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Citation

Published Version
doi:10.1002/ajh.23934

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Combination therapy with a Tmprss6 RNAi-therapeutic and the oral iron chelator deferiprone additively diminishes secondary iron overload in a mouse model of β-thalassemia intermedia

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β-thalassemias result from diminished β-globin synthesis and are associated with ineffective erythropoiesis and secondary iron overload caused by inappropriately low levels of the iron regulatory hormone hepcidin. The serine protease Tmprss6 attenuates hepcidin production in response to iron stores. Hepcidin induction reduces iron overload and mitigates anemia in murine models of β-thalassemia intermedia. To further interrogate the efficacy of an RNAi-therapeutic downregulating Tmprss6, β-thalassemic Hbbthalassemic animals on an iron replete, an iron deficient, or an iron replete diet also containing the iron chelator deferiprone were treated with Tmprss6 siRNA. We demonstrate that the total body iron burden is markedly improved in Hbbthalassemic animals treated with siRNA and chelated with oral deferiprone, representing a significant improvement compared to either compound alone. These data indicate that siRNA suppression of Tmprss6, in conjunction with oral iron chelation therapy, may prove superior for treatment of anemia and secondary iron loading seen in β-thalassemia intermedia.

Introduction

The thalassemias result from a quantitative defect in the production of α- or β-globin chains. β-Thalassemia is caused by the partial or complete lack of β-globin synthesis resulting in a variably severe anemia and/or red blood cell abnormalities. In its most severe form, β-thalassemia major, affected patients have profound secondary iron overload largely due the requirement for chronic red blood cell transfusional support. Patients with β-thalassemia intermedia, a less severe form, do not require chronic transfusions (i.e., nontransfusion dependent thalassemia), but still develop iron overload as a consequence of chronic suppression of the synthesis of the iron regulatory hormone hepcidin by ineffective erythropoiesis [1,2]. In both cases, complications of iron overload, including liver disease, cardiomyopathy, and endocrinopathies, are the most significant contributors to morbidity and mortality in these individuals. A mouse model of β-thalassemia intermedia, Hbbthalassemic, has a heterozygous deletion of both the β-minor and β-major hemoglobin genes [3]. Similar to their human counterparts, these animals have moderately severe, transfusion-independent hypochromic, microcytic anemia associated and ineffective erythropoiesis resulting in splenomegaly, hepcidin suppression, and secondary iron overload [4–7].

Tmprss6 (matriptase-2) is a serine protease expressed predominantly in the liver that down-regulates hepcidin transcription by inhibiting an iron-responsive bone morphogenetic protein-mothers against decapentaplegic (BMP-SMAD) signaling pathway [8]. Germline mutations in Tmprss6 in both mice and humans result in iron-refractory iron deficiency anemia (IRIDA) due to chronic inappropriate overexpression of hepcidin relative to liver iron stores [9]. Modulating hepcidin expression by targeting the BMP-SMAD signaling pathway in hepatocytes may be an effective modality for treating iron disorders, particularly those associated with a relative hepcidin deficiency or excess. For example, deletion of Tmprss6 in the Hfe cell mouse model of hereditary hemochromatosis results in elevated expression of hepcidin, leading to an amelioration of the iron overload phenotype [10]. Likewise, induction of hepcidin or iron deficiency in β-thalassemic animals using several different methods not only mitigates the iron overload, but also ameliorates the anemia through an incompletely understood mechanism [11–13]. Most recently, we, and others, have demonstrated that suppression of Tmprss6 mRNA expression with lipid nanoparticle (LNP)-formulated siRNAs or antisense oligonucleotides (ASOs) increases hepcidin, leading to diminished iron uptake and recycling and improved erythropoiesis and anemia in β-thalassemic

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Additional Supporting Information may be found in the online version of this article.

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Conflict of interest: TR, JSB, and KF are employees and/or stockowners of Alnylam Pharmaceuticals; MDF receives research funding from Alnylam Pharmaceuticals; MW is a founder, chief executive officer, and stock owner in Intrinsic LifeSciences; PJS has no relevant conflict of interest.

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Contract grant sponsor: Alnylam Pharmaceuticals.

Received for publication: 22 December 2014; Accepted: 24 December 2014


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animals [14,15]. These preclinical data suggest that modulation of hepcidin expression could be a substitute for or an adjunct to iron chelation therapy in patients with thalassemia. To further investigate this possibility, we treated Hbb\(^{th3/1}\) animals with an LNP-formulated RNAi therapeutic targeting Tmprss6 in combination with deferiprone, an orally bioavailable iron chelator [16]. We found that siRNA combined with oral deferiprone therapy is superior to monotherapy with dietary iron deficiency, iron chelator, or Tmprss6 siRNA for the reduction of hepatic iron stores, while still maintaining some of the beneficial effects on anemia.

### Methods

Other than as noted below, all mouse strains and methods are identical to those previously described [14]. Mice were born and housed in the barrier facility at Boston Children’s Hospital under approved protocols, and were raised on Prolab RMH 3000 diet (Lab Diet, 380 ppm iron) until 6 weeks of age, when the diet was switched to an iron replete (50 ppm) control diet, iron deficient (3–5 ppm iron), or control diet supplemented with deferiprone (50 ppm iron, 0.125% deferiprone). A: Liver Tmprss6 mRNA assessed by quantitative real-time PCR and normalized to β-actin (Actb). B: ELISA serum hepcidin analysis (ng/ml). C: Serum transferrin saturation (%). D: Serum iron (μg/dl). E: Nonheme liver iron (μg/g). F: 3,3'-diaminobenzidine (DAB)-enhanced iron stain of liver sections. G: Total spleen iron (μg). Error bars are ±SEM. (n = 4–6 for each group) Student's t-test P-values: ****P < 0.001, ***P < 0.005, **P < 0.01, and *P < 0.05. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### Results

LNP-Tmprss6 siRNA plus oral deferiprone therapy diminishes secondary iron overload in murine β-thalassemia intermedia

We determined whether modulating systemic iron stores by dietary restriction or pharmacological chelation could add to the beneficial effects of Tmprss6 siRNA therapy on Hbb\(^{th3/1}\) anemia and iron phenotypes. To do so, we treated wild type and Hbb\(^{th3/1}\) animals with biweekly LNP-Tmprss6 siRNA injections in conjunction with an iron
replete synthetic control diet (50 ppm iron), a low iron diet (3–5 ppm iron), or an iron replete diet containing the orally bioavailable chelator deferiprone (50 ppm iron, 0.125% wt/wt chelator). In wild-type animals, we observed qualitatively similar effects on gene expression and iron metabolism, including $\text{TMPRSS6}$ suppression and the induction of systemic iron deficiency, as we did previously (Supporting Information Fig. 1) [14]. As expected, LNP-$\text{TMPRSS6}$ treatment of $\text{HBBth3/1}$ animals on all diets suppressed $\text{TMPRSS6}$ mRNA expression and up-regulated serum hepcidin (Fig. 1A,B). Furthermore, treated animals had decreased transferrin saturation and serum iron (Fig. 1C,D), with the largest and most significant changes seen in animals on the diet containing deferiprone. When compared to control siRNA-treated animals on the same diet, nonheme liver iron was diminished only in animals treated with deferiprone (Fig. 1E), possibly due to the relatively low iron (50 ppm), which is only approximately two times the recommended adult murine daily requirement, in the formulated test diets compared to conventional mouse chow (380 ppm iron) that we employed previously [14]. In comparison to the control diet, LNP-$\text{TMPRSS6}$ treated animals on either an iron deficient diet or diet with added chelator had lower liver iron levels, with the latter having a greater effect. In $\text{HBBth3/1}$ animals, this change in liver iron can be visualized on DAB-enhanced Perls’ stained tissue sections as decreased periportal hepatocellular iron (Fig. 1F).

LNP-$\text{TMPRSS6}$ siRNA plus oral deferiprone therapy improves erythropoiesis and splenomegaly in murine $\beta$-thalassemia intermedia

In earlier studies, we observed that $\text{TMPRSS6}$ siRNA treatment induces a mild microcytic, hypochromic anemia in wild-type animals [14]. We observed the same phenotype here, regardless of the diet employed (Supporting Information Fig. 2). However, in slight contrast to published studies [11], treatment of $\text{HBBth3/1}$ mice with an

Figure 2. Erythropoietic parameters in $\text{HBBth3/1}$ animals treated with LNP-$\text{TMPRSS6}$ siRNA and dietary iron deficiency or chelation. Animals were treated as in Figure 1. A: Hemoglobin (g/dl). B: Red blood cell count. C: Mean red blood cell volume (fl). D: RBC morphology on Wright’s stained peripheral blood smears. E: Fraction of reticulocytes (%). F: Absolute reticulocyte count. G: Serum erythropoietin levels (pg/ml). H: Spleen weight (g). Error bars are ±SEM. (n = 4–6 for each group) Student’s t-test $P$-values: ****$P < 0.001$, ***$P < 0.005$, **$P < 0.01$, and *$P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
experiments were conducted. For example, it is possible that the rela-
more iron-restricted baseline dietary conditions under which the current
on erythropoiesis, it is likely that many of the deviations are due to the
Figure 2D). These changes are readily appreciated on peripheral
blood smears, which demonstrate replacement of the anisopikilocytosis of
the baseline phenotype with a relatively monotonous population of
evenly sized and shaped hypochromic microcytes (Fig. 2D).

Despite the relative beneficial effect of the addition of deferiprone to
LNP-Tmprss6 treatment on the Hbb\textsuperscript{th3/1} anemia, some parameters sug-
gest that the addition of chelator at the dose employed mitigates some
of the other positive effects of hepatic-induced iron restriction. For
example, while the fraction of RBCs that are reticulocytes (Fig. 2E) is
deprecated, there is no decrease in the absolute number of reticulocytes
(Fig. 2F).

For the moment, however, despite the decrease in serum erythropoietin
is most significant in deferiprone-treated animals, changes in spleen size
are not as profound as in animals on an iron deficient diet alone (Fig.
2G,H). Finally, consistent with the reduction in splenomegaly, there was
partial restoration of splenic architecture in LNP-Tmprss6 treated ani-
also maintained on deferipron (Supporting Information Fig. 3).

Discussion

Taken together, these data demonstrate that combined LNP-Tmprss6
siRNA and oral deferiprone chelator therapy is superior in reducing
hepatic iron stores compared to dietary iron deficiency with or without
LNP-Tmprss6 treatment, or chelator therapy alone. However, in this
murine model and at these siRNA and chelator dosages, the addition of
chelation appears to partially attenuate several of the ancillary beneficial
effects, including amelioration of the anemia and splenomegaly, observed
in previous studies. Although this could be a direct effect of the chelator
on erythropoiesis, it is likely that many of the deviations are due to the
more iron-restricted baseline dietary conditions under which the current
experiments were conducted. For example, it is possible that the rela-
tively high concentrations of iron found in most mouse chis are inhib-
itory to thalassemic erythropoiesis, thus worsening the baseline
phenotype and exaggerating any positive effects of hepatic induction
therapy. On the other hand, there is also evidence that too much hep-
idin and iron restriction is equally detrimental to thalassemic hematologi-
cal phenotypes [11]. For this reason, it will be prudent ultimately, if this
is to be translated to the clinic, to titrate both modalities to each patient
to achieve individualized chelation and hematological effects.

In addition to the beneficial effects on the anemia, combination ther-
apy with long-acting hepatic induction therapy, such as TMPRSS6
siRNA, has several distinct theoretical advantages over chelation alone.
First, hepatic-induced down-regulation of ferroportin sequesters iron in
macrophages may limit its toxicity by reducing plasma “free” (e.g., non-
transferrin bound) iron, particularly when plasma chelators are at their
nadir between doses. Second, the effect of hepaticin on ferroportin
expression in duodenal enterocytes responsible for iron absorption limits
the ongoing absorption of iron—an effect not achieved by chelators
alone. This mechanism of action will likely be critical in individuals with
nontransfusional iron overload, where intestinal absorption due to hepici-
din suppression is the key pathogenetic mechanism. Nevertheless, even
in the transfusion-dependent patient, it has been shown that hepatic
levels, which are ordinarily increased, vary pre- and post-transfusion;
hepatic levels increase after transfusion, presumably due to the suppres-
sion of hepatic synthesis by anemia, hypoxia, and ineffective hemato-
poiesis that increase as the hemoglobin drops in the interval between
transfusions [2,18]. Thus, while the physiological changes induced by
hepatic induction therapy, as with TMPRSS6 siRNA, are ideally suited
to the patient with transfusion-independent, iron-loading anemias, they
may equally benefit patients on chronic transfusion programs.

Acknowledgments

Authors would like to thank Dean Campagna and Daniel Kierstead
for excellent technical assistance and members of the Fleming labora-
tory for helpful discussions.

Author Contributions

PS, JSB, KF and MDF conceived and designed the murine experi-
ments; PJS analyzed the mouse data and wrote the manuscript; TR
completed the tail injections; and JSB, KF and MDF designed
research and wrote the manuscript. MW and colleagues developed
and performed the serum hepatic assays.

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