Mitapivat Improves Transfusion Burden and Reduces Iron Overload in Thalassemic Mice

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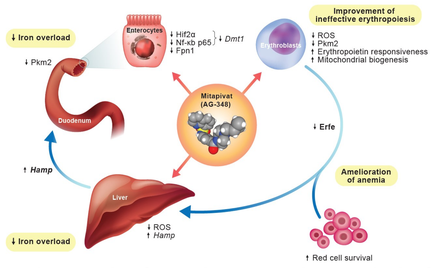
Background

- **Mitapivat (AG-348) is an oral allosteric activator of red blood cell pyruvate kinase[1]**
- **Recently, we showed that mitapivat improves anemia and iron homeostasis in a mouse model of β-thalassemia intermedia (Hbb$^{th3/+}$) *via* a multi modal action[2]**



- ***Ad interim* results from the extension period of a phase 2 trial of a phase-2 trial with mitapivat in non transfusion dependent thalassemic (NTDT) patients demonstrates sustained long-term increase in Hb (≥ 1 g/dL) with improvement of hemolysis and ineffective erythropoiesis** (Kuo et al.abstract # 576; ASH 2021).
- **Mitapivat might be a potential therapeutic option also for TDT patients since transfusion burden severely impact patients' quality of life.[3]**
- **To address this question, we exposed Hbb$^{th3/+}$ mice to chronic transfusion with or without mitapivat treatment. We also evaluated the effect of mitapivat associated with deferiprone (DFP), an oral iron chelator, on hematologic parameters.**

Methods

Mouse strains and design of the study. 3-4 months old female mice of C57BL6/J, as wild-type controls (WT), and Hbb$^{th3/+}$ mice (β-thal), as mouse model of β-thalassemia intermedia, were used in the present study.[2] Whenever indicated mice were treated by oral gavage with Mitapivat (AG-348) or vehicle at the dosage of 50 mg/Kg twice daily (BID) up to 71 days. For the transfusion study, Hbb$^{th3/+}$ mice, treated with mitapivat (50 mg/Kg BID) or vehicle for 10 days, were transfused with 400 µL washed heathy RBCs at 40-45% Hematocrit (Hct).[4] We chose Hb 10.5 g/dL as transfusion threshold for our β-thal mouse model. Whenever indicated deferiprone (DFP) was administered to Hbb$^{th3/+}$ mice treated with mitapivat (50 mg/Kg BID) in drinking water at the dosage of 1.25 mg/ml.[5] For nonterminal and terminal blood collection, mice were anesthetized by isoflurane inhalation and blood was collected by retro-orbital venipuncture using heparinized microcapillary tubes. Hemoglobin was manually determined by staining with Drabkin's reagent (Sigma-Aldrich, St. Louis, MO) followed by spectrophotometric analysis at 540 nm. Reticulocytes and circulating erythroblasts were measured either by Sysmex XN-1000 Hematology Analyzer (Sysmex Corporation, Japan) or by flow cytometric analysis using CD71-PE (Thermo Fisher Scientific, Waltham, USA) staining as previously reported.

Flow Cytometric Analysis of Mouse Erythroid Precursors from bone marrow and spleen was carried out using the CD44-Ter119 gating strategy as previously described.[2] The following antibodies were used: anti-CD16/CD32 blocking agent, anti–CD44-FITC, CD71-PE, Ter119-APC, CD45 APC-eFluor 780, GR1 APC-Cy7, and CD11b APC-Cy7 (all from eBiosciences, Thermo Fisher Scientific, USA). Samples were acquired using the FACSCanto II flow cytometer (Becton Dickinson) and analyzed with FlowJo software version 10 (Tree Star).[2]

In vivo Erythrophagocytosis was determined by flow cytometry as previously described [6]. Spleen macrophages (MΦ) from WT and Hbb$^{th3/+}$ mice were stained with anti-F4/80 antibody and anti-Ter-119 antibody to detect ingested RBCs. Phagocytosis was assessed as the percentage of double positive (F4/80$^+$/Ter-119$^+$) cells. **Macrophage M1 and M2 polarization** was determined using the specific M1 marker CD80 and M2 CD206 antibodies on spleen MΦ.[6]
Liver and spleen iron content were analyzed either using the Perls prussian blue staining or measured using the bathophenanthroline method as previously described.[2]

Statistical Analysis: Data were analyzed using either the one-way analysis of variance (ANOVA) and the Two-tailed unpaired Student t test. A difference with a p<0.05 was considered significant.

Results

Mitapivat reduces the need of transfusion in β-thal mice

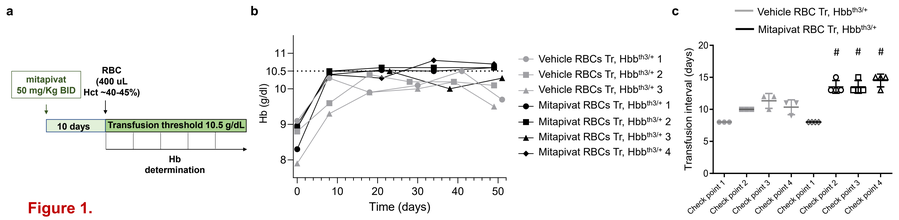


Figure 1.

a. Design of the study. β-thal mice (Hbb$^{th3/+}$), treated with vehicle or mitapivat (50 mg/Kg BID), were transfused with 400 µL washed heathy RBCs (RBC Tr.) at 40-45% hematocrit (Hct) (Park et al Blood. 2020). Hb 10.5 g/dL was chosen as transfusion threshold.

b. Overtime Hb changes in transfused β-thal mice treated with either vehicle or mitapivat (50 mg/Kg BID).

c. Mitapivat treated β-thal mice showed greater sustained rise in Hb from baseline compared to vehicle treated β-thal mice. This results in longer length of time between transfusion (mitapivat β-thal mice 13.8±1.0 days *vs* vehicle β-thal mice 10.5±1.0 days, n= 4 and n=3; # P<0.05 compared to vehicle treated animals). This was associated with a drop in both reticulocyte and peripheral circulating erythroblasts in both vehicle and mitapivat treated β-thal mice (data not shown).

In β-thal mice, mitapivat beneficially impacts spleen iron-overload by reduction of transfusion burden and amelioration of β-thal ineffective erythropoiesis

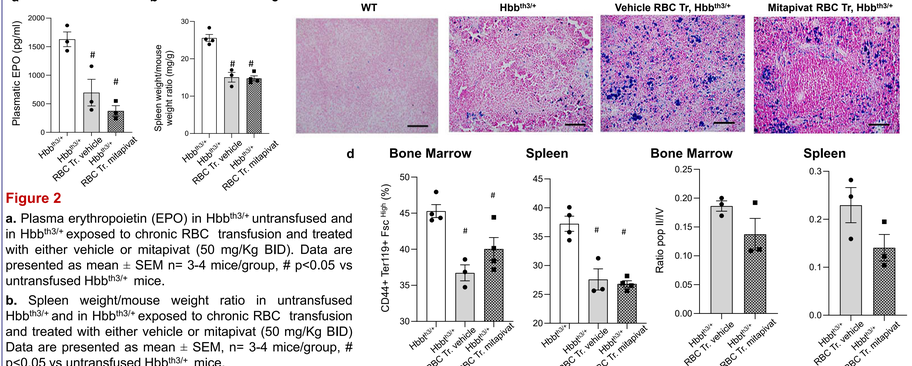


Figure 2

a. Plasma erythropoietin (EPO) in Hbb$^{th3/+}$ untransfused and in Hbb$^{th3/+}$ exposed to chronic RBC transfusion and treated with either vehicle or mitapivat (50 mg/Kg BID). Data are presented as mean ± SEM n= 3-4 mice/group, # p<0.05 vs untransfused Hbb$^{th3/+}$ mice.

b. Spleen weight/mouse weight ratio in untransfused Hbb$^{th3/+}$ and in Hbb$^{th3/+}$ exposed to chronic RBC transfusion and treated with either vehicle or mitapivat (50 mg/Kg BID) Data are presented as mean ± SEM, n= 3-4 mice/group, # p<0.05 vs untransfused Hbb$^{th3/+}$ mice.

c. Perl's stained sections of spleens from WT, untransfused and transfused Hbb$^{th3/+}$ mice treated with either vehicle or mitapivat (50 mg/Kg BID), showing a reduced spleen iron overload in mitapivat treated transfused β-thal mice compared to vehicle transfused β-thal animals. One representative image from 6 with similar results.

d. Flow-cytometric analysis of the erythropoietic activity and the maturation pattern of the erythroid precursors (immature-Pop II/mature –Pop IV erythroblasts) from the bone marrow (**left panel**) and spleen (**right panel**) in transfused β-thal mice treated with vehicle (Hbb$^{th3/+}$ RBC Tr. vehicle) or mitapivat (50 mg/Kg BID, Hbb$^{th3/+}$ RBC Tr. mitapivat) compared to non transfused mice (Hbb$^{th3/+}$). Data are mean ± SEM (*N*= 3-4 mice/group). # P < 0.05 compared to untransfused β-thal mice.

In transfused β-thal mice, Mitapivat induces a pro-resolving profile of splenic macrophages

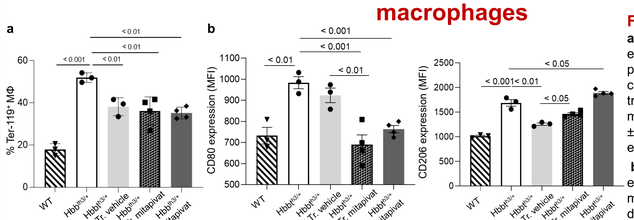


Figure 3

a. Flow cytometric analysis of the *in vivo* splenic erythrophagocytosis, assessed as the percentage of F4/80$^+$/Ter-119$^+$ double positive cells in spleen from WT, untransfused or transfused β-thal mice treated with mitapivat (50 mg/Kg BID) or vehicle. Data are shown as mean ± SEM (n= 3-4 mice/group). No cumulative effect of the two treatments was observed.

b. Flow cytometric analysis of the surface expression of the M1 marker CD80 and the M2 marker CD206 on spleen macrophages (MΦ) data are mean ± SEM (n= 3-4 mice/group). (MFI, mean fluorescence intensity)

Mitapivat treated transfused β-thal mice show reduced liver iron accumulation and modulation of Dmt1 IRE expression in duodenum

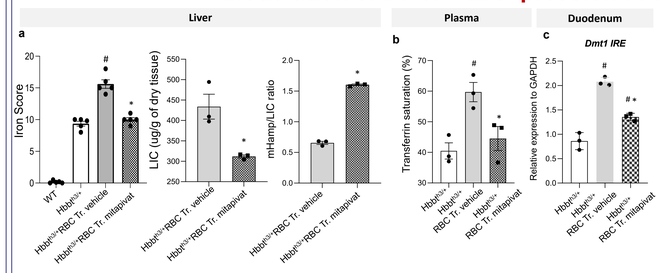


Figure 4

a. Perls staining (left panel), non-heme liver iron content (LIC- middle panel) and mHamp/LIC ratio in the liver from transfused β-thal mice treated with either vehicle or mitapivat (50 mg/Kg BID).
b. Serum Transferrin saturation (%) in untransfused and transfused β-thal mice treated with either vehicle or mitapivat (50 mg/Kg BID).
c. mRNA expression of *Dmt1*-iron response element (IRE) by qRT-PCR on duodenums from untransfused and transfused β-thal mice treated with vehicle or mitapivat.

Data are mean ± SEM (N= 3 mice/group). # P<0.05 compared to untransfused β-thal mice. * P<0.05 compared to vehicle.

Mitapivat induced improvement of anemia is maintained in beta-thal mice treated with deferiprone (DFP)

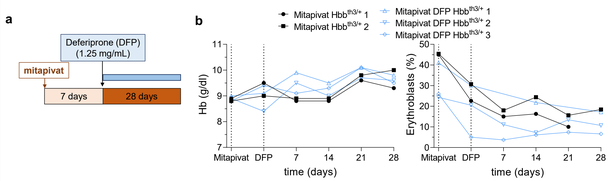


Figure 5

a. Design of the study. Mitapivat (50 mg/Kg BID) was administrated in combination with deferiprone (DFP), an oral iron chelator (1.25 mg/ml) to β-thal (Hbb$^{th3/+}$) mice.
b. Hemoglobin **(Hb, left panel)** and circulating erythroblasts **(%, right panel)** in β-thal mice treated with either mitapivat or mitapivat+DFP. Data are presented for single mouse at the different time points.

Conclusions

- We generated a model for chronic RBC transfusion in β-thal mice.
- In this model, we show that mitapivat:
  - Increases the length of time between transfusion, resulting in decreased spleen and liver iron overload.
  - Induces a pro-resolving profile of spleen macrophages
  - Improves iron homeostasis by targeting *Dmt1* expression

In conclusion, mitapivat reduces the transfusion burden in a mouse model of transfused β-thalassemia, which could potentially impact the management of iron chelation and anemia in TDT.

References

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Disclosures

PAK and LD - AgiosPharmaceuticals,Inc- Current Employment and Current holder of *stock options* in a privately-held company
LDF - F. Hoffmann-La Roche Ltd – Consultancy; Novartis - Consultancy

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