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# Ex vivo activation of pyruvate kinase by tebapivat reduces sickling and red blood cell adhesion in sickle cell disease

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## INTRODUCTION

Pyruvate kinase (PK) activators are currently being explored for patients with sickle cell disease (SCD).[1-3] Recently, we demonstrated that increased red blood cell (RBC) density is associated with reduced PK activity and a greater sickling tendency.[4] Additionally, we identified correlations between PK thermostability, sickling tendency and RBC adhesion to laminin.[4] To better understand the therapeutic response of PK activators, we studied the response of RBCs from patients with SCD with different densities on ex vivo PK activation by the novel PK activator tebapivat.

## AIM

To explore how activation of PK by tebapivat improves red blood cell characteristics including RBC adhesion of low dense RBCS and high dense RBCs of patients with SCD.

## METHOD

RBCs from individuals with HbSS without recent transfusion were obtained for ex-vivo treatment with 5uM and 50uM of tebapivat. RBCs were purified and separated into low-density (LD) and high-density (HD) subpopulations using a percoll gradient (70%).

Total purified RBCs, LD and HD subpopulations were diluted in patient's own plasma (hematocrit 0.22L/L) and incubated (14-16h at 4°C, 1h at RT, 3h at 37°C) with either vehicle control (0.1% DMSO) or tebapivat (5µM, 50µM). After incubation the following assays were performed:

- PK activity (enzymatic activity)
- PK thermostability (enzymatic activity at $53^0$ C)
- ATP and 2,3-DPG levels (Targeted LC-MS/MS)
- Point of sickling (PoS: $pO_2$ at which sickling starts during deoxygenation), was assessed using oxygen gradient ektacytometry (RR Mechatronics).
- RBC adhesion to laminin was measured with a laminin-coated (Biolamina) microfluidic flow assay (IBIDI VI).
- Blood viscosity was assessed with a cone-plate viscometer (Brookfield).

A one-way ANOVA or Friedman test was performed to assess statistical significance (p=<0.05).

## RESULTS

RBC samples form six patients with HbSS (median age 32 years [range 18-46], n=3 female, n=3 on hydroxyurea) were included. PK activity and PK thermostability significantly increased in total, LD and HD RBCs treated with 5µM and 50µM tebapivat (Figure 1A-B). The increase in PK activity was more pronounced in LD compared to HD RBCs (5µM tebapivat 1.7 vs 0.8 U/gHb, p=0.031, 50µM tebapivat 1.6 vs 0.8 U/gHb, p=0.063), while both densities showed similar improvements in PK thermostability. Additionally, ex vivo incubation with tebapivat significantly reduced 2,3-DPG and increased ATP levels, thereby improving the ATP/2,3-DPG ratio. In LD RBCs, 5µM tebapivat significantly lowered 2,3-DPG levels without altering ATP, whereas RBCs with HD showed a significant 2,3-DPG decrease at 50µM tebapivat and ATP increases at both concentrations (data not shown). The ATP/2,3-DPG ratio improved similarly in both subpopulations (Figure 1C). The p50, PoS and RBC adhesion to laminin decreased significantly with tebapivat in total, and RBCs with LD and HD, though not in all conditions (Figure 2A-C). The reduction in PoS was more pronounced in RBCs with HD than in LD, but did not reach statistical significance (50µM tebapivat: -2.6 vs -7.3mmHg, p=0.094). The absolute decrease of adhered RBCs was most pronounced in RBCs with HD (50µM tebapivat: -4.1 cells vs -1.8 cells, p=0.031). Blood viscosity remained stable across treatments (data not shown).

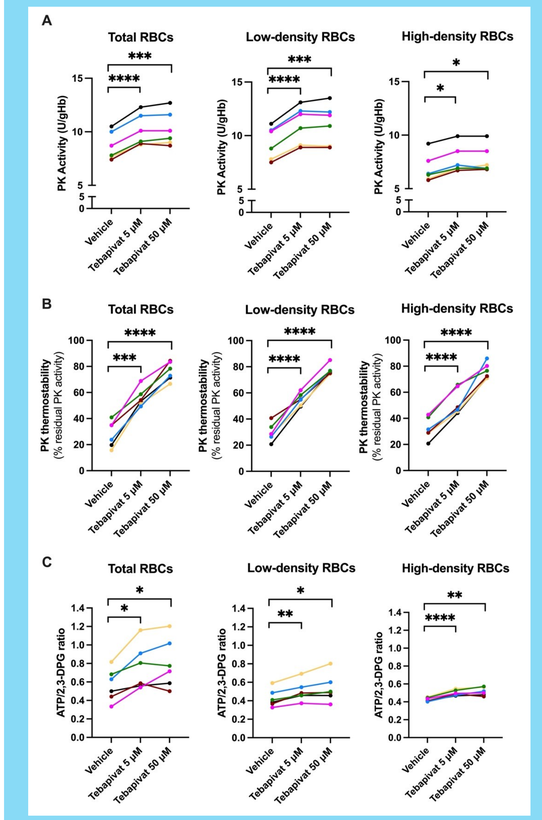


***Figure 1. Ex vivo treatment with tebapivat improves PK activity, PK thermostability and ATP/2,3-DPG ratio in different HbSS RBC subpopulations.*** *Each color represents a unique non-transfused HbSS patient. The black, red and yellow lines represent the patients on hydroxyurea treatment. (A-B) Ex vivo incubation with 5µM or 50µM tebapivat showed a significant increase in pyruvate kinase (PK) activity and PK thermostability compared to vehicle in total, low-density and high-density RBCs. PK thermostability, expressed as % residual PK activity, was measured on lysates after one-hour incubation at 53°C. (C) Adenosine triphosphate (ATP)/2,3-diphosphoglycerate (2,3-DPG) increased in all conditions. ns=p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. Vehicle: 0.1% DMSO.*

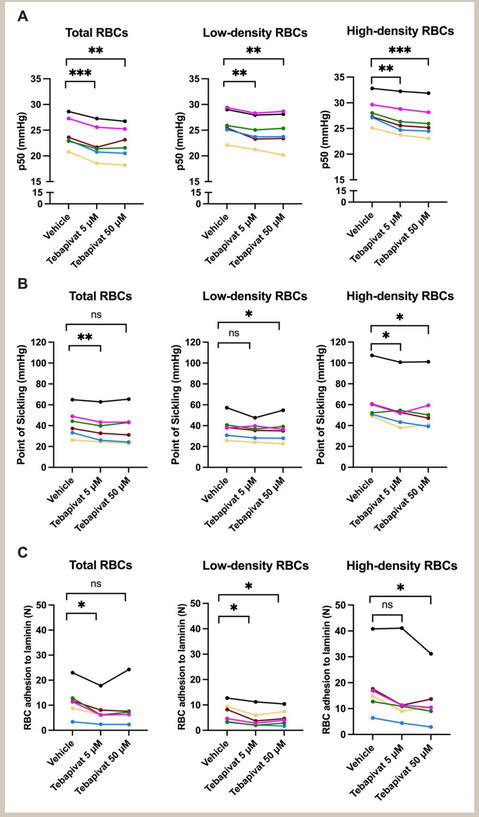


***Figure 2. Ex vivo treatment with tebapivat increases oxygen affinity and decreases sickling and RBC adhesion to laminin in different HbSS RBC subpopulations.*** *Each color represents a unique non-transfused HbSS patient. The black, red and yellow lines represent the patients on hydroxyurea treatment. (A) p50 decreases significantly in all conditions, thereby increasing the oxygen affinity. (B) The point of sickling decreases significantly at 5µM tebapivat in total RBCs, at 50µM tebapivat in low-density RBCs, and at both concentrations in high-density RBCS. (C) RBC adhesion to laminin decreases significantly at 5µM tebapivat in total RBCs, at both concentrations in low-density RBCs, and at 50µM tebapivat in high-density RBCs. Abbreviations: RBC, red blood cell. ns=p>0.05,*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. Vehicle: 0.1% DMSO.*

## CONCLUSIONS

- Ex vivo treatment with tebapivat improved RBC metabolic and functional properties, including reduced sickling tendency in all RBC (sub)populations.
- Both LD and HD RBCs responded to ex vivo treatment with tebapivat.
- A significant reduction in RBC adhesion to laminin was observed indicating that PK activation improves SCD pathophysiology outside of RBC metabolism.
- Our findings improve our understanding of the effects of PK activation in SCD and support the potential clinical benefit of tebapivat therapy in SCD.

## REFERENCES

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