Mitapivat Ameliorates in vitro Human β Thalassemic Erythroid Maturation Index and Modulates the Expression of the Cytoprotector Peroxiredoxin-2

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INTRODUCTION

- β-thalassemia (β-Thal) is characterized by chronic hemolysis and ineffective erythropoiesis, due to severe oxidation related to the unbalance in globin chain synthesis, resulting in block of erythroid maturation.[1,2]
- In β-Thal, chronic oxidation and increased generation of methemoglobin up-regulate glycolysis. This is consistent with persistent expression of pyruvate kinase (PK)M2 in both erythroblasts and RBC from mouse, as well as in human β-Thal red cells.[3,4]
- Recently, we reported that mitapivat, a pyruvate kinases activator, improves ineffective erythropoiesis and ameliorates anemia in a mouse model for β-Thal. [4,5]
- The results of a phase 2 proof-of concept study (NCT03692052) in non-transfusion-dependent thalassemic patients supports the beneficial effects of mitapivat on thalassemias. [6]

OBJECTIVES

To determine whether Pyruvate Kinase activation *via* mitapivat might affect key cytoprotective systems.

METHODS

In vitro cell cultures of human erythoid precursors from CD34⁺ cells: We studied CD34+ derived erythroblasts from either healthy controls or β-thal (cod β^{039}) patients (n=5). Light-density mononuclear cells were obtained by centrifugation on Lymphoprep (STEMCELL Technologies) density gradient, as previously described.[4] The CD34⁺ cells were positively selected by anti-CD34-tagged magnetic beads (MiniMACS columns; Miltenyi Biotech) according to the manufacturer's protocol. The recovery was more than 90% CD34⁺ cells, as determined by flow cytometry. CD34⁺ cells were grown at a density of 10^5 cells/mL in α-MEM supplemented with 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, 10^{-6} mol/L hydrocortisone, 10^{-3} g/L nucleotide, 25 × 10^{-3} mg/L gentamicin, 10^{-4} mol/L 2-mercaptoethanol, 1% deionized BSA, 1 μg/mL cyclosporine A (all from Sigma Aldrich), and 30% fetal bovine serum (GIBCO) using a two step based procedure. The following recombinant cytokines were added to the media: 3 U/mL recombinant human (rH) EPO (Janssen-Cilag), 20 ng/mL rH stem cell factor, and 10 ng/mL rH interleukin-3 (both PeproTech). Whenever indicated mitapivat was added to the culture medium at 5, 7, 10, 13 days of culture (final concentration: 2 μM). Cell samples were collected for cell counting and determination of cell viability.[4]

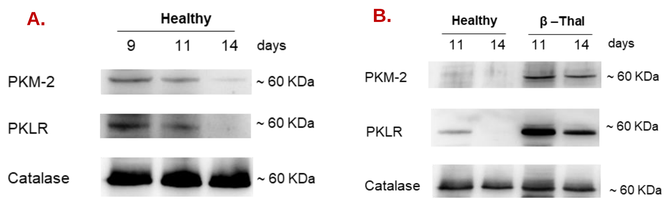
Flow cytometric analysis of erythroid precursors: The maturation profile of the erythroid precursors was analysed by flow cytometric analysis using the following antibodies: anti-CD71 APC and anti CD36-FITC (both from Thermo Fisher Scientific), anti glycophorin A-PE (BD Biosciences). All the analyses were performed with the flow cytometer FACSCanto I (Becton Dickinson). The biparametric scatter plots were analyzed with FlowJo software version 10.8.1 (Becton Dickinson). [4]

qRT-PCR on erythroblasts: Messenger RNA (mRNA) was isolated and reverse transcribed into high-purity complementary DNA (cDNA) using μMACS One-step cDNA Kit according to the manufacturer's instructions (Miltenyi Biotec). We started from 500 000 cells collected at day 11th and 14th of culture. One-fiftieth of the reactions were added to appropriate wells of the PCR plates. qRT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using Applied Biosystems Model 7900HT Sequence Detection System. All PCR reactions were performed in triplicate. Relative gene expression was calculated using the 2-ΔCt method, in which Ct indicates cycle threshold, the cycle number where the fluorescent signal reaches the detection threshold. ΔCt was computed by calculating the difference of the average Ct between the test gene and the internal control gene, HbA1 (hemoglobin subunit alpha 1).[7]

Western blot analysis: 1.5 million of cells collected at day 11th and 14th of culture were lysed with ice cold lysis buffer (25 mM bicine, 1.5% Triton X-100, 1 mM EDTA, 1 mM NaF, Roche protease inhibitor cocktail, 1 mM Na_3VO_4). After 3 "freeze and thaw" cycles, samples were centrifuged at 12 000 g for 30 min at 4° C. The supernatants, containing proteins, were further solubilized adding 0.1% SDS, 1 mM DTT (final concentration) and Laemli buffer (SB: 50mM Tris pH 6.8, 2% SDS, 10% Glycerol, 100mM β-mercaptoethanol, Bromophenol Blu). Samples were analysed by SDS-PAGE followed by western blot analysis using specific antibodies anti-Pklr (Santa Cruz Biotechnology, USA), anti-Pkm2 and HSP70 (Cell Signaling Technologies), anti Prx-2 (kindly gifted by Ho Zoo Chae, School of Biological Science and Technology, Chonnam National University, Gwangju, Korea). Catalase was used as protein loading control. Secondary donkey anti-rabbit IgG and anti-mouse IgG HRP conjugates (GE Healthcare Life Sciences), secondary donkey anti-goat HRP (Santa Cruz Biotechnology) were used. Blots were developed using the Luminata Forte Chemiluminescent HRP Substrate from Merck KGaA, and images were acquired using the Alliance Q9 Advanced Chemiluminescence Imager (Uvitec, Cambridge, UK). [8]

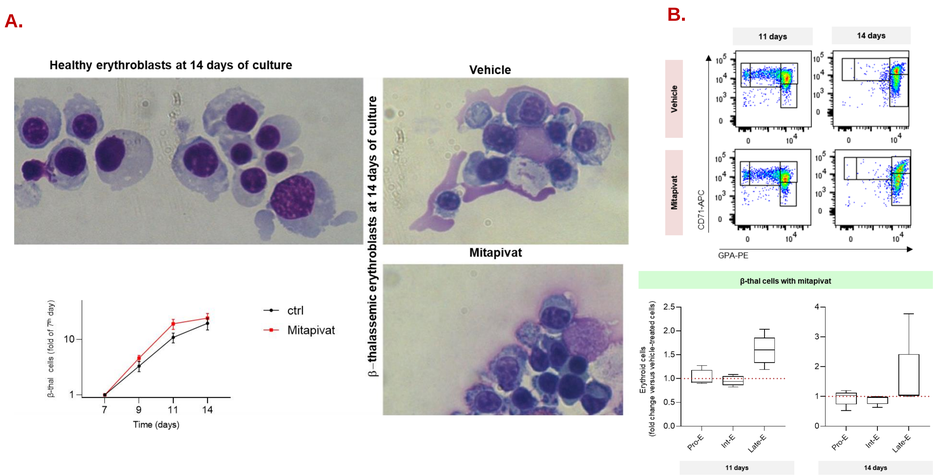
RESULTS

Erythroblasts from β-Thal patients (cod β^{039}) show compensatory persistence of PKM2 expression in the late phases of erythropoiesis



Western blot analysis with specific antibodies against PKM2 and PKLR of in vitro CD34+ derived erythroid precursors from **(A)** healthy controls, analysed at 9, 11 and 14 days of culture and **(B)** healthy controls and β-Thal patients (cod β^{039}), analyzed at 11 and 14 days of culture. Catalase was used as loading control. One representative immunoblots of 3 others with similar results.

Mitapivat beneficially impacts in vitro human β-Thal erythropoiesis

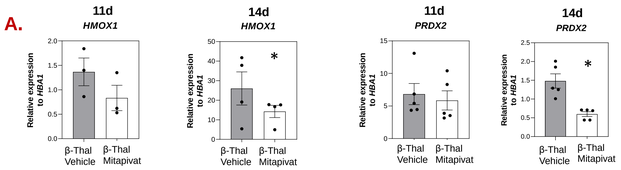


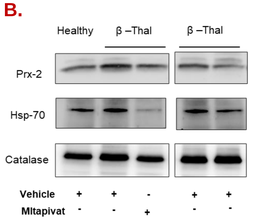
A. The morphologic analysis of erythroid precursors shows amelioration of irregular nuclear shape and chromatin condensation β-Thal erythroblasts when compared to vehicle treated cells. Hematoxylin/Eosin staining for erythroblast morphology (upper panel) of CD34+ derived erythroid precursors, at 14 days of culture, from healthy controls and β-thal patients (cod β039) *in vitro* treated with vehicle or mitapivat (2 μM). One representative image from 5 with similar results is shown. Original magnification 100×. Proliferation (lower panel) of CD34+ derived erythroid precursors from β-thal patients (cod β039) treated with vehicle or mitapivat (2 μM). Data are mean ± SEM (*n* = 5).

B. Mitapivat promotes increased in poly/orthochromatic erythroblasts (Late-E) at 11 and 14 days of culture. Representative flow cytometric plots (left panel) and cumulative differentiation profile (right panel) of β-thalassemic erythroid precursors, at 11 and 14 days of culture, treated with vehicle (DMSO) or mitapivat (2 μM). Box and whiskers plots from 5 different patients showing the increased amount of the late erythroblasts . CD36, glycophorin-A (GPA), and CD71 were used as surface markers to identify the following homogenous cell populations: erythroid colony-forming unit (CFU-E), pro-erythroblasts (Pro-E), basophilic erythroblasts (Int-E), and polychromatic/orthochromatic erythroblasts as late erythroblasts (Late-E).

In human β-thal erythropoiesis, mitapivat decreases the stress induced up-regulation of the cyto-protective systems

In β-Thal erythropoiesis, we previously showed that peroxiredoxin-2 (Prx-2) is a key anti-oxidant and cytoprotective system, acting as back-up mechanism in coordination with the redox-related transcriptional factor Nrf2.[9,10] This results in modulation of ARE-genes such as heme-oxygenase-1 (HMOX1), which is involved in heme catabolism.





Mitapivat treated β-Thal erythroblasts displayed down-regulation of HMOX-1 and PRDX-2 genes (A). qRT-PCR of CD34+ derived erythroid precursors, at 11 and 14 days of culture, from β-thal patients (cod β039) *in vitro* treated with vehicle or mitapivat (2 μM). HBA1 served as constitutive gene Data are mean±SEM (n=3-5) * p<0.05 compared to vehicle treated cells.

At 14 days of cuture, the reduction in the expression of Prx-2 was confirmed by Western-blot analyses (B). We also found reduced expression of heat shock-protein70 (HSP70), which was previously reported to protect β-Thal against the oxidation mediated by free a-globin chain.[7,11] Western-blot analysis of CD34+ derived erythroid precursors, at 14 days of culture, from β-thal patients (cod β^{039}) Catalase served as loading control in the Wb. One representative of other 3 with similar results.

CONCLUSION(S)

- **Human β-Thal erythroblasts show a compensatory persistence of PKM2 expression in erythropoiesis**
- **Mitapivat improves human β-Thal ineffective erythropoiesis**
- **Mitapivat treated β-Thal erythroblasts displayed down-regulation of cytoprotective systems such as Prx-2 and HMOX-1, supporting an amelioration of β-Thal cell environment.**

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