FT-4202, an oral PKR activator, has potent antisickling effects and improves RBC survival and Hb levels in SCA mice

Archana Shrestha,1 Mengna Chi,1,* Kimberly Wagner,1,* Astha Malik,2 Jennifer Korpik,3 Adam Drake,4 Keertik Fulzele,4 Sylvie Guichard,4 and Punam Malik1,5

1Division of Experimental Hematology and Cancer Biology, 2Division of Gastroenterology, Hepatology and Nutrition, and 3Erythrocyte Diagnostic Laboratory, Cancer and Blood Diseases Institute (CBDI), Cincinnati Children’s Hospital Medical Center (CCHMC), Cincinnati, OH; 4FORMA Therapeutics, Inc, Watertown, MA; and 5Division of Hematology, CBDI, CCHMC, Cincinnati, OH

Key Points

• FT-4202, an oral investigational agent, increases the hemoglobin S O2 affinity to reduce sickling and improve RBC membrane deformability.
• Two-week administration of FT-4202 improves RBC survival and Hb levels in SCA mice.

Sickle cell anemia (SCA) results from an abnormal sickle hemoglobin (HbS). HbS polymerizes upon deoxygenation, resulting in red blood cell (RBC) sickling and membrane damage that cause vaso-occlusions and hemolysis. Sickle RBCs contain less adenosine triphosphate and more 2,3-diphosphoglycerate than normal RBCs, which allosterically reduces hemoglobin (Hb) oxygen (O2) affinity (ie, increases the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen [P50]), potentiating HbS polymerization. Herein, we tested the effect of investigational agent FT-4202, an RBC pyruvate kinase (PKR) activator, on RBC sickling and membrane damage by administering it to Berkeley SCA mice. Two-week oral FT-4202 administration was well tolerated, decreasing HbS P50 to levels similar to HbA and demonstrating beneficial biological effects. In FT-4202–treated animals, there was reduced sickling in vivo, demonstrated by fewer irreversibly sickled cells, and improved RBC deformability, assessed at varying shear stress. Controlled deoxygenation followed by reoxygenation of RBCs obtained from the blood of FT-4202–treated mice showed a shift in the point of sickling to a lower partial pressure of oxygen (pO2). This led to a nearly 30% increase in RBC survival and a 1.7g/dL increase in Hb level in the FT-4202–treated SCA mice. Overall, our results in SCA mice suggest that FT-4202 might be a potentially useful oral antisickling agent that warrants investigation in patients with SCA.

Introduction

Sickle cell anemia (SCA) is caused by a mutated HBB gene, resulting in the production of sickle hemoglobin (HbS). Upon deoxygenation, HbS polymerizes into long fibers, causing red blood cell (RBC) sickling and membrane damage, resulting in vaso-occlusions and hemolysis, and end-organ damage. Additionally, sickle RBCs have elevated 2,3-diphosphoglycerate (2,3-DPG) levels compared with normal RBCs,1-3 effects we also observe in an ongoing clinical study of FT-4202 in healthy subjects and patients with SCA.3,4 This occurs secondary to the anemia, hypoxia, and excessive adenosine signaling that is seen in SCA.5-8 2,3-DPG allosterically reduces hemoglobin (Hb) oxygen (O2) affinity (ie, it increases the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen [P50]) by binding to the Hb tetramer and stabilizing its T (tense)/deoxygenated form, which further promotes HbS polymerization.1,9 More elevated levels of 2,3-DPG correlate with higher sickling,10-15 although this was not observed in the early studies.16,17

Inhibiting HbS polymerization and reduction of sickling can occur via several mechanisms. Voxelotor (GBT440) modulates Hb O2 affinity by covalently binding α-globin and stabilizing oxyhemoglobin.18,19 Increasing the activity of 2,3-DPG phosphatase decreases 2,3-DPG and reduces HbS polymerization in
Figure 1. Oral administration of FT-4202 improves RBC parameters in the BERK mouse model. 2,3-DPG (A) and ATP (B) concentration in blood of BERK mice fed with control or FT-4202 chow for 2 weeks. (A-B) n = 17 mice per group. (C) P50 curve/oxygen equilibrium curve (OEC) showing the HbS P50 level in the blood of BERK assessed after 2 weeks of FT-4202 vs control chow administration. The oxygen dissociation curves are plotted using the average values of partial pressure of oxygen (pO2) and the percentage of oxygen saturation at each point of 4 control BERK mice (red line) and 4 FT-4202 BERK mice (blue line). Dashed arrows mark the P50 values for each group. (D) P50 levels. (C-D) n = 4 mice per group. (E) Representative peripheral blood smears showing ISCs (black arrow) stained with modified Wright’s stain; scale bar, 10 μM. (F) Irreversibly sickled cells (ISCs) quantification in control and FT-4202 BERK mice. Images of the blood smears were taken using a Nikon Eclipse Ti inverted microscope,
sickle RBCs in vitro. A different approach of reducing 2,3-DPG in RBCs is by increasing RBC pyruvate kinase (PKR) activity, increasing substrate utilization through the glycolytic cycle, which also generates adenosine triphosphate (ATP) (supplemental Figure 1). Mitapivat is a PKR agonist that was shown to improve the hemolytic anemia associated with pyruvate kinase deficiency.

FT-4202 is an activator of PKR that decreases 2,3-DPG levels, and hence would increase the HbS O2 affinity to reduce sickling. Moreover, activation of PKR by FT-4202 would also increase glycolytic ATP production, the energy currency of the cell that increases RBC membrane deformability by improving RBC hydration. Here, we determined the tolerability of daily oral FT-4202 in vivo, and its effects on sickle RBC 2,3-DPG levels, sickling, membrane deformability, and survival in an SCA mouse model.

**Methods**

Berkeley SCA [Tg(HueminLCRx1GISTA23Hba0/0 Hbb0/0)]28 (BERK) mice were either fed FT-4202 or control chow for 2 weeks in 4 cohorts. Health status, weight, and cell consumption were determined 3 times per week. Three cohorts were injected with sulfo-NHS-biotin 1 week into treatment, and RBC survival was assessed over the next week with serial bloods while on treatment. One cohort was only bled before and after 2 weeks of treatment to obtain complete blood and reticulocyte counts. Mice were exsanguinated after 2 weeks of treatment. Blood was processed for (a) RBC levels of 2,3-DPG and ATP, (b) irreversibly sickled RBCs (ISCs) by ImageJ analysis, (c) kinetics of experimentally induced sickling and RBC membrane deformability via Lorca Oxygenscan (RR Mechatronics, Zwaag, The Netherlands), (d) P50 by Hemox Analyzer (TCS Scientific Corp, New Hope, PA), and (e) plasma levels of FT-4202 by liquid chromatography with tandem mass spectrometry, bilirubin, and lactate dehydrogenase (LDH) by colorimetry. Animal experiments were performed using institutional animal care and use committee–approved protocols. Detailed methodology is available in supplemental Methods.

**Results and discussion**

BERK mice fed FT-4202 chow (FT-4202 BERK) consumed a similar amount of food, and had similar weights and survival, compared with BERK mice fed control chow (control BERK) throughout the 2-week period, showing that oral FT-4202 was well tolerated by FT-4202 BERK mice (supplementary Figure 2A-C). After 2 weeks of FT-4202 administration, plasma FT-4202 levels were 7702 ± 796 ng/mL in FT-4202 BERK mice. Levels of 2,3-DPG were significantly decreased and ATP levels were significantly increased in FT-4202 BERK vs control BERK mice (Figure 1A-B), which were associated with a significantly lower P50 in FT-4202 BERK compared with control BERK mice (Figure 1C-D; supplemental Figure 3). Indeed, P50 in FT-4202 BERK mice was similar to P50 of normal RBCs. Although the oral dose and the exposure of FT-4202 in BERK mice was higher than in the ongoing human trial, it was well tolerated and the pharmacodynamic effect on 2,3-DPG levels and P50 was similar.

Repeated cycles of HbS polymerization in microvasculature and its depolymerization in lungs damage RBC membranes, which results in membrane loss and ISCs. Indeed, blood smears showed significantly reduced numbers of ISCs in the FT-4202 BERK compared with control BERK mice (Figure 1E-F). To demonstrate the in vivo effect of FT-4202 on HbS polymerization and RBC membrane health, we assessed the sickling kinetics and membrane deformability after 2 weeks of treatment in BERK mice using Lorca Oxygenscan. FT-4202 BERK mice showed an improved Oxygenscan curve when compared with control BERK mice (Figure 1G): the point of sickling (PoS) in the FT-4202 BERK mice occurred at a much lower pO2 of 30 mm Hg compared with 37 mm Hg in controls (P < .002). The minimum elongation index (EImin) was also significantly improved in FT-4202 BERK than in control BERK mice (P < .03), whereas the maximum EI (EImax) showed an improved trend in FT-4202 BERK (0.52 ± 0.01) vs control BERK mice (0.47 ± 0.02; P = .07). In contrast, in normal mice, there was no change in EI with deoxygenation/reoxygenation, as is expected (supplemental Figure 4). Taken together, RBCs from FT-4202 BERK mice had a reduced propensity to sickle. Next, we assessed RBC membrane deformability across a gradient of shear stress under normoxia: FT-4202 BERK mice had improved EI at shear stress > 3 to 9 Pa vs controls (P < .03; Figure 1H) and this effect was more pronounced after 1 cycle of deoxygenation/reoxygenation (P < .01-.001; Figure 1I).

Sickling-induced membrane damage increases hemolysis, reducing RBC lifespan in mice and humans with SCA. Indeed, hemolysis...
was significantly reduced in FT-4202 BERK mice, reflected in reduced bilirubin ($P < .0001$) and LDH levels ($P < .0002$) (Figure 2A-B). BERK mice have an extremely short RBC lifespan coupled with high RBC turnover as indicated by $50\%$ reticulocytes. NHS-biotin tracing revealed a $28.5\%$ increase in the RBC half-life to $1.8$ vs $1.4$ days in the FT-4202 BERK vs control BERK mice, respectively ($P < .004$; Figure 2C). In fact, RBC half-life inversely correlated with the percentage of ISCs in BERK FT-4202 mice (supplemental Figure 6). Reduced hemolysis and increased RBC survival was accompanied by increased RBC counts ($P < .002$), Hb levels ($P < .0003$), and hematocrit fractions ($P < .003$; Figure 2D-F) in FT-4202 BERK mice at 2 weeks. However, the reticulocyte counts were only minimally reduced (Figure 2G), which may be a mouse erythropoiesis-specific phenomenon, due to the inherently high reticulocytes ($50\%$) in BERK mice. It is conceivable that the increased HbS $O_2$ affinity results in a compensatory increase in RBC production. Notably, FT-4202 administration increased HbS $O_2$ affinity to levels well within the physiological range, similar to that of HbA, and hence should not cause tissue hypoxia. Furthermore, the improved Hb should not increase vaso-occlusions as RBCs have a reduced propensity to sickle and have reduced hemolysis. Thus, the increased Hb in FT-4202 BERK mice is consistent with improved RBC survival (Figure 2C) even with mildly increased HbS $O_2$ affinity (Figure 1C-D; supplemental figure).
Biochemical effects translated to significant biological benefits with increasing ATP levels in SCA RBCs. In SCA mice, these FT-4202 has potential multimodal effects of reducing 2,3-DPG and improving membrane deformability, and reduced Po2. S. In addition, we show that oral FT-4202 was well tolerated by SCA mice. A parallel FT-4202 human phase 1 study in healthy subjects and patients with sickle cell disease is ongoing (NCT03815695), and the preliminary data thus far are consistent with the mouse studies.3,4

Acknowledgments

The authors thank Jeff Bailey and Victoria Summey (Comprehensive Mouse and Cancer Core) for assistance with mouse procedures, Scarlett Ripberger and the Erythrocyte Diagnostic Laboratory at CCHMC for their help with P50 measurements, Theodisia Kalfa, Katie Seu, and Rose Fessler for technical assistance with Llorca Oxygenscan, Alexander G. Miethke for help with hemolysis assays, and Sydney Felker and Oluwabukola Gbotosho for help with blood sample processing. The authors also thank the Research Flow Cytometry Core at CCHMC for their services.

This work was supported by Forma Therapeutics, Inc.

Authorship

Conflict-of-interest disclosure: A.D. is a shareholder in Forma Therapeutics, Inc. K.F. is a current employee of, and a shareholder in, Forma Therapeutics, Inc. S.G. is a current employee of, and a shareholder in, Forma Therapeutics, Inc, and is a shareholder in AstraZeneca. P.M. holds patents with, and receives royalties from, Aruvant Sciences and CSL Behring, and has provided consultancy services to Aruvant Sciences and Forma Therapeutics, Inc. The remaining authors declare no competing financial interests.

ORCID profiles: A.M., 0000-0001-6957-8044; S.G., 0000-0003-1143-2805; P.M., 0000-0002-8942-2108.

Correspondence: Punam Malik, Division of Experimental Hematology and Cancer Biology, Cincinnati Children’s Hospital Medical Center, ML 7013, 3333 Burnet Ave, Cincinnati, OH 45229; e-mail: punam.malik@cchmc.org.

References

1. Charache S, Grisolia S, Fiedler AJ, Hellegers AE. Effect of 2,3-diphosphoglycerate on oxygen affinity of blood in sickle cell anemia. J Clin Invest. 1970;49(4):806-812.
2. Poillon WN, Kim BC, Labotka RJ, Hicks CU, Kark JA. Antisickling effects of 2,3-diphosphoglycerate depletion. Blood. 1995;85(11):3289-3296.
3. Kalfa T, Kuppers F, Telen M, et al. Phase 1 single (SAD) and multiple ascending dose (MAD) studies of the safety, tolerability, pharmacokinetics (PK) and pharmacodynamics (PD) of FT-4202, an allosteric activator of pyruvate kinase-R, in healthy and sickle cell disease subjects [abstract]. Blood. 2019;134(suppl 1). Abstract 616.
4. Estepp JH, Kalfa T, Saraf S, et al. Phase 1 single (SAD) and multiple ascending dose (MAD) study of the safety, pharmacokinetics (PK) and pharmacodynamics (PD) of FT-4202, a PKR-activator, in healthy and sickle cell disease subjects [abstract]. HemaSphere. 2020;4(suppl 1):709. Abstract EP1531.
5. Zhang Y, Dai Y, Wen J, et al. Detrimental effects of adenosine signaling in sickle cell disease. Nat Med. 2011;17(1):79-86.
6. Liu H, Adebiyi M, Liu RR, et al. Elevated ecto-5′-nucleotidase: a missing pathogenic factor and new therapeutic target for sickle cell disease. Blood Adv. 2018;2(15):1957-1968.
7. Sun K, D’Alessandro A, Ahmed MH, et al. Structural and functional insight of sphingosine 1-phosphate-mediated pathogenic metabolic reprogramming in sickle cell disease. Sci Rep. 2017;7(1):15281.
8. Sun K, Zhang Y, Bogdanov MV, et al. Elevated adenosine signaling via adenosine A2B receptor induces normal and sickle erythrocyte sphingosine kinase 1 activity. Blood. 2015;125(10):1643-1652.
9. MacDonald R. Red cell 2,3-diphosphoglycerate and oxygen affinity. Anaesthesia. 1977;32(6):544-553.
10. Poillon WN, Robinson MD, Kim BC. Deoxygenated sickle hemoglobin. Modulation of its solubility by 2,3-diphosphoglycerate and other allosteric polyanions. J Biol Chem. 1985;260(26):13897-13900.
11. Poillon WN, Kim BC, Welty EV, Walder JA. The effect of 2,3-diphosphoglycerate on the solubility of deoxyhemoglobin S. Arch Biochem Biophys. 1986;249(2):301-305.
12. Poillon WN, Kim BC. 2,3-Diphosphoglycerate and intracellular pH as interdependent determinants of the physiologic solubility of deoxyhemoglobin S. Blood. 1990;76(5):1028-1036.
13. Rogers SC, Ross JG, d’Avignon A, et al. Sickle hemoglobin disturbs normal coupling among erythrocyte O2 content, glycolysis, and antioxidant capacity. Blood. 2013;121(9):1651-1662.
14. Jensen FB. The dual roles of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of local blood flow. *J Exp Biol.* 2009;212(pt 21):3387-3393.

15. Safo MK, Kato GJ. Therapeutic strategies to alter the oxygen affinity of sickle hemoglobin. *Hematol Oncol Clin North Am.* 2014;28(2):217-231.

16. Beutler E, Paniker NV, West C. The effect of 2,3-DPG on the sickling phenomenon. *Blood.* 1971;37(2):184-186.

17. Swerdlow PH, Bryan RA, Bertles JF, Poillon WN, Magdoff-Fairchild B, Milner PF. Effect of 2, 3-diphosphoglycerate on the solubility of deoxy-sickle hemoglobin. *Hemoglobin.* 1977;1(6):527-537.

18. Vichinsky E, Hoppe CC, Ataga KI, et al; HOPE Trial Investigators. A phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med.* 2019;381(6):509-519.

19. Oksenberg D, Dufu K, Patel MP, et al. GBT440 increases haemoglobin oxygen affinity, reduces sickling and prolongs RBC half-life in a murine model of sickle cell disease. *Br J Haematol.* 2016;175(1):141-153.

20. Grace RF, Rose C, Layton DM, et al. Safety and efficacy of mitapivat in pyruvate kinase deficiency. *N Engl J Med.* 2019;381(10):933-944.

21. Henry ER, Cellmer T, Dunkelberger EB, et al. Allosteric control of hemoglobin S fiber formation by oxygen and its relation to the pathophysiology of sickle cell disease. *Proc Natl Acad Sci USA.* 2020;117(26):15018-15027.

22. Kung C, Hixon J, Kosinski PA, et al. AG-348 enhances pyruvate kinase activity in red blood cells from patients with pyruvate kinase deficiency. *Blood.* 2017;130(11):1347-1356.

23. Shima M, Miyashima K, Yawata Y. Increased calcium uptake in the red cells of un- splenectomized patients with hereditary spherocytosis: significant contribution of reticulocytosis. *Clin Chim Acta.* 1984;142(2):183-192.

24. Ptaszy C, Brion CM, Manci E, et al. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. *Science.* 1997;278(5339):876-878.

25. Brown RC, Cruz K, Kalfa TA, et al. FT-4202, an allosteric activator of pyruvate kinase R, demonstrates proof of mechanism and proof of concept after a single dose and after multiple daily doses in a phase 1 study of patients with sickle cell disease [abstract]. *Blood.* 2020;136(suppl 1):19-20.

26. Allan D, Limbrick AR, Thomas P, Westerman MP. Release of spectrin-free spicules on reoxygenation of sickled erythrocytes. *Nature.* 1982;295(5850):612-613.

27. Manci EA, Hillery CA, Bodian CA, Zhang ZG, Lutty GA, Coller BS. Pathology of Berkeley sickle cell mice: similarities and differences with human sickle cell disease. *Blood.* 2006;107(4):1651-1658.

28. Yudin J, Verhovsek M. How we diagnose and manage altered oxygen affinity hemoglobin variants. *Am J Hematol.* 2019;94(5):597-603.