RNAi-Mediated Inhibition of Tmprss6 Ameliorates Anemia and Secondary Iron Overload in a Mouse Model of β-Thalassemia Intermedia and Decreases Iron Overload in Hfe<sup>−/−</sup> Mice

PJ Schmidt,<sup>1</sup> AK Sendamarai,<sup>1</sup> I Toudjarska,<sup>2</sup> T Racio,<sup>2</sup> JS Butler,<sup>2</sup> MD Fleming,<sup>1</sup> D Burmicot<sup>2</sup>

<sup>1</sup>Department of Pathology, Children's Hospital Boston and Harvard Medical School, Boston, MA; <sup>2</sup>Alnylam Pharmaceuticals Inc., Cambridge, MA, USA
β-Thalassemia Intermedia (TI), an inherited hemoglobinopathy caused by partial loss of β-globin synthesis, is characterized by anemia, extramedullary hematopoiesis and ineffective erythropoiesis as well as secondary iron overload. Hereditary hemochromatosis (HH) is most frequently caused by mutations in HFE and is marked by excess uptake of dietary iron with concomitant tissue iron overload. In both diseases, increased iron absorption is due to suppressed levels of the liver hormone, Hepcidin (encoded by HAMP). The membrane serine protease Matriptase-2 (encoded by Tmprss6) attenuates BMP-mediated HAMP induction by cleaving the BMP co-receptor, Hemojuvelin. Previously, it has been shown that elevating HAMP expression by genetic inactivation of Tmprss6 reduces disease severity in the Hbb\textsuperscript{αα−/−} mouse model of TI and prevents iron overload in Hfe\textsuperscript{−/−} mice.\textsuperscript{11} Therefore, a therapeutic approach comprising specific inhibition of Tmprss6 could prove efficacious in TI and HH.

Here we show that systemic administration of a potent lipid nanoparticle (LNP) formulated siRNA directed against Tmprss6 leads to >80% inhibition of Tmprss6 mRNA in the livers of Hbb\textsuperscript{αα−/−} and Hfe\textsuperscript{−/−} mice with concomitant >2-fold elevation in HAMP expression. In the TI model mice, Tmprss6 silencing leads to ~30% reductions in serum iron and non-heme liver iron. In Hfe\textsuperscript{−/−} mice, serum iron and non-heme liver iron are similarly reduced, and Perl staining of peri-portal iron is diminished. Remarkably, the partial iron restriction induced by Tmprss6 inhibition in Hbb\textsuperscript{αα−/−} mice leads to dramatic improvements in the hematological aspects of the disease phenotype. Thus, the severity of the anemia is decreased as evidenced by an approximately 1 g/dL increase in total hemoglobin and a 50% decrease in circulating Erythropoietin levels. As in the human disease, Hbb\textsuperscript{αα−/−} mice exhibit the hallmarks of ineffective erythropoiesis including sphenomegaly, decreased erythrocyte survival and marked reticulocytosis. Treatment with LNP formulated Tmprss6 siRNA leads to a dramatic 2-3 fold decrease in spleen size, a 3-4 fold decrease in reticulocyte counts and a >7-day increase in RBC half-life. Histological analysis of spleens from Tmprss6 siRNA treated animals reveals a restoration of normal splenic architecture, as well as a reduction in the number of Tfr1-positive erythrocyte precursors in the spleen. The overall quality of erythropoiesis in treated animals is improved as evidenced by the near normalization of blood smears.

Taken together, these data demonstrate that RNAi-mediated silencing of liver Tmprss6 elevates HAMP expression and reduces iron overload in both TI and HH model mice. More significantly, Tmprss6 siRNA treatment significantly ameliorates all aspects of the disease phenotype in the TI mouse model. These results support the development of an RNAi therapeutic targeting Tmprss6 for the treatment of TI, HH and potentially other disorders characterized by excess iron absorption.
Results

Figure 1. TMPRSS6: A negative regulator of the iron regulatory hormone Hepcidin

Hamp1, the gene encoding Hepcidin, is positively regulated by a specialized branch of the BMP/SMAD signaling pathway. The membrane protein Hemjuvin (HJV) is a BMPR co-receptor, unique to the Hepcidin regulatory pathway, that positively regulates Hamp1 mRNA expression. TMPRSS6, a membrane bound serine protease, negatively regulates Hamp1 mRNA expression by cleaving HJV, thereby rendering the BMPR complex inactive. The Hepcidin expression pathway is also positively regulated by the HFE protein. HFE is a membrane bound receptor that, in the presence of holo-transferrin, activates Hamp1 expression via the BMP/SMAD signalling pathway. In hereditary hemochromatosis (HH), genetic defects in Hfe, the gene encoding for the HFE protein, abrogate its function, thereby rendering it a less potent activator of Hamp1 mRNA expression. Figure adapted from reference 3.
Figure 2. Suppression of Tmprss6 mRNA by a single dose of LNP-Tmprss6 increases Hamp1 mRNA expression and reduces transferrin saturation

(A) Wild-type (WT) mice were intravenously (IV) administered varying concentrations of lipid nanoparticle formulated Tmprss6 siRNA (LNP-Tmprss6) or a control siRNA targeting the luciferase gene (LNP-Luc). After 24 hours of treatment, Tmprss6 and Hamp1 mRNA were quantified by real-time quantitative PCR (qPCR) using β-actin (Actb) mRNA levels as an internal reference standard. To compare relative mRNA levels between different dose levels, the mRNA levels described above were normalized to the respective mean relative mRNA levels measured in the control LNP-Luc treated animals. (B) Four-week time course analysis of Tmprss6 mRNA, Hamp1 mRNA, and transferrin saturation (%) in WT mice after a single IV injection of PBS, control formulation LNP-Luc (0.3 mg/kg), or LNP-Tmprss6 (0.3 mg/kg). Ratios are expressed ± SEM; n=5 in each group.
In experiments evaluating the effect of LNP-Tmprss6 in HH and TI mouse models (Hfe<sup>P<sup>1</sup> and Hbb<sup>TI</sup>, respectively), six-week-old female mice received either one (Two Week) or three (Six Week) IV injections of the respective test article (PBS, LNP-Luc, or LNP-Tmprss6). In both experimental paradigms, animals were harvested 14 days after the final dose administered in the respective experiment.
Six-week-old WT or HH (Hfe-) female mice were IV administered PBS, control formulation LNP-Luc, or LNP-Tmprss6 (1 mg/kg). Mice received either one injection (Two Week) or three injections (Six Week) according to the schedules described in Figure 3. Relative Tmprss6 (A) and Hmpt1 (B) mRNA were quantified and expressed as described in Figure 2 (n=4 or 5 for each genotype). Analysis of (C) serum transferrin saturation, (D) serum iron, (E) non-heme liver iron, and (F) total spleen iron are depicted (mean value ± SEM, n=5 for each genotype). p-values were calculated using Student’s t-test. ****p < 0.001, ***p < 0.005, *p < 0.01 and +p < 0.05. (G) Analysis of liver non-heme iron deposition by Perls Prussian blue stain (10X magnification).
Figure 5. Administration of LNP-Tmprss6 induces microcytic anemia in the HH mouse model

(A) Hemoglobin, (B) mean cell volume (MCV) and (C) reticulocyte mean cell hemoglobin (CHR) were measured in female WT and HH (Hfe<sup>-/-</sup>) mice using the same treatment schedule as described in Figure 4. Data are presented as mean ± SEM; n=5 for each genotype. p values were calculated using Student’s t test. ****p < 0.001, ***p < 0.005, **p < 0.01 and *p < 0.05.
Figure 6. LNP-Tmprss6 diminishes secondary iron overload in T1 mouse model

Six-week-old female WT or T1 (Hbb^t10) mice were administered PBS, control formulation LNP-Luc (1 mg/kg), or LNP-Tmprss6 (1 mg/kg). Mice received either one injection (Two Week) or three injections (Six Week) according to the schedules described in Figure 3. Tmprss6 (A) and Hamp1 (B) mRNA was quantified and expressed as described in Figure 2 (n=4 or 5 for each genotype). Analysis of (C) serum transferrin saturation (%), (D) serum iron (μg/dl), (E) non-heme liver iron and (F) total spleen iron are depicted (mean values ± SEM, n=5 or 6 for each genotype). p-values were calculated using Student’s t-test. ****p < 0.0001, ***p < 0.001, **p < 0.01 and *p < 0.05.
Figure 7. LNP-Tmprss6 ameliorates anemic status in T1 mouse model

The red blood cell parameters serum hemoglobin (A), mean cell volume (B), and reticulocyte mean cell hemoglobin, CHr (C) for animals treated as described in Figure 6. Serum erythropoietin (EPO) levels, a measure of erythropoietic drive, are depicted (D). Data are presented as mean ± SEM, n=5 or 6 for each genotype. p values were calculated using Student’s t test. ****p < 0.001, ***p < 0.005, **p < 0.01 and *p < 0.05.
Figure 8. LNP-Tmprss6 ameliorates anemic status by improving RBC quality

(A) Total spleen weight, (B) red blood cell count (RBC), and (C) reticulocyte count (Retic) of animals treated as described in Figure 6 are shown. (D) RBC survival lifetime of mice in various treatment groups (WT, LNP-Luc, n=5; WT, LNP-Tmprss6, n=4; Hbb\textsuperscript{α/α}, LNP-Luc, n=2; Hbb\textsuperscript{α/α}, LNP-Tmprss6, n=3). Lifetime measurements made using a biotinylated probe and streptavidin-Alexa488 detection system. In contrast to the experiments described in Figure 6, mice in this experiment received a total of 4 doses, once every two weeks (two doses prior to RBC collection and two more doses during RBC observation period). (E) RBC membrane-bound globins were analyzed using TAU gel electrophoresis. The number below each lane, the Hb-α/Hb-β ratio, is a measure of the relative intensity of α-globin (Hb-α) as compared to β-globin (Hb-β). Globins from the WT soluble fraction have been employed as a standard. Data are presented as mean ± SEM. P values were calculated using Student’s t test. ****p < 0.0001 and ***p < 0.005. (F) Histological analysis of RBCs by Wright-Giemsa staining of whole blood from WT and Hbb\textsuperscript{α/α} animals treated for 14 or 42 days (Two Week and Six Week, respectively) with the indicated test article (100X magnification).
Summary

- LNP-Tmprss6 is a novel siRNA formulation developed for iron overload disorders characterized by abnormally low Hepcidin levels such as β-Thalassemia Intermedia (TI) and Hereditary Hemochromatosis (HH)
  - Designed to increase Hepcidin levels by suppressing the expression of Tmprss6, a negative regulator of Hamp1 expression in hepatocytes
- LNP-Tmprss6 ameliorates secondary iron overload in mouse models of HH and TI
  - RNAi-mediated suppression of Tmprss6 mRNA levels leads to increased Hamp1 mRNA expression, ultimately leading to reductions in transferrin saturation, serum iron concentration, and non-heme liver iron concentration
- In the TI mouse model, LNP-Tmprss6 ameliorates the anemic state, improves hematological parameters, and reduces extramedullary hematopoiesis
  - Increase in total serum Hemoglobin levels
  - Reduction in hypoxia, as inferred from a reduction in serum EPO levels
  - Reduction in ineffective erythropoiesis, as inferred from the reduction in reticulocyte count
  - Normalization of RBC morphology and lifespan
  - Correction of Hb-a/Hb-β imbalance
- These results demonstrate that RNAi-mediated suppression of Tmprss6 may be a generally viable therapeutic strategy for treating iron overload disorders associated with abnormally low Hepcidin levels
  - The added benefits of Tmprss6 reduction observed in the TI mouse model suggest this approach may be particularly well-suited to treating individuals with β-Thalassemia Intermedia
- Future direction:
  - Further dissect mechanism underlying hematological improvement in the TI mouse model following RNAi-mediated suppression of Tmprss6
  - Develop an siRNA-GalNAc conjugate suitable for once weekly subcutaneous administration

References


Disclosures

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