Activating pyruvate kinase improves red blood cell integrity by reducing Band3 tyrosine phosphorylation

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INTRODUCTION

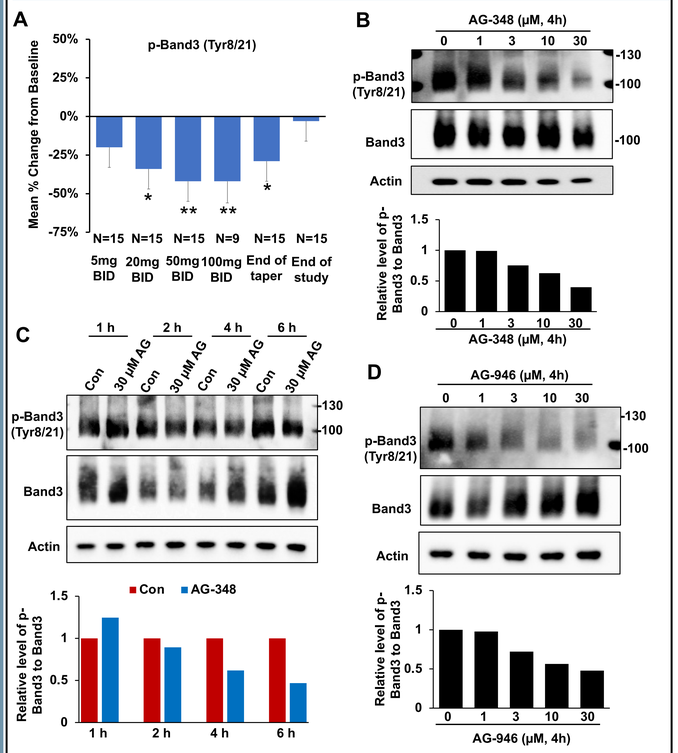
In a Phase 1 study we established proof of concept for activating pyruvate kinase (PK) in sickle cell disease (SCD) as a viable therapeutic approach.[1] Mitapivat (AG-348), a PK activator, increased ATP and decreased 2,3-DPG levels in a dose-dependent manner accompanied by improved hematologic parameters, increased oxygen affinity, and reduced sickling in HbSS patients. The Hb increase was sustained after stopping mitapivat until end of study (EOS) at 4 weeks, suggesting an improvement in RBC survival. Mitapivat has been shown to improve oxidative stress by increasing GSH/GSSG ratio.[2] In SCD, elevated oxidative stress inhibits RBC tyrosine (Tyr) phosphatases that normally maintain the RBC Band3 tyrosines in an unphosphorylated state, and studies have shown that band 3 Tyr is highly phosphorylated in SCD red cells. Tyr-phosphorylation of Band3 (Tyr-p-Band3) destabilizes membrane integrity and reduces RBC deformability.[3] We hypothesize that PKR activators, such as mitapivat and AG-946 (a novel activator of PKR), improve the membrane integrity via regulating Tyr-p-Band3.

METHODS

- Of the 16 patients in the Phase1 study, frozen whole blood samples were evaluable in 15, from which RBC ghosts (membranes) at the different time points (baseline and following 2 weeks of treatment at each dose level) were isolated. RBC ghosts from these 15 subjects were analyzed for Tyr-p-Band3 and intact PTP1B by Western blotting using a specific phospho-Tyr Band3 and PTP1B antibody, respectively.
- *Ex vivo* experiments were conducted by treating RBCs from HbSS patients with different concentrations of AG-348 or AG-946, for different times at 37°C.
- To investigate the effect of AG-348 on kinases and phosphatases, HbSS RBCs were treated with different concentrations of AG-348 with or without Syk kinase inhibitor-fostamatinib (Fosta).
- HbSS RBCs were treated with 30 μM mitapivat for 2h, 4h, 6h, and 8h and ATP levels were detected using CellTiter-Glo® 2.0 Cell Viability Assay.
- Immunoprecipitation was performed to measure the interaction between Band3 and PTP1B using PTP1B antibody.

RESULTS

Pyruvate kinase activators decrease Tyr-p-Band3.



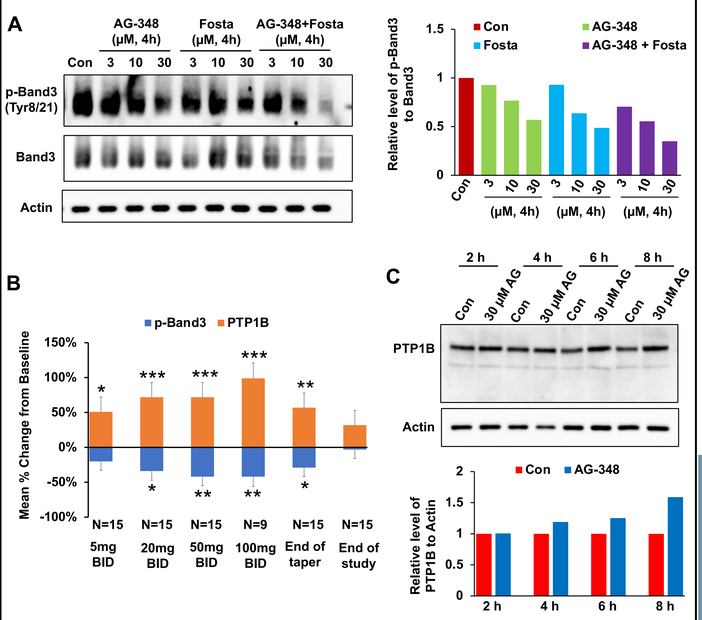
A) Mean change in Tyr-p-Band3 from baseline. Mitapivat decreased Tyr-p-Band3 in a dose-dependent manner in the 15 subjects followed by a return to near baseline by End of Study

B, C) AG-348 decreased Tyr-p-Band3 in (B) dose- and (C) time-dependent manner *ex vivo* .

D) AG-946, a novel activator of PK, decreased Tyr-p-Band3 in a dose-dependent manner *ex vivo.*

*P<0.05, **P<0.01.

Mitapivat (AG-348) decreases Tyr-p-Band3 by increasing PTP1B activity.



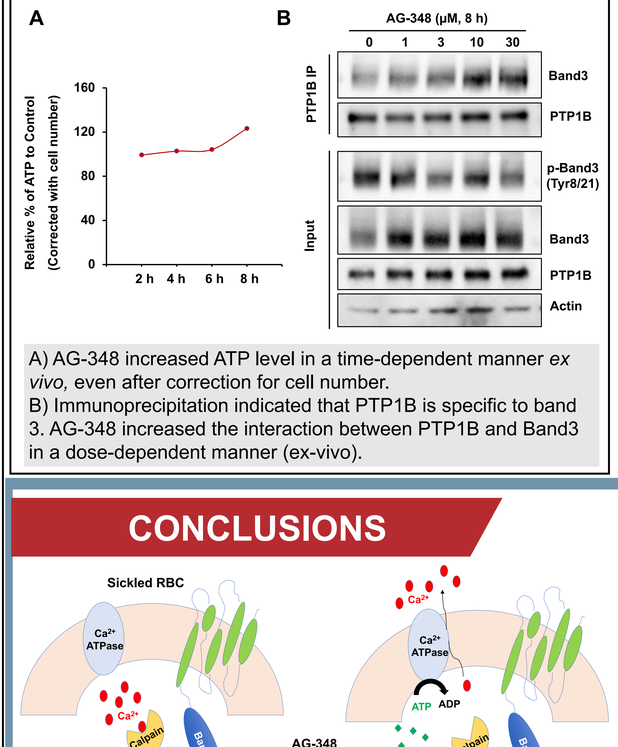
A) Combination of AG-348 with fostamatinib (Fosta) showed enhanced effect on the reduction of Tyr-p-Band3, which suggests that AG-348 may affect the activity of constitutive phosphatase-PTP1B.

B) Mean change in PTP1B from baseline. Mitapivat increased intact PTP1B associated with RBC membrane in a dose-dependent manner.

C) AG-348 increased the protein level of intact PTP1B associated with cell membrane in a time-dependent manner *ex vivo (*HbSS RBCs*)*.

*P<0.05, **P<0.01, ***P<0.001.

Mitapivat (AG-348) regulates PTP1B activity by increasing ATP level.



A) AG-348 increased ATP level in a time-dependent manner *ex vivo,* even after correction for cell number.

B) Immunoprecipitation indicated that PTP1B is specific to band 3. AG-348 increased the interaction between PTP1B and Band3 in a dose-dependent manner (ex-vivo).

CONCLUSIONS

A recent study showed that PTP1B is the major phosphatase that maintains Band3 tyrosine in an unphosphorylated state.[4] PTP1B could be cleaved into an inactive form by calpain, which needs to be activated by calcium. The balance of calcium in RBC relies on the activity of the plasma membrane Ca^{2+} ATPase (PCMA) pump, which is fueled by ATP. Our results suggest that in enhancing PKR activity, mitapivat leads to an increase in intracellular ATP which increases activity of the PCMA pump, and an increased efflux of intracellular calcium. A reduction in intracellular Ca^{2+} reduces the activity of calpain, and thereby suppresses calpain's cleavage of phosphatase-PTP1B, resulting in reduction of Band3 Tyr-phosphorylation. ATP increase is thus a key mechanism of mitapivat's beneficial effect on the hemoglobin increase in SCD.

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