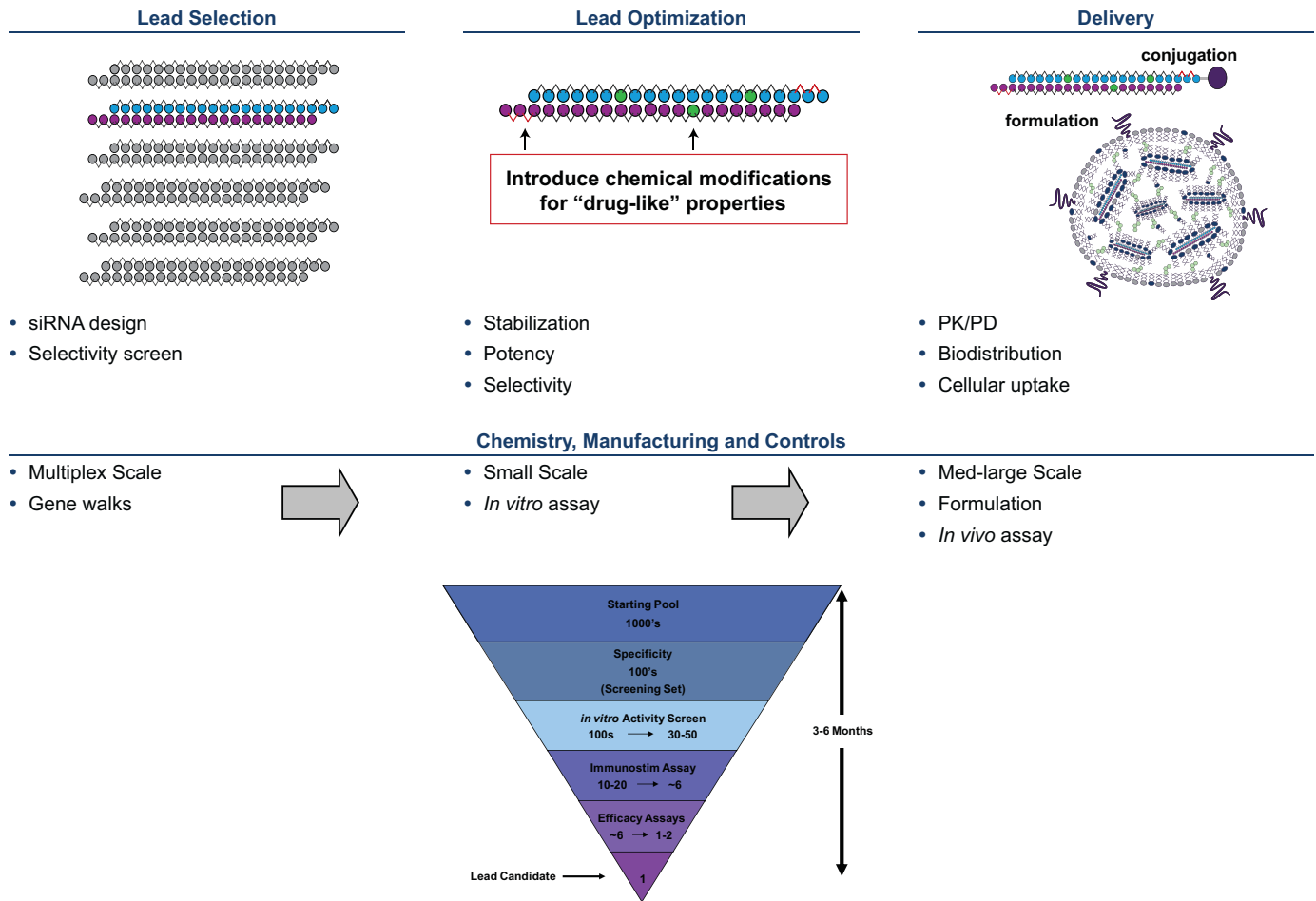
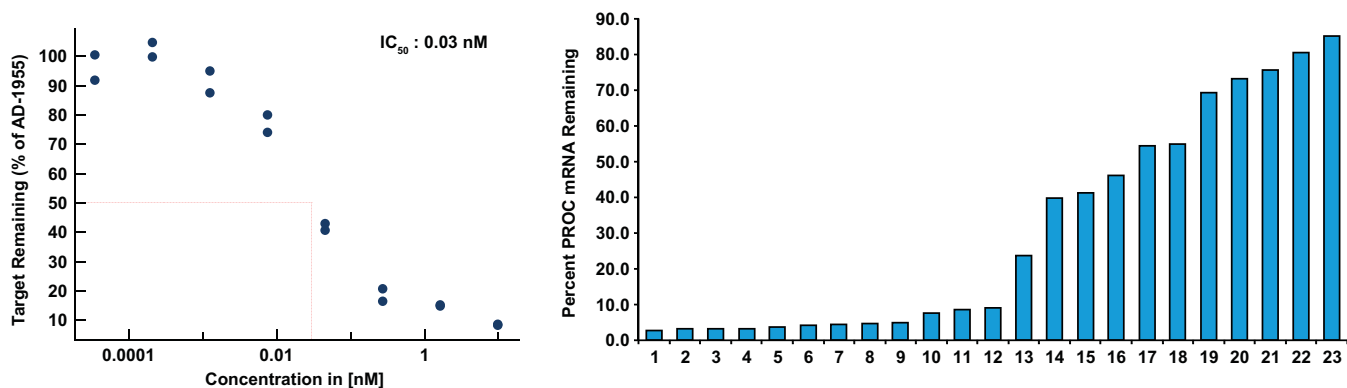
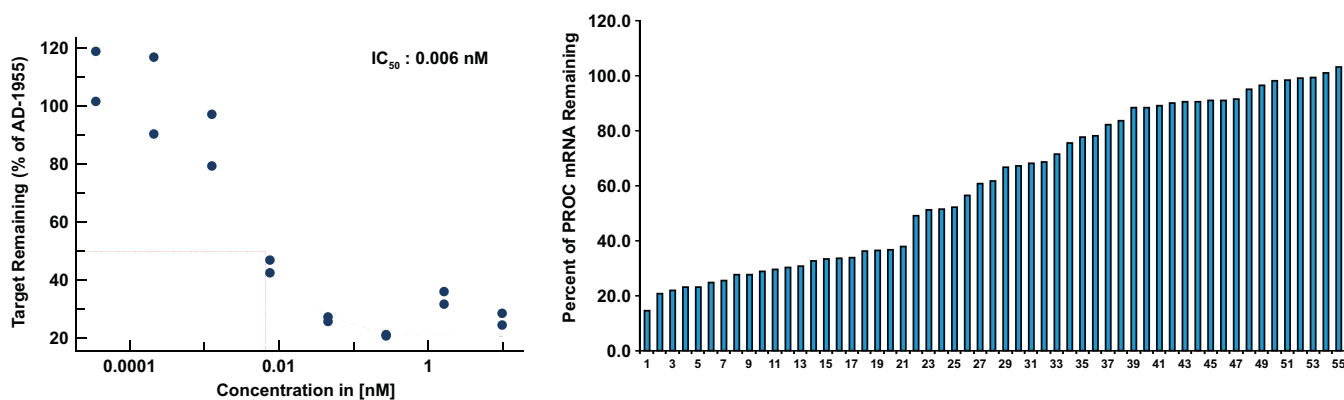


Figure 4. Protein C siRNA lead selection

A Selection of protein C rodent sequences *in vitro*



B Selection of protein C human/cynomolgus sequences *in vitro*



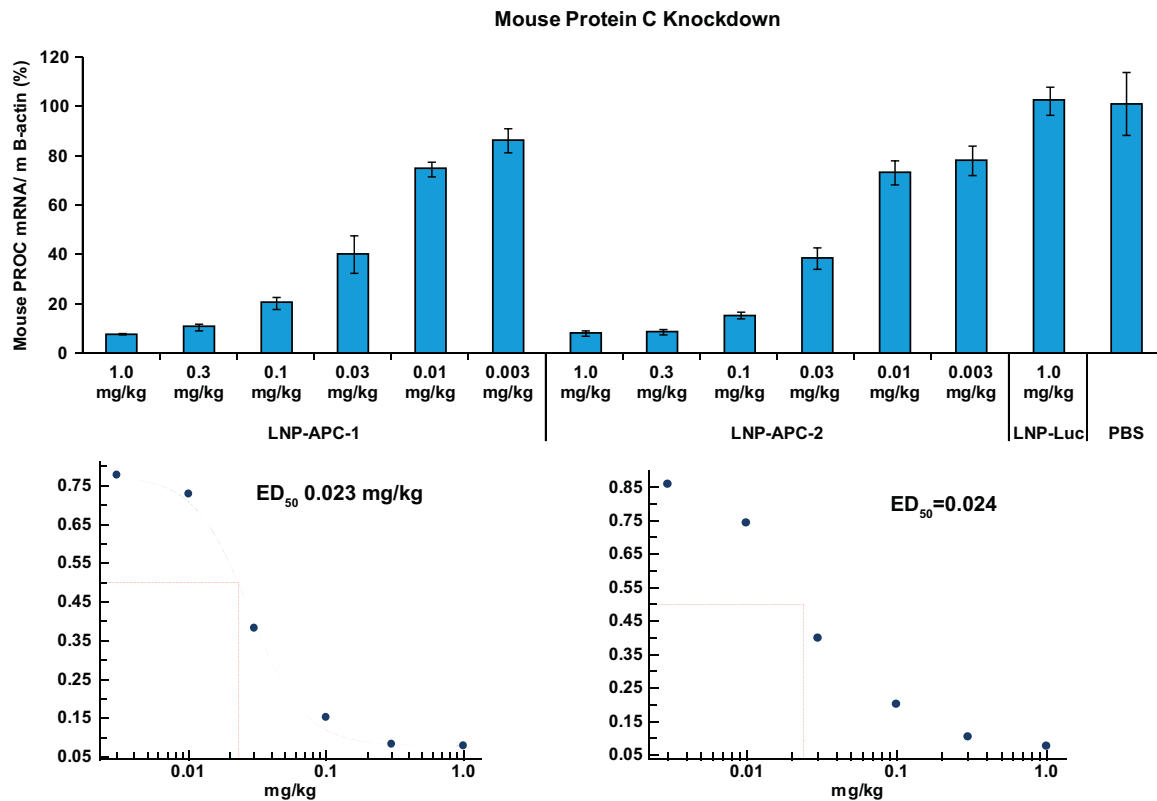
Protein C siRNA lead selection. We have designed potent siRNA sequences against human, cynomolgus monkey and mouse and rat PROC mRNA. Chemically-modified siRNAs were screened by transfection in tissue culture cells. The human/cynomolgus sequences were screened in HEP3B hepatoma cells. The mouse/rat sequences were screened in primary mouse hepatocytes.

(A) Selection of protein C rodent sequences *in vitro*. The waterfall graph represents *in vitro* screening of rodent sequences at single dose of 10 nM in primary mouse hepatocytes using RNAiMax transfection reagent. Potent sequences were screened for IC_{50} in primary hepatocytes. Typically two highly potent sequences with a single picomolar IC_{50} and minimal off-target effect are selected for further investigation in animal models. The IC_{50} curve of the rodent lead molecule is presented- IC_{50} is 30 pM.

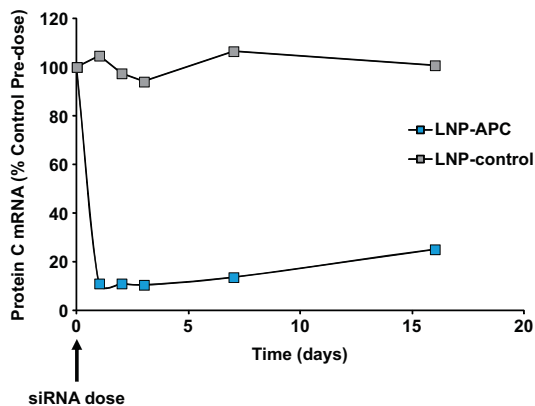
(B) Selection of protein C human/cynomolgus sequences *in vitro*. The waterfall graph represents *in vitro* screening of human/cynomolgus sequences at single dose of 10 nM in HEP3B using RNAiMax transfection reagent. Potent sequences were screened for IC_{50} in HEP3B cells. The IC_{50} curve of the human/cynomolgus lead siRNA is presented- IC_{50} is 6 pM. We achieve potent silencing in wild-type C57BL/6 mice after single dose of 0.3 mg/kg that is durable for weeks. 24h post dose mRNA is reduced by 90%. Reduction of more than 75% was sustained more than two weeks. 7 week old male C57BL/6 were injected siRNAs, formulated in lipid nano particles (LNP). Animals received a single dose via tail vein IV of LNP-APC-1 or LNP-Luc control (siRNA targeting the non-mammalian gene Luciferase) or PBS. Animals were sacrificed at indicated times post dose. Liver mRNA expression was performed using Taqman analyses. N=10 per group. Data represent mean +/- s.d.

Figure 5. Protein C siRNA rodent lead selection *in vivo*

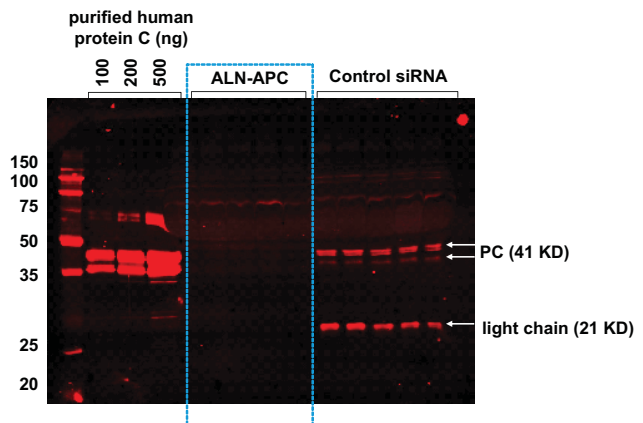
A



B Potent and durable PROC silencing



C Significant reduction in plasma protein C levels



Protein C silencing in wild-type C57BL/6 mice is potent and durable. (A) We achieve potent silencing in wt C57BL/6 with multiple siRNAs with ED₅₀ 20 ug/kg. 7 week old male C57BL/6 were injected siRNAs, formulated in lipid nanoparticles (LNP). Animals received a single dose via tail vein IV of LNP-APC-1 or -2 or LNP-Luc control (siRNA targeting the non-mammalian gene luciferase) or PBS. Animals were sacrificed 24h post dose. Liver mRNA expression was performed using Taqman analyses. N=5 per group. Data represent mean +/- s.d. **(B)** Protein C siRNA demonstrates potent suppression of mRNA after single dose of 0.3 mg/kg that is durable for weeks. **(C)** ALN-APC virtually eliminates protein C protein from circulation three days post single dose with 0.3 mg/kg

Conclusions

- ALN-APC is a novel siRNA lipid nanoparticle formulation being developed for treatment of Hemophilia
- Potent siRNAs, with single-digit pM IC₅₀s, targeting protein C have been generated
- Efficacy of ALN-APC has been demonstrated in wild type mice
 - Dose dependent silencing of PROC mRNA with maximum silencing of greater than 90% and ED₅₀ of approximately 20 ug/kg was achieved
 - Silencing is durable with more than 75% reduction of PROC mRNA levels for more than two weeks
 - Reduction of mRNA to 10% of control levels leads to virtually undetectable levels of circulating protein C protein, based on Western blot
- Studies of ALN-APC in mouse models of Hemophilia are in progress

References

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Conflict of Interest

IT, TR, JB, JA, JH, SM, BRB, DWS, DB, JM, AV, AA are employees and/or stockholders of Alnylam Pharmaceuticals

ZC has no relevant conflict of interest to disclose