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

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

Correspondence: cmayeur@mgh.harvard.edu
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Supplemental Appendix for the manuscript:

Oral administration of a Bone Morphogenetic Protein (BMP) type I receptor inhibitor prevents the development of anemia of inflammation

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, USA

[2] Therapeutics for Rare and Neglected Diseases (TRND) Program, National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH), Rockville, USA

[3] Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, USA

[4]Cardiovascular Research Center, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, USA

[5]Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, USA

Correspondence should be addressed to

Claire Mayeur, MD; E-mail: cmayeur@mgh.harvard.edu

Methods

All experiments using mice were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. We studied 8- to 10-week-old C57BL/6 mice fed a regular diet (Prolab® 5P75 Isopro® 3000). LDN-193189, 4-(6-(4-(piperazin-1-yl) phenyl) pyrazolo [1,5-a]pyrimidin-3-yl) quinoline, was synthesized as a hydrochloride salt[1] and dissolved in citric acid (pH=3.1).

Measurement of LDN-193189 levels

Serum, liver, and muscle LDN-193189 levels were measured using ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS). LDN-193189 was extracted by mixing serum or tissue homogenate with acetonitrile. Mass spectrometric analysis was performed on a Waters Xevo TQ-S triple quadruple instrument using electrospray ionization in positive mode with the selected reaction monitoring (SRM). SRM transition for LDN-193189 was 407.2/350.1. The separation was performed on a Waters Acquity UPLC system using an Acquity BEH C18 column (50 x 2.1 mm, 1.7 µ). The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in acetonitrile. The retention time of LDN-193189 was 0.9 minute, and the total run time was 2.5 minutes.

Pharmacokinetic parameters were calculated with the mean LDN-193189 serum levels of 3 mice at each time point, using Phoenix 6.2.0.495 (WinNonLin) software.

Modeling AI using turpentine injections

Eight-week-old female mice received a subcutaneous intrascapular injection of turpentine (5 mL/kg) weekly for three weeks[2, 3]. Seven days after the last injection, mice were anesthetized, and blood was obtained by retro-orbital puncture[3], after which mice were euthanized, and liver tissues were collected to measure hepatic hepcidin gene expression. Complete blood counts were measured using a HemaVet Veterinary Analyzer (Heska). Serum IL-6 levels were measured using the mouse IL-6 ELISA Kit (R&D, Cat. No M6000B). Serum iron levels and transferrin saturations were determined using the Iron/UIBC Kit (Genzyme) following the manufacturer's protocol.

Hepatic mRNA levels

Total RNA was extracted from liver tissues and quantitative RT-PCR (qRT-PCR) was performed, as previously described[3], to measure levels of 18s rRNA and mRNAs encoding mouse hepcidin and Id1.

Measurement of phosphorylated BMP-responsive Smads

Proteins were extracted from liver samples, and immunoblots were performed as previously described[4], using antibodies directed against total Smad1 (Life Span) and phosphorylated Smad1/5 (Cell Signaling, S463/465).

Statistical methods

All values were expressed as mean±SD. Data were analyzed using one-

way ANOVA with post-hoc testing using the least squares method, when applicable. Statistical significance was considered for $p<0.05$.

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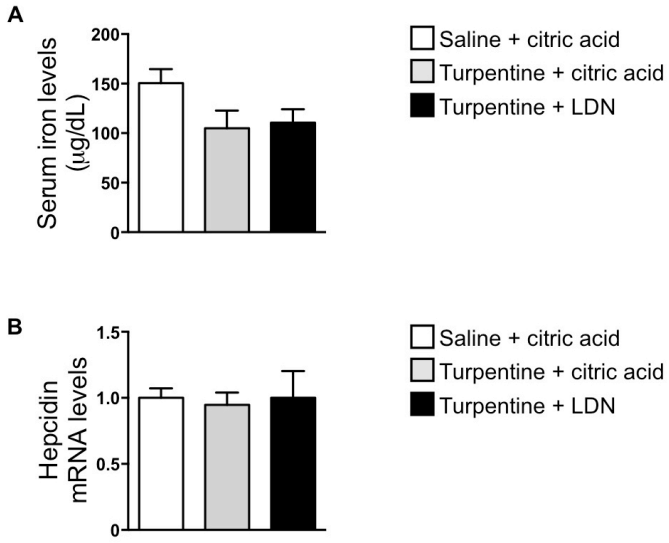
Supplemental Figure



Supplemental Figure 1. Serum iron levels and hepatic hepcidin gene expression, in turpentine-challenged mice. Eight-week-old C57BL/6 female mice were injected weekly with turpentine (5 mL/kg, subcutaneously) during three weeks. Mice were treated daily with either LDN-193189 (LDN, 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, and 24 hours after the last administration of LDN or vehicle, mice were sacrificed. (**A**) Blood was collected to measure serum iron levels (One-way Anova p=0.09). (**B**) Livers were harvested, RNA was extracted, and levels of mRNAs encoding for hepcidin were measured by qRT-PCR (One-way Anova p=0.9).