Mitapivat Ameliorates Red Cell Features and Decreases Anemia in Band 4.2$^{-/-}$ Mice, A Model of Hereditary Spherocytosis

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INTRODUCTION

- Hereditary Spherocytosis (HS) is the most common non-immune inherited hemolytic anemia with a prevalence going from 1: 2,000 to 1: 5,000 births.[1]
- HS is due to mutation on genes encoding for red cell membrane or cytoskeleton proteins such as ankyrin, band 3, band 4.1, band 4.2 or α-, β-spectrin. [2]
- In HS, reduced ATP content has been linked to abnormal red cell features, contributing to the red cell membrane mechanical instability. In addition, relative PK deficiency has been reported in HS red cells. [3]
- Mitapivat (AG-348) is an oral small-molecule activator of pyruvate kinase (PK), which modulates red cell ATP content. [4]
- Two phase 3 clinical trials of Mitapivat in adult patients with PK deficiency demonstrated early and sustained increase in Hb levels. [5a/b]
- Recently, we reported that Mitapivat improves ineffective erythropoiesis and ameliorates anemia of a mouse model for β-thalassemia. This beneficially impacts iron homeostasis throughout erythroferrone/hepcidin axis and modulation of DMT1 expression in enteric cells. [6]

OBJECTIVE(S)

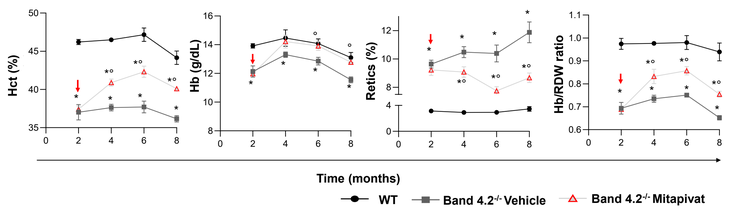
To Understand whether the activation of PK by Mitapivat might affect anemia and red cell features in a mouse model for HS (band 4.2$^{-/-}$ mice)

METHOD(S)

- **Mouse strains and design of the study.** Epb42^{tm1Llp}/LlpJ (band 4.2$^{-/-}$) mice were obtained from Jackson laboratory and breeding was performed in the animal facility (CIRSAL) of the University of Verona. 3-8 months old Epb42^{tm1Llp}/LlpJ and C57BL6/J, as control, female mice were studied. Mice fed with standard diet (Control) or Standard diet containing 1200 ppm Mitapivat (corresponding to a dosage of 200 mg/Kg/d) for 6 months was used. The Institutional Animal Experimental Committee of University of Verona (CIRSAL) and the Italian Ministry of Health approved the experimental protocol (56DC9.12). Hemoglobin (Hb) and hematocrit (Hct) were manually determined as previously reported. [6] RDW and reticulocytes were obtained by Sysmex XN-1000 Hematology Analyzer (Sysmex Corporation, Japan). Lactate dehydrogenase and creatinine were evaluated using standard biochemical assays. [6]
- **Flow cytometric analysis of circulating red cells.** RBCs from whole blood were either analyzed to determine exposure of phosphatidylserine (PS) groups using the eBioscience Annexin V Apoptosis Detection Kit PE (Thermo Fisher Scientific), or positivity to naturally occurring antibodies using a Donkey anti-Mouse IgG Highly Cross-Adsorbed Antibody, Alexa Fluor 488 (Thermo Fisher Scientific) as previously described. [7-8]
- **Osmotic stability assays** was performed by flow cytometric analysis as previously described. [9] Briefly, the same number of RBCs were incubated in lysis solutions (192 mOsm) for 10 minutes at 37°C. Lysis was stopped with 4x volume of quenching solution and AccuCheck Counting Beads were added (Thermo Fisher Scientific). Cells with normal flow cytometric FSC/SSC profiles were considered to be intact. The ratio of intact cells to beads was used to calculate the absolute number of cells and percent of lysis was determined by normalization to the 320 mOsm control condition (0% of lysis).
- **In vivo Erythrophagocytosis** was determined by flow cytometry as previously described. [10] Spleen macrophages (MΦ) from WT and Band 4.2$^{-/-}$ 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 polarization** was determined using the specific M1 marker CD80 antibody on spleen MΦ.
- **Liver iron content** was analyzed either using the Perls prussian blue staining or measured using the bathophenanthroline method as previously described. [6] **Liver western blot analysis** was performed as previously described (Matte et al JCI 2021). The following antibodies were used: anti Phospho- Ser536 NF-κb p65 (pNF-κb p65), NF-κb p65 (clone C22B4) from Cell Signaling Technology; Phospho Ser40 NRF2 (pNRF2, clone EP1809Y), NRF2 (clone EP1809Y), STAT3 (clone C-20) and GAPDH from Santa Cruz Biotechnology, Phospho-Tyr705 pSTAT3 (clone EP2147Y) from AbCam. [6]

RESULTS

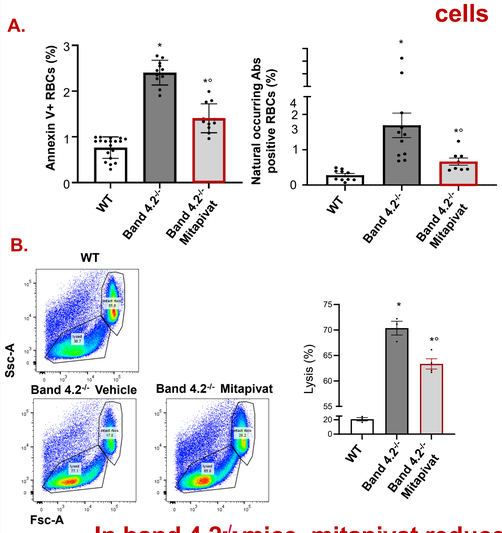
Mitapivat ameliorates anemia and reduces hemolysis in band 4.2$^{-/-}$ mice



A. Hematocrit (Hct), Hemoglobin (Hb), Reticulocytes, Hb/RDW ratio in WT and band4.2$^{-/-}$ mice treated with vehicle or Mitapivat. The red arrow indicates the initiation of treatment. Data are means ± SEM (*n* = 5-7).

B. Plasma LDH and bilirubin, known markers of hemolysis. Data are mean ± SEM (N=5). * P < 0.05 compared to WT; ° P< 0.05 compared to Vehicle treated band 4.2$^{-/-}$ mice .

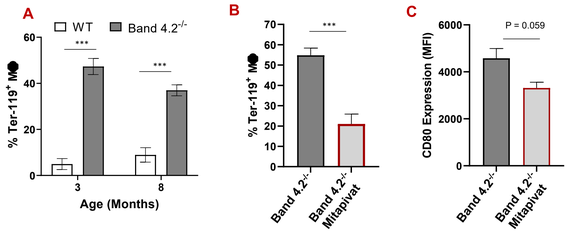
Mitapivat improves membrane mechanical stability of band 4.2$^{-/-}$ mouse red cells



A.Mitapivat reduces the amount of PS+ red cells and the natural occuring antibody anti-band 3.
Percent of phosphatidyl serine positive (PS) RBCs measured with the Annexin-V assay **(left panel)** and Natural occurring Antibodies (Abs) positive RBCs **(right panel)** in WT and Band4.2$^{-/-}$ mice treated with vehicle or Mitapivat. Results are means ± SEM (n= 7-19). * P < 0.05 compared to WT; ° P< 0.05 compared to Vehicle treated Band 4.2$^{-/-}$ mice .

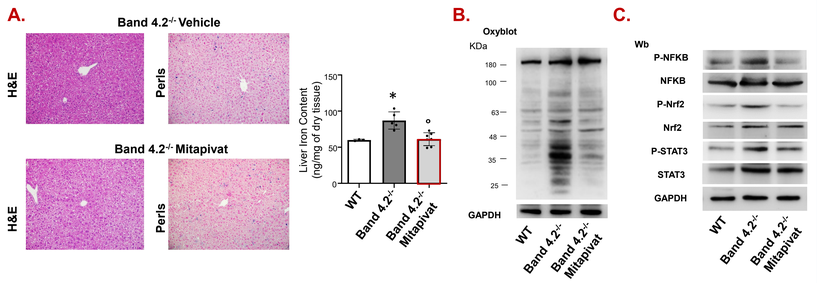
B. Mitapvat improves band 4.2$^{-/-}$ red cell osmotic fragility.
Representative scatter plots **(left panel)** and percent of red cells lysis (Lysis %) at 192 mOsm determined by flow cytometry WT and Band4.2-/- mice were treated with either Vehicle or Mitapivat. Results are mean ± SEM from 3-4 mice/group. * P < 0.05 compared to WT; ° P< 0.05 compared to Vehicle treated Band4.2$^{-/-}$ mice .

In band 4.2$^{-/-}$ mice, mitapivat reduces erythrophagocytosis and macrophage M1 polarization



A-B. In vivo splenic erythrophagocytosis in WT and Band 4.2$^{-/-}$ mice treated with either Vehicle or Mitapivat, determined by flow cytometry. Phagocytosis was assessed as the percentage of F4/80$^+$/Ter-119$^+$ double positive cells. Results are mean ± SEM (n= 3-4 mice/group). *** P < 0.001. **C**. surface expression of the M1 marker CD80 on spleen MΦ from Band 4.2$^{-/-}$ mice treated with Vehicle or Mitapivat. Results are mean ± SEM (n=7-10 mice/group).

In band 4.2-/- mice, mitapivat reduces liver iron overload and related liver oxidative stress



A.Hematoxylin and Eosin (H&E) and iron staining (Perl's Prussian blue) of livers from band4.2$^{-/-}$ mice treated with vehicle or mitapivat. One representative image from 6 with similar results (left panel). **Quantification of the non-Heme liver iron content in WT and band4.2$^{-/-}$ mice treated with vehicle or mitapivat using the bathophenanthroline staining method (right panel panel).** Data are mean ± SEM (*n* = 6). * *P* < 0.05 compared with WT mice and ° *P* < 0.05 compared with vehicle-treated mice. **B. Oxyblot analysis of soluble fractions of liver proteins from WT and band4.2$^{-/-}$ mice treated with vehicle or mitapivat.** Proteins were analyzed with 10% SDS-PAGE. GAPDH as loading control. **C. Western blot analysis with specific antibodies against phospho (p) NF-κb p65, NF-κb p65, pNRF2, and NRF2, pSTAT3, STAT3 of liver from WT and Hbb$^{th3/+}$ mice with vehicle or mitapivat treatment.** GAPDH as loading control.

CONCLUSIONS

- **In band 4.2-/- mice, Mitapivat:**
  - **Reduces chronic hemolysis**
  - **Improves membrane mechanical stability**
  - **Decreases erythrophagocytosis with associated pro-resolving profile of spleen macrophages.**
- **Mitapivat reduces liver iron-overload, resulting in improvement of liver proteins oxidation and decrease activity of redox related transcriptional factors.**
- **Mitapivat might represent a potential therapy to be explored in patients with HS.**

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CONTACT INFORMATION

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