

Supplemental data

Title

Oral ferroportin inhibitor ameliorates ineffective erythropoiesis in a model of β -thalassemia

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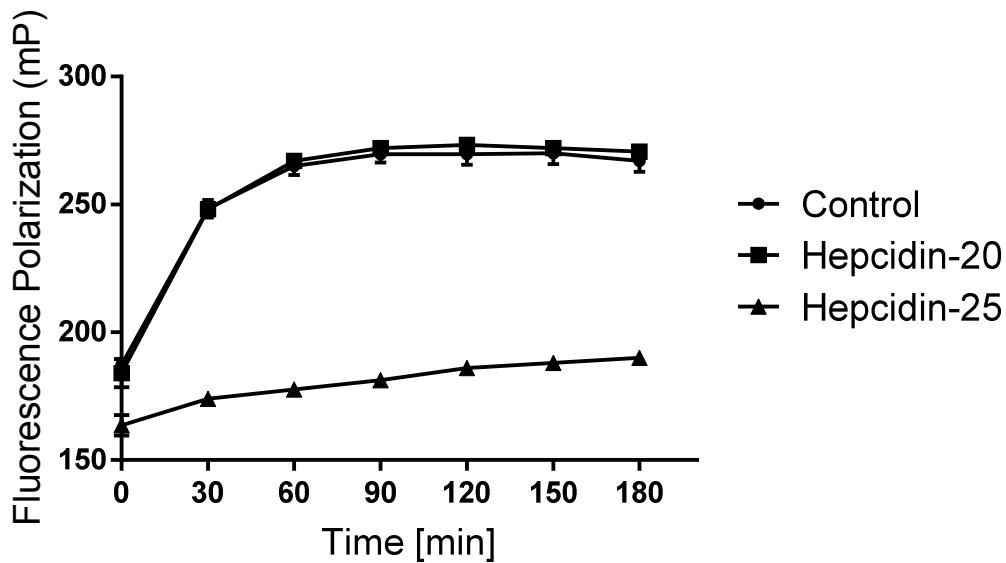
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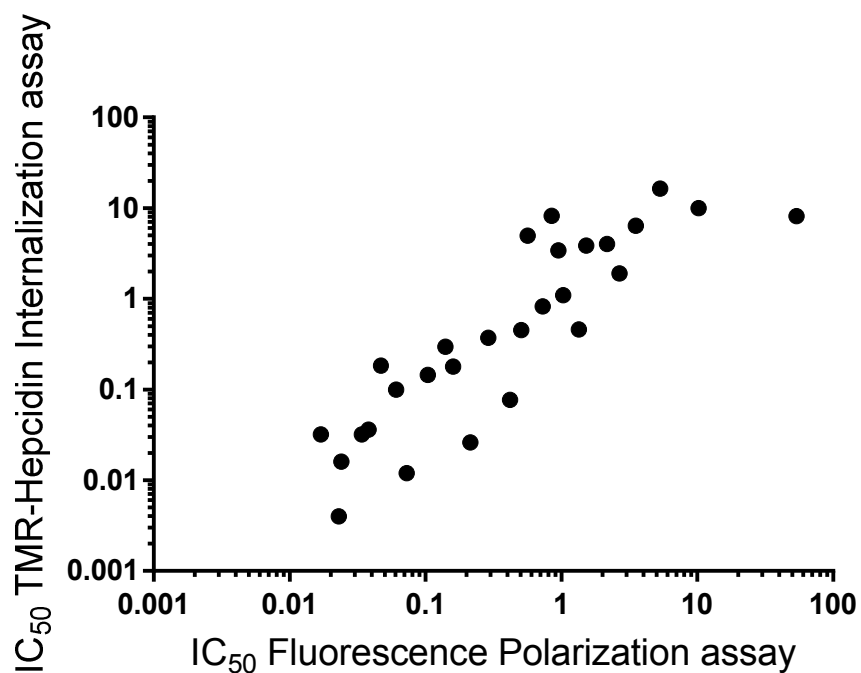
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Conflict of interest statement

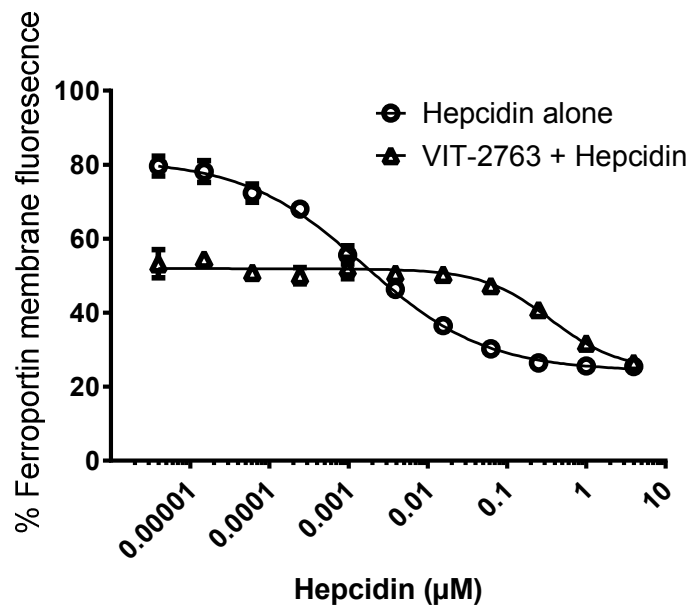
All authors are employees of Vifor (International) Ltd. and might own equities. VM and FD are inventors in patents related to the publication.



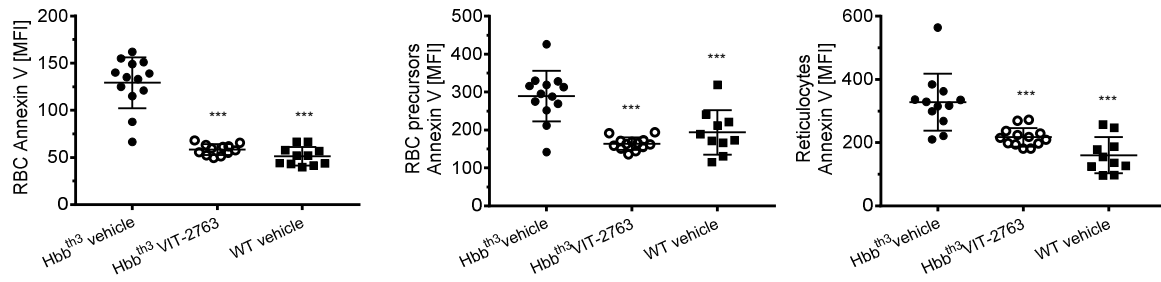
Supplemental Figure 1. Competition of unlabeled hepcidin-20 (human hepcidin lacking the first 5 N-terminal amino acids) and hepcidin-25 (full-length human hepcidin) with TMR-hepcidin for binding to purified yeast ferroportin in fluorescence polarization assay. Hepcidin-20 (4 μ M, Bachem), hepcidin-25 (4 μ M, Bachem) or buffer only (Control) were added to a mixture of TMR-hepcidin (10 nM) and purified yeast ferroportin (0.55 μ M), mean values with standard deviations are shown, $n=3$ per time point. The fluorescence polarization was measured in a time course with 30 min intervals. Detailed description of the assay is provided in the Materials and Methods section of the manuscript.



Supplemental Figure 2. Correlation analysis of the potencies (IC_{50}) of structurally related ferroportin inhibitors measured in J774 TMR-hepcidin internalization assay and TMR-hepcidin fluorescence polarization assay, mean values of IC_{50} are shown, $n=2-10$ for different compounds. Nonparametric Spearman correlation analysis with 95% confidence interval showed a significant correlation ($p<0.001$, $r = 0.8909$) between the potencies of ferroportin inhibitors in both assays. Detailed description of the assays is provided in the Materials and Methods section of the manuscript.



Supplemental Figure 3. VIT-2763 competed with hepcidin for ferroportin internalization in MDCK cells constitutively expressing human ferroportin with a fluorescent HaloTag. Cells were pre-incubated with either medium only or VIT-2763 at a constant concentration of 1 μ M for 15 minutes before adding serial dilutions of unlabeled hepcidin (4nM - 4 μ M) for 18h. The ferroportin-associated membrane fluorescence in MDCK cells was quantified as described in the Materials and Methods section of the manuscript. EC_{50} (hepcidin alone) = 1.6 nM; EC_{50} (VIT-2763 + hepcidin) = 356 nM, mean and standard deviations for each hepcidin concentrations are shown, n=4.



Supplemental Figure 4. VIT-2763 reduced apoptosis markers on RBC and their precursors in blood. Erythrocyte populations were identified as RBC (left graph, Ter119^{hi}CD71^{neg}), RBC precursors (middle graph, Ter119^{hi}CD71^{int}) and reticulocytes (right graph, Ter119^{hi}CD71^{hi}) using flow cytometry. Annexin V staining was performed to detect membrane phosphatidylserine on cells as an apoptosis marker. The mean fluorescence intensity (MFI) of Annexin V was quantified by flow cytometry. Dot plots with values for individual mice are shown, mean and standard deviations of the Annexin V MFI are shown, n=10-13 per group. Statistical analysis were performed by comparing all treatment groups to the Hbb^{th3/+} vehicle group using one-way ANOVA with Dunnett's multiple comparison test.

Materials and Methods

Acute hypoferremia in C57BL/6 mice

8-weeks old female C57BL/6 mice received a single i.p. dose of hepcidin (5 mg/kg) or were dosed orally with either VIT-2763 at 60 mg/kg or 0.5% methylcellulose as a vehicle. Animals were sacrificed at 1, 3, 6 and 16h hours post dose and serum iron was analyzed.

PK and PD in rats

6-week old male Sprague-Dawley rats were dosed with VIT-2763 either i.v. (1 mg/kg) or orally (30 mg/kg) and blood samples were withdrawn from jugular vein at 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24 hours post dosing. Plasma levels of VIT-2763 were analyzed by LC-MS/MS. PK parameters were calculated for individual animal by non-compartmental model with Phoenix software version 7.0.

Blood, serum and tissue analysis

Complete blood counts were analyzed using Idexx ProCyt analyzer (Idexx Bioresearch). Serum EPO levels were determined in duplicates using mouse EPO DuoSet ELISA (R&D Systems) according to manufacturer's instructions. Serum iron levels were determined in triplicates using MULTIGENT Iron assay (Abbott Diagnostics). Total organ iron and ^{58}Fe concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) and mass spectrometry (ICP-MS), respectively.

Measurement of RBC lifespan

Male and female Hbb^{th3/+} and WT mice 8-12 weeks of age were treated with vehicle (0.5% methylcellulose) or VIT-2763 (only Hbb^{th3/+} mice) bid for 7 weeks. Three weeks after treatment start, mice were injected i.v. into the tail vein with 1 mg per mouse of NHS-PEG4-Biotin reagent (Pierce) on three consecutive days. Twenty-four hours after the last injection, one drop of blood was collected from tail vein and incubated with Alexa Fluor 488-conjugated streptavidin (Invitrogen). The fraction of biotin-labeled RBCs was analyzed by flow cytometry (BD FACSCanto™ II) weekly for 4 weeks after the initial biotin injection. FlowJo[®] software (FlowJo, LLC, version 10.1) was used for data analysis.

Histology

Spleens were fixed in 10% buffered formalin and embedded in paraffin. De-paraffinized sections of tissue were stained for non-heme ferric iron deposition with diaminobenzidine (DAB)-enhanced Perls stain. Consecutive sections were stained with hematoxylin and eosin (HE). Images were acquired with 40x objective (NA 0.95) using an Olympus VS120 Virtual Slide Microscope.

Detection of membrane α -globins in erythroid cells

Blood samples were collected and membrane cytoskeletons were prepared as previously described (52). Briefly, the erythrocyte ghosts were washed three times by resuspending in hypotonic lysis buffer and centrifugation at 21'000xg. Membrane lipids were extracted in 50 mM sodium borate pH 8, 1mM EDTA, 0.5% Triton X-100 (Sigma-Aldrich) and protease inhibitors. After a last 30 min centrifugation at 30'000xg the supernatant was completely removed and the triton-insoluble pellet corresponding to the membrane cytoskeletons was snap frozen and analyzed using TAU polyacrylamide gel, as previously described (53).

Quantitative PCR (qPCR)

Erfe, *Hamp* and *Hmox1* mRNA levels were determined using TaqMan (Fam132b: Mm00557748_m1, Thermo Scientific) and SYBR Green (*Hamp*: qMmuCED0050768, *Hmox1*: qMmuCID0040051, Bio-Rad Laboratories) gene expression assays on a LightCycler 480 II instrument (Roche Diagnostics) according to manufacturer's instructions. Transcript abundance was calculated by comparison with a reference gene *Gusb* (TaqMan: Mm01197698_m1, Thermo Scientific. SYBR Green: qMmuCED0046361, Bio-Rad Laboratories).

References

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