Oral administration of a bone morphogenetic protein type I receptor inhibitor prevents the development of anemia of inflammation

Anemia of inflammation (AI), the second most frequent form of anemia, complicates inflammatory and chronic diseases.[1] Although typically mild to moderate, AI is associated with increased morbidity and mortality.[1] One of the mechanisms contributing to the development of AI is a persistent stimulation of hepcidin synthesis, a critical regulator of iron homeostasis, by inflammatory cytokines, including interleukin-6 (IL-6).[2] Hepcidin synthesis occurs mainly in the liver and is predominantly regulated at a transcriptional level.[2] Bone morphogenetic protein (BMP) signaling induces transcription of the gene encoding hepcidin (HAMP).[3] Activation of the BMP pathway induces the phosphorylation of regulatory SMAD proteins (SMADs 1, 5, and 8), which activate the expression of target genes, such as the genes encoding the inhibitor of DNA binding 1 (ID1) and hepcidin.[2,4] Treatment of AI is typically directed to curing the underlying disease, which is not always feasible. Current adjuvant therapies, including intravenously-administered iron, erythropoiesis-stimulating agents, and erythrocyte transfusions, have limited efficacy and important side effects.[5-7] Research has focused on the development of new therapeutic agents targeting hepcidin,[8] including inhibitors of hepcidin synthesis. Among them, LDN-193189, a BMP type I receptor inhibitor that is a derivative of dorsomorphin, has been reported to increase hemoglobin levels in animal models of AI.[9-11] However, in experimental anemia models, LDN-193189 was administered by intraperitoneal injection, which may limit its applicability to treatment of patients with AI.

We sought to determine if LDN-193189 is biologically available when administered orally and whether oral administration of LDN-193189 is sufficient to prevent the development of AI. Although other dorsomorphin derivatives, such as DMH1, have been developed, we chose to study LDN-193189 because its pharmacokinetics[12] and efficacy after parenteral administration have been studied by several groups in different disease models, including anemia of inflammation in mice.[9-11]

To evaluate the *in vivo* disposition kinetics and oral bioavailability of LDN-193189, mice received a single dose of LDN-193189 (3 mg/kg) by gavage or intravenously (IV) by tail vein injection, and blood was collected after various times to measure serum LDN-193189 levels (Table 1 and *Online Supplementary Appendix*). Following a single IV administration, LDN-193189 had a moderate clearance (CL) of 39 mL/min/kg and a large steady-state volume of distribution (V_{dss}) of 6.9 L/kg, suggesting that LDN-193189 penetrates extensively into tissues. As expected, the peak concentration (C_{max}) was higher when LDN-193189 was administered intravenously than when it was administered orally. The area under the concentration-time curve from time zero to infinity ($AUC_{(0\text{-}inf)}$) was greater when LDN-193189 was administered by IV injection than when it was orally-administered. As anticipated, the half-life ($t_{1/2}$) of serum LDN-193189 levels was not impacted by the route of administration.

To assess the dose-dependency of orally-administered LDN-193189 to increase serum LDN-193189 levels, blood was collected at various times after mice received a dose of LDN-193189 (1, 3, or 10 mg/kg), and serum LDN-193189 concentrations were measured (Table 1). The *in vivo* exposure (C_{max} and $AUC_{(0\text{-}inf)}$) increased in a dose-dependent manner. The half-life of LDN-193189 was independent of the orally-administered dose. Comparison of the $AUC_{(0\text{-}inf)}$s revealed that, after oral administration, LDN-193189 bioavailability was 54%-83%.

To study the tissue exposure of orally-administered LDN-193189, serum, liver, and muscle were harvested at various times after mice received a dose of LDN-193189 (1 mg/kg) by gavage, and LDN-193189 levels were measured (Table 1 and *Online Supplementary Appendix*). The *in vivo* exposure (C_{max} and $AUC_{(0\text{-}inf)}$) was higher in liver than in serum and muscle. The tissue-to-serum AUC ratio was approximately 2.3 for the liver and 0.79 for the muscle. These results suggest that orally-administered LDN-193189 accumulates in the liver.

To confirm that orally-administered LDN-193189 was able to inhibit hepatic BMP signaling, we measured phosphorylation of BMP-responsive Smads (Smads 1 and 5) and expression of genes encoding Id1 and hepcidin. Mice were administered LDN-193189 (1 mg/kg) by gavage, 1-24 h prior to euthanasia (*Online Supplementary Appendix*). Administration of LDN-193189 rapidly reduced Smad1/5 phosphorylation, an effect that persisted up to 4 h (Figure

Table 1. LDN-193189 pharmacokinetic properties.

Administration mode (dose, mg/kg)	IV (3)	PO (10)	PO (3)	PO (1)	PO (1)	PO (1)
		Serum			Liver	Muscle
$AUC_{(0\text{-}inf)}$ (ng°h/mL)	1280	3480	695	356	822	300
$t_{1/2}$ (h)	3.7	3.6	3.7	3.4	3.0	1.7
T_{max} (h)	NA	0.5	0.5	1.5	0.2	0.5
C_{max} (ng/mL serum or ng/g tissue)	1183	683	177	93	227	97
CL (mL/min/kg)	39	NA	NA	NA	NA	NA
V_{dss} (L/kg)	6.9	NA	NA	NA	NA	NA
Bioavailability (%)	NA	82	54	83	NA	NA

C57BL/6 male mice (n=3/group) received a single dose of LDN-193189 either by intravenous injection (IV, 3 mg/kg) or by gavage (PO, 1, 3, or 10 mg/kg). After various durations (0.083-24 h), blood, liver, and muscle were collected to obtain LDN-193189 levels, allowing the measurement of the peak concentration (C_{max}) and the peak time (T_{max}), as well as the calculation of the half-life ($t_{1/2}$), the bioavailability, the area under the concentration-time curve from time zero to infinity ($AUC_{(0\text{-}inf)}$), the clearance (CL), and the volume of distribution (V_{dss}). After IV administration, Cmax was measured 5 min after administration. In tissues, C_{max} was defined by the highest LDN-193189 concentration measured in the liver or muscle tissues within 24 h after LDN-193189 administration. NA: not applicable.

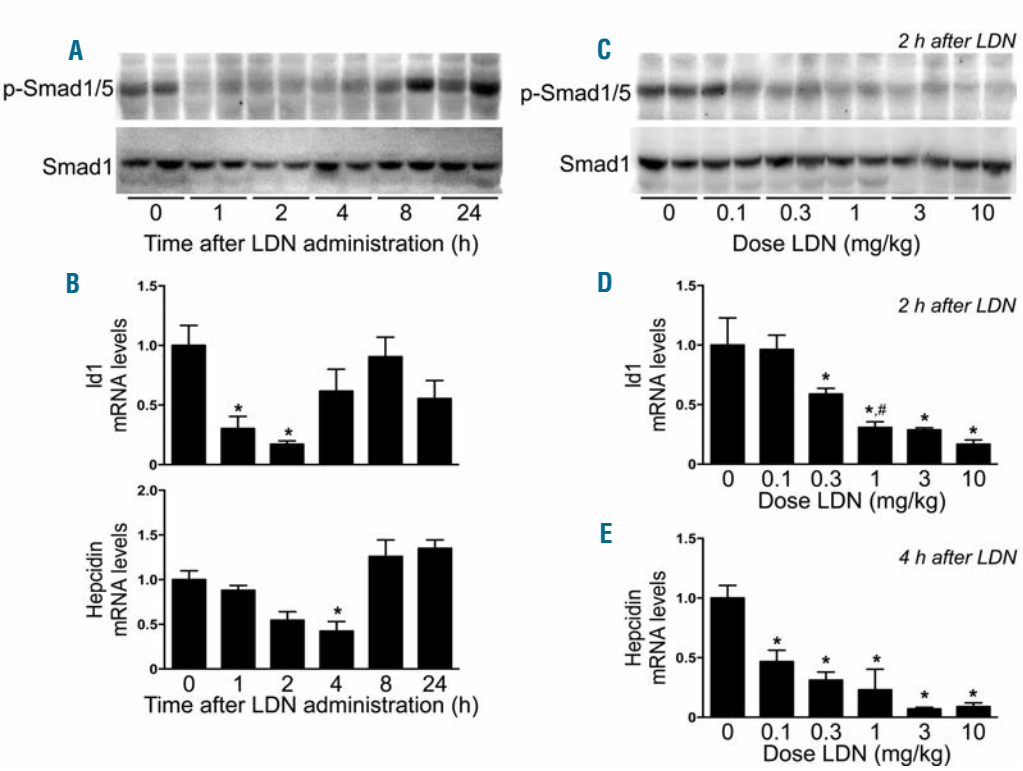

Figure 1. Oral administration of LDN-193189 inhibits hepatic Id1 and hepcidin gene expression in a time- and dose-dependent manner. C57BL/6 female mice (n=3-4/group) received a dose of LDN-193189 (LDN, 1 mg/kg), by gavage, at various times (1- 24 h) prior to sacrifice. After euthanasia, livers were harvested. (A) Proteins were extracted, and levels of total Smad1 and phosphorylated Smad1/5 (p-Smad1/5) were determined by immunoblots. (B) RNA was extracted to measure the levels of mRNAs encoding Id1 (upper panel) and hepcidin (lower panel) by qRT-PCR (one-way ANOVAs $P<0.007$; *$P<0.03$ *vs.* time 0). Two hours after oral administration of a dose of LDN-193189 (doses ranging from 0.1 to 10 mg/kg) or vehicle (citric acid), mice were euthanized, and livers were harvested. (C) Proteins were extracted, and levels of Smad1 and p-Smad1/5 were determined. (D) RNA was extracted, and levels of mRNAs encoding for Id1 were measured by qRT-PCR (one-way Anova $P<0.0001$; *$P<0.04$ *vs.* mice treated with citric acid; #$P<0.05$ mice treated with LDN-193189 0.3 *vs.* 1 mg/kg). (E) Four hours after administration of increasing doses of LDN-193189, mice were euthanized, and livers were harvested. RNA was extracted, and levels of mRNAs encoding for hepcidin were measured by qRT-PCR (one-way Anova $P<0.0001$; *$P<0.002$ *vs.*mice treated with citric acid).

1A). Similarly, orally-administered LDN-193189 decreased hepatic Id1 gene expression after 1 h with a nadir at 2 h, whereas hepcidin mRNA levels progressively decreased and were lowest at 4 h (Figure 1B).

To assess the dose-dependency of orally-administered LDN-193189 to inhibit BMP signaling, mice received LDN-193189 by gavage at doses ranging from 0.1 to 10 mg/kg. Two hours later, mice were sacrificed, and their livers were harvested. LDN-193189 inhibited Smad1/5 phosphorylation at doses above 0.3 mg/kg (Figure 1C). Orally-administered LDN-193189 at doses above 0.3 mg/kg decreased hepatic Id1 gene expression (Figure 1D), whereas hepcidin mRNA levels did not decrease (*data not shown*). Four hours after oral administration of LDN-193189, hepatic hepcidin gene expression was decreased at doses of 0.1 mg/kg or over (Figure 1E). Taken together, these results demonstrate that orally-administered LDN-193189 inhibits hepatic BMP signaling and hepcidin gene expression. Moreover, we observed that hepatic BMP signaling was sensitive to inhibition by oral LDN-193189, with as little as 0.1 mg/kg able to reduce hepcidin mRNA levels. The ability of orally-administered LDN-193189 to inhibit hepatic BMP signaling at such a low dose could be explained by the accumulation of LDN-193189 in the liver, as reflected by the hepatic tissue-to-serum AUC of 2.3.

To determine whether the levels of LDN-193189 achieved after oral administration were sufficient to increase hemoglobin levels in AI, mice were challenged with turpentine, a well-established animal model of AI, which induces hepatic hepcidin gene expression in an IL6-dependent manner.[13] Mice were injected with turpentine weekly for three weeks,[9,14] and were treated daily with LDN-193189 (1 mg/kg) or vehicle (citric acid) by gavage (*Online Supplementary Appendix*). As controls, mice received injections of saline weekly and received vehicle by gavage daily. Because LDN-193189 does not affect erythropoiesis,[9] we did not include a group of mice that received weekly injections of saline and that were treated daily with LDN-193189. Seven days after the last injection of turpentine, and 24 h after the last administration of LDN-193189, mice were anesthetized, blood was collected to measure complete blood counts, serum IL-6 levels and serum iron levels. Mice were then euthanized to collect liver samples to measure hepatic hepcidin gene expression. Mice injected with turpentine developed sterile abscesses and systemic inflammation with increased granulocyte and platelet counts and serum IL-6 levels (Figure 2A). Administration of LDN-193189 did not modulate the development of inflam-

mation, as reflected by similar increases in granulocyte and platelet counts and serum IL-6 levels in turpentine-challenged mice treated with LDN-193189 and vehicle. Mice challenged with turpentine and treated with vehicle developed AI with a decrease in hemoglobin levels, mean corpuscular volumes (MCVs), and erythrocyte counts (Figure 2B). Hemoglobin levels, MCVs, and erythrocyte counts were greater in turpentine-challenged mice treated with LDN-193189 than in turpentine-challenged mice treated with vehicle. Treatment of turpentine-challenged mice with LDN-193189 increased, but did not fully normalize hemoglobin levels. This partial effect of LDN-193189 has been observed in turpentine and other models of AI, when LDN-193189 was given by intraperitoneal injection.[9-11,15]

In turpentine-challenged mice, serum iron levels tended to be lower than in control mice and were not corrected by LDN-193189 treatment (*Online Supplementary Figure S1A*). In contrast, Steinbicker *et al.*[9] reported that, in turpentine-challenged mice, administration of LDN-193189 at a dose of 3 mg/kg increased serum iron levels. These results suggest that a dose of LDN-193189 of more than 1 mg/kg might be required to normalize serum iron levels. A week after the last injection of turpentine, there was no change in hepatic hepcidin gene expression in turpentine-challenged mice treated with vehicle (*Online Supplementary Figure S1B*). These results are consistent with previous observations of Prince *et al.*[14] In addition, hepatic hepcidin mRNA levels were similar in turpentine-challenged mice treated with LDN-193189 or vehicle (*Online Supplementary Figure S1B*). In this study, mice were sacrificed 24 h after the last administration of LDN-193189 when hepatic hepcidin gene expression was no longer inhibited (Figure 1B).

In summary, we report that LDN-193189 is orally bioavailable. Oral administration of a BMP type I receptor inhibitor is effective in inhibiting hepatic BMP signaling and reducing hepcidin gene expression, as well as development of anemia in an animal model of AI. These results suggest that LDN-193189 or other dorsomorphin-related molecules may represent a novel orally-effective therapy for patients with AI.

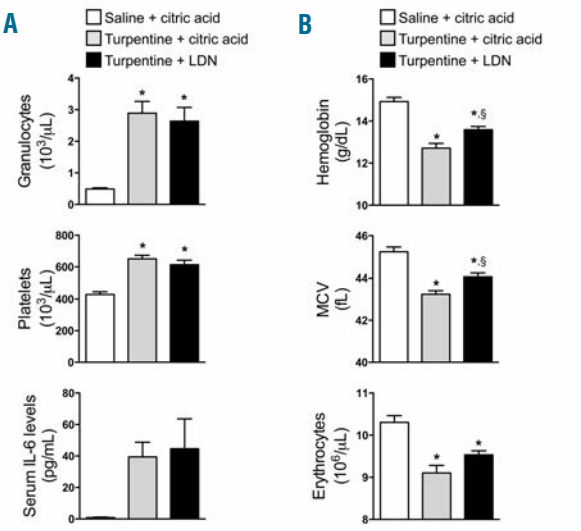

Figure 2. Orally-administered LDN-193189 increases hemoglobin levels in turpentine-challenged mice. Eight-week-old C57BL/6 female mice were injected weekly with turpentine (5 mL/kg, subcutaneously) during three weeks. Simultaneously, mice were treated daily with either LDN-193189 (1 mg/kg, black bars, n=11) or vehicle (gray bars, n=10). White bars represent control female mice challenged with saline weekly and treated with vehicle (citric acid) daily (n=11). Seven days after the last injection of turpentine or saline, mice were sacrificed. Blood was collected to measure complete blood counts and serum IL-6 levels. Turpentine injections induced systemic inflammation with (A) an increase of granulocyte (upper panel) and platelet counts (middle panel) (one-way Anovas *P*<0.0001 and **P*<0.0001 *vs.* control mice), as well as an increase in serum IL-6 levels (lower panel). (B) In vehicle-treated mice, turpentine injections induced AI with a decrease of hemoglobin levels (upper panel), mean corpuscular volumes (MCVs, middle panel), and erythrocyte counts (lower panel) that was attenuated by oral administration of LDN-193189 (one-way Anovas *P*<0.0001 and **P*<0.0001 *vs.* control mice; §*P*<0.006 *vs.* mice injected with turpentine and treated with vehicle).

Claire Mayeur,[1] Starsha A. Kolodziej,[1] Amy Wang,[2] Xin Xu,[2] Arthur Lee,[2] Paul B. Yu,[3] John Shen,[2] Kenneth D. Bloch,[1,4] and Donald B. Bloch[1,5]

[1]Anesthesia Center for Critical Care Research of the Department of Anesthesia, Critical Care, and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA; [2]Therapeutics for Rare and Neglected Diseases (TRND) Program, National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH), Rockville, MD, USA; [3]Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA; [4]Cardiovascular Research Center, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA; and [5]Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

Funding: this work was supported by a grant from NIDDK (R01DK082971 KDB), the NIH BrIDGs Program (1X01NS070702), the NIH TRND Program, and the Fondation LeDucq. The authors thank the Division of Veterinary Research, NIH, for their help in conducting the pharmacokinetics experiments

Correspondence: cmayeur@mgh.harvard.edu

doi:10.3324/haematol.2014.111484

Key words: anemia of inflammation, BMP inhibitor, hepcidin.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Weiss G, Goodnough LT. Anemia of chronic disease. N Engl J Med. 2005;352(10):1011-1023.
2. Ganz T. Systemic iron homeostasis. Physiol Rev. 2013;93(4):1721-41.
3. Babitt JL, Huang FW, Wrighting DM, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet. 2006;38(5):531-539.
4. Nohe A, Keating E, Knaus P, Petersen NO. Signal transduction of bone morphogenetic protein receptors. Cell Signal. 2004;16(3):291-299.
5. Daoud E, Nakhla E, Sharma R. Q: Is iron therapy for anemia harmful in the setting of infection? Cleve Clin J Med. 2011;78(3):168-170.
6. Macdougall IC, Fishbane S, Duliege AM. Peginesatide for anemia in chronic kidney disease. N Engl J Med. 2013;368(16):1553-1554.
7. Swedberg K, Young JB, Anand IS, et al. Treatment of anemia with darbepoetin alfa in systolic heart failure. N Engl J Med. 2013; 368(13):1210-1219.
8. Fung E, Nemeth E. Manipulation of the hepcidin pathway for therapeutic purposes. Haematologica. 2013;98(11):1667-1676.
9. Steinbicker AU, Sachidanandan C, Vonner AJ, et al. Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. Blood. 2011;117(18):4915-4923.
10. Theurl I, Schroll A, Sonnweber T, Nairz M, Theurl M, Willenbacher W, et al. Pharmacologic inhibition of hepcidin expression reverses anemia of chronic inflammation in rats. Blood. 2011;118(18):4977-

4984.
11. Theurl M, Nairz M, Schroll A, et al. Hepcidin as a predictive factor and therapeutic target in erythropoiesis-stimulating agent treatment for anemia of chronic disease in rats. Haematologica. 2014; 99(9):1516-1524.
12. Yu PB, Hong CC, Sachidanandan C, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol. 2008;4(1):33-41.
13. Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest. 2004;113(9):1271-1276.
14. Prince OD, Langdon JM, Layman AJ, et al. Late stage erythroid precursor production is impaired in mice with chronic inflammation. Haematologica. 2012;97(11):1648-1656.
15. Sun CC, Vaja V, Chen S, et al. A hepcidin lowering agent mobilizes iron for incorporation into red blood cells in an adenine-induced kidney disease model of anemia in rats. Nephrol Dial Transplant. 2013; 28(7):1733-1743.