Etavopivat, a Pyruvate Kinase Activator in Red Blood Cells, for the Treatment of Sickle Cell Disease

Patricia Schroeder,1 Keertik Fulzele,1 Sanjeev Forsyth, Maria D. Ribadeneira, Sylvie Guichard, Erik Wilker, C. Gary Marshall, Adam Drake, Rose Fessler, Diamantis G. Konstantinidis, Katie G. Seu, and Theodosia A. Kalfa

Forma Therapeutics, Watertown, Massachusetts (P.S., K.F., M.D.R., S.G.); Tango Therapeutics, Cambridge, Massachusetts (E.W.); Repare Therapeutics, Cambridge, Massachusetts (C.G.M.); Ichnos Sciences, Epalinges, Switzerland (A.D.); Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio (R.F., D.G.K., K.G.S., T.A.K.); and Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, Ohio (T.A.K.)

Received May 20, 2021; accepted December 21, 2021

ABSTRACT

Etavopivat is an investigational, oral, small molecule activator of erythrocyte pyruvate kinase (PKR) in development for the treatment of sickle cell disease (SCD) and other hemoglobinopathies. PKR activation is proposed to ameliorate the sickling of SCD red blood cells (RBCs) through multiple mechanisms, including reduction of 2,3-diphosphoglycerate (2,3-DPG), which consequently increases hemoglobin (Hb)-oxygen affinity; increased binding of oxygen reduces sickle hemoglobin polymerization and binding. In addition, PKR activation increases adenosine triphosphate (ATP) produced via glycolytic flux, which helps preserve membrane integrity and RBC deformability. We evaluated the pharmacodynamic response to etavopivat in nonhuman primates (NHPs) and in healthy human subjects and evaluated the effects in RBCs from patients with SCD after ex vivo treatment with etavopivat. A single dose of etavopivat increased 2,3-DPG in NHPs and healthy subjects. Hb-oxygen affinity was significantly increased in healthy subjects after 24 hours. After daily dosing of etavopivat over 5 consecutive days in NHPs, ATP was increased by 38% from baseline. Etavopivat increased Hb-oxygen affinity and reduced sickling in RBCs collected from patients with SCD with either homozygous hemoglobin S or hemoglobin S and C disease. Collectively, these results demonstrate the ability of etavopivat to decrease 2,3-DPG and increase ATP, resulting in increased Hb-oxygen affinity and improved sickle RBC function. Etavopivat is currently being evaluated in clinical trials for the treatment of SCD.

SIGNIFICANCE STATEMENT

Etavopivat, a small molecule activator of the glycolytic enzyme erythrocyte pyruvate kinase, decreased 2,3-diphosphoglycerate in red blood cells (RBCs) from nonhuman primates and healthy subjects and significantly increased hemoglobin (Hb)-oxygen affinity in healthy subjects. Using ex vivo RBCs from donors with sickle cell disease (SCD) (homozygous hemoglobin S or hemoglobin S and C genotype), etavopivat increased Hb-oxygen affinity and reduced sickling under deoxygenation. Etavopivat shows promise as a treatment for SCD that could potentially reduce vaso-occlusion and improve anemia.

This study was funded by Forma Therapeutics, Inc. (Watertown, MA). P.S., S.F., M.D.R., and S.G. are employees of Forma Therapeutics and may own shares in Forma Therapeutics. K.F. is a previous employee of Forma Therapeutics and reports equity ownership. A.D. is a previous employee of Forma Therapeutics and owns shares in Forma Therapeutics. T.A.K. receives research funding from Forma Therapeutics and from Agios Pharmaceuticals and honoraria from Agios Pharmaceuticals for participation in Steering Committee. E.W., C.G.M., R.F., D.G.K., and K.G.S. have nothing to declare.

Part of this work was previously presented as follows: Estep JH, Kalfa T, Saraf S, Kim H, Bienatt L, Malik P, Cruz K, Brown RC, Fessler R, Kuypers FA, et al. (2020) 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. European Hematology Association Annual Congress; 2020 Jun 11–21; held virtually.

†P.S. and K.F. contributed equally to this work as co-first authors.

This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: AUC0–24, area under the concentration time curve from time 0 to 24 hours after dosing; EI, elongation index; Hb, sickle hemoglobin; HbSC, hemoglobin S and C; HbSSc, homozygous hemoglobin S; HU, hydroxyurea; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NHP, nonhuman primate; PD, pharmacodynamics; PK, pharmacokinetics; PKR, erythrocyte pyruvate kinase; pO2, partial pressure of dissolved oxygen; P50, the pO2 at which hemoglobin is 50% saturated with oxygen; PoS, point of sickling; RBC, red blood cell; SCD, sickle cell disease; VOS, vaso-occlusive crisis.

Introduction

Sickle cell disease (SCD) is an autosomal recessive blood disorder caused by a single point mutation in the beta-globin gene, resulting in the expression of sickle hemoglobin (HbS). HbS tends to polymerize when deoxygenated in the low oxygen tension of capillaries or even in arterioles. HbS polymerization alters red blood cell (RBC) morphology and negatively affects its function (Sundd et al., 2019). The two most common SCD genotypes are due to homozygosity for the beta-S mutation (p.Glu6Val) (HbSS) or compound heterozygosity for beta-S and beta-C (p.Glu6Lys) (HbSC) (da Guarda et al., 2020). The formation of cytoplasmic HbS polymers causes the RBCs to adopt a rigid, sickle-like shape that is the defining characteristic of SCD (Kato et al., 2018). Sickled RBCs display abnormal

210
rheological properties, aggregate, and can lead to microcapillary blockage causing tissue hypoxia, which is experienced as a painful vaso-occlusive crisis (VOC). Repeated HbS polymerization and depolymerization with changing oxygen pressure, as well as oxidative damage, alters the RBC membrane, ultimately leading to a significantly shortened RBC lifespan (i.e., hemolysis) (Sundd et al., 2019).

Despite increased reticulocyte production, this decreased RBC lifespan results in anemia. The physiologic metabolic response of RBCs to anemia is to increase 2,3-diphosphoglycerate (2,3-DPG), thereby promoting the release and offloading of oxygen from hemoglobin (Hb) to tissues (Charache et al., 1970). This shift to lower oxygen affinity promotes HbS deoxygenation and consequently polymerization, leading to RBC sickling. Resulting changes in the RBC membrane, such as expression of phosphatidyl serine on the cell surface, lead to altered interaction with other blood cells and the endothelium. Together with the release of proinflammatory RBC contents upon hemolysis, these changes elicit an inflammatory response, including the activation of white blood cells and increased adhesion of sickle RBCs and white blood cells to the endothelium (Telen, 2007), resulting in the sudden, severe, and painful VOC episodes. VOC is the leading diagnosis associated with emergency department visits for patients with SCD (Yusuf et al., 2010); upwards of 40% of such emergency department visits can result in hospital admission (Lanzkron et al., 2010). VOCs also lead to secondary complications, such as acute chest syndrome, that cause end-organ damage and premature death in patients with SCD (Novelli and Gladwin, 2016).

Erythrocyte pyruvate kinase (PKR) is the RBC-expressed isoform of the key glycolytic enzyme pyruvate kinase. PKR catalyzes the last and rate-limiting step of glycolysis from phosphoenol-pyruvate to pyruvate while generating adenosine triphosphate (ATP) from adenosine diphosphate. In patients with pyruvate kinase deficiency, decreased PKR activity results in anemia (Zanella et al., 2005), whereas activation of PKR improves RBC survival and Hb levels (Grace et al., 2019). We hypothesized that PKR activation above normal levels would alter several steps of the glycolytic pathway utilizing glucose to generate pyruvate. Increased PKR activity would be expected to decrease production of 2,3-DPG and increase production of ATP within RBCs (Koralkova et al., 2014; Bianchi et al., 2019). PKR is therefore a logical target to activate for treatment of SCD, as a decrease in 2,3-DPG levels would increase the affinity of Hb for oxygen, lowering the potential for HbS polymerization and RBC sickling.

Furthermore, an increase in ATP synthesis can enhance overall RBC function and health. Reduced ATP within RBCs adversely affects ion homeostasis, contributing to cell dehydration (Ortiz et al., 1990; Gallagher, 2017), membrane damage (de Jong et al., 2001; Soupen and Kuypers, 2006; Weiss et al., 2012), and impaired RBC deformability (Clark et al., 1980), all of which contribute to the pathophysiologic features of SCD. By attenuating these adverse effects, an increase in ATP synthesis via PKR activation within sickle RBCs would potentially have the dual beneficial effect of addressing the chronic anemia associated with SCD and reducing the incidence of VOC.

Here, we describe the cellular effects of etavopivat (FT-4202), a potent, selective, orally bioavailable PKR activator (Ericsson et al., 2020). Using oral dosing studies in nonhuman primates (NHPs) and healthy subjects, we demonstrate that etavopivat decreases 2,3-DPG and increases ATP in RBCs. We also present the results of a series of pharmacodynamic (PD) assays after in vivo treatment of healthy RBCs and ex vivo treatment of sickle RBCs with etavopivat, supporting the relevance of these biomarkers when evaluating response to this investigational agent. This work demonstrates the translational value of measurements adopted in preclinical and early clinical development, which are indicative of RBC health. We present target engagement data (single 700-mg dose) from the first-in-human clinical trial that provides a platform of evidence supporting further clinical development of etavopivat for the treatment of SCD.

Materials and Methods

In Vivo Studies

Procedures in Cynomolgus Monkeys. The pharmacokinetics (PK) and PD of etavopivat were assessed in male cynomolgus monkeys. The decision to use male cynomolgus monkeys was based on availability only. There were no PK/PD gender differences observed during preclinical multiple-dose safety studies. Animals (AlphaGene-Sis Inc., Yemassee, SC) were of Chinese origin, in good health (non-naive), and housed, cared for, and acclimated to study procedures in compliance with the testing facility Institutional Animal Care and Use Committee Guidelines and Standard Operating Procedure (Biomere, MA). NHP experiments were approved by the Institutional Animal Care and Use Committee in Biomere, MA, and conducted according to the National Research Council’s Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

A spray-dried dispersion of etavopivat was delivered in a vehicle solution containing 0.5% hydroxypropyl methylcellulose and was administered to monkeys by oral gavage. In a single-dose experiment, monkeys (n = 4) received etavopivat at a dose of 50 mg/kg. In a multiple-dose experiment, animals received a dose of 0.5% hydroxypropyl methylcellulose (control); n = 3) or a dose of etavopivat at 3, 8, or 22 mg/kg (n = 4 per group) once daily on 5 consecutive days. The dose volume for all groups was 5 ml/kg. For the single-dose experiment, whole blood samples (~0.5 ml) for determination of plasma concentrations of etavopivat and whole blood 2,3-DPG and ATP were collected predose, 30 minutes postdose, and 2, 4, 6, 8, 12, and 24 hours postdose. For the multiple-dose experiment, blood samples were collected 1, 6, 12, and 24 hours postdose on day 1 (~0.5 ml) and predose, 30 minutes postdose, and 1, 2, 4, 6, 8, 12, and 24 hours postdose on day 5 (~0.8 ml).

Single-Dose Plasma PK and PD of Etavopivat in Healthy Subjects. Key aspects of the first-in-human study of etavopivat are summarized below. The study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the relevant institutional review board. Subjects who took part in the study were healthy volunteers aged 18 to 60 years inclusive and provided written informed consent.

The single-ascending dose portion of the study was a phase 1, single-center, randomized, placebo-controlled, double-blind dose escalation study to assess the safety, tolerability, PK, and PD after a single oral dose of etavopivat in healthy subjects (ClinicalTrials.gov identifier: NCT03815695). Placebo and etavopivat were administered orally to subjects after overnight fasting. Successively escalated dose levels of etavopivat at 200, 400, 700, and 1000 mg were evaluated. For each sequential dose cohort, eight subjects were randomly assigned 2:6 to receive a single oral dose of placebo or etavopivat, respectively. Blood samples were collected pre- and post-etavopivat dosing at serial time points for PK/PD assessment. Etavopivat plasma concentrations were measured using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.

Procedures in Healthy Subjects. Single-dose plasma PK and PD of etavopivat were evaluated in healthy subjects. Subjects were enrolled in a single-dose pharmacodynamic study after overnight fasting. Subjects who took part in the study were healthy volunteers aged 18 to 60 years inclusive and provided written informed consent.

Procedures in NHPs. Following multiple-dose safety studies in NHPs, single-dose plasma PK and PD of etavopivat were evaluated in NHPs. Animals were selected on the basis of their availability at the time of the study, with randomization to administration of either a single dose of etavopivat or placebo. NHPs were given a single oral dose of etavopivat or placebo, and blood samples were collected predose, 30 minutes postdose, and 2, 4, 6, 8, 12, and 24 hours postdose.

All studies were performed in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).
were measured in whole blood by generating oxygen equilibrium curves as described below. Noncompartmental PK analysis of etavopivat was conducted using Phoenix WinNonlin (Certara, Princeton, NJ).

In Vitro Studies

**Activation of PKR in Human Red Blood Cells.** The activation of wild-type PKR by etavopivat in mature human RBCs ex vivo was evaluated in purified RBCs purchased from Research Blood Components (Watertown, MA). Varying concentrations of etavopivat stock solutions (2.5 μl) were added to 22.5 μl of washed human RBCs (0.75 million) in the range of 2 nM to 30 μM in Roswell Park Memorial Institute Medium (Corning Inc., Corning, NY) and incubated for 3 hours at 37°C. Cells were spun at 1500 rpm for 5 minutes, and the supernatant was removed. Cells were washed twice using 200 μl of PBS and a 5-minute 1500 rpm spin cycle. After the second wash, the RBC pellet was lysed for 30 minutes in 200 μl of assay buffer. After a 5-minute spin at 1500 rpm, lysis was assayed using a Biocinor (Militpas, CA) Pyruvate Kinase Assay (Catalog # K709-100).

**Whole Blood from Patients with SCD.** Whole blood samples from female and male patients homozygous for HbS (HbSS) or compound heterozygous for HbS and HbC (HbSC) were collected in vacutainers with K$_2$-EDTA as an anticoagulant, at Cincinnati Children’s Hospital, with informed consent and in accordance with an Independent Review Board-approved protocol. Whole blood samples (250 μl) from normal control donors (HbAA) and patients with SCD were washed twice with 1 ml Hanks’ Balanced Salt Solution (Thermo Fisher Scientific, Wal- tham, MA) containing 10 mM HEPES, 10 mM MgCl$_2$, and 5.5 mM glucose at pH 7.4 (Buffer 1) and centrifuged at 400 × g for 5 minutes. Supernatant was removed by aspiration. The RBC pellet was resuspended in 1 ml of Buffer 1 and centrifuged for 5 minutes at 800 × g to pack the cells. Supernatant was aspirated and packed RBCs were resuspended to 20% (v/v) (20% hematocrit) with Buffer 1 and incubated for 3 hours at 37°C. Cells were spun at 1500 rpm for 5 minutes, and the supernatant was removed. Cells were washed twice using 200 μl of PBS and a 5-minute 1500 rpm spin cycle. After the second wash, the RBC pellet was lysed for 30 minutes in 200 μl of assay buffer. After a 5-minute spin at 1500 rpm, lysis was assayed using a Biocinor (Militpas, CA) Pyruvate Kinase Assay (Catalog # K709-100).

**Etavopivat in Dimethyl Sulfoxide (DMSO) or Etavopivat-Treated RBCs.** To evaluate changes in the deformability of RBCs with or without previous etavopivat treatment as a function of oxygen pressure under steady shear stress. 200 × 10$^6$ RBCs from patients with SCD treated ex vivo with DMSO or etavopivat, as detailed above, were suspended in 5 ml of Oxy Iso (osmolality 282–286 mOsm/kg; pH 7.35–7.45) at room temperature. The RBC suspension was injected into a rotating wall cylinder maintained at 37°C with a fixed shear stress of 30 Pascal. Deoxygenation and reoxygenation of the RBC suspension were achieved by gradual entry of nitrogen gas and ambient air, respectively. One oxygenscan consisted of approximately 80 measurements of the elongation index (EI) during one round of deoxygenation (1300 seconds) and was followed by reoxygenation (280 seconds). Oxygen saturation in the RBC suspension was calculated every 20 seconds based on quenching of the signal using a luminophore oxygen sensor. Ambient air Po$_2$ in the cup of the device started at approximately 150 mm Hg and was gradually lowered to below 20 mm Hg of oxygen. The Po$_2$ in oxygenscan is calculated as the Po$_2$ (mm Hg) at which the EI falls below 5% of the maximum EI (EI$_{max}$) during deoxygenation and indicates the oxygen pressure at which the polymerization of HbS starts to show effects on RBC deformability.

**Statistics**

Statistical analysis was performed using Prism, Version 8.4 (GraphPad Software, San Diego, CA). Paired $t$-test or Wilcoxon tests were used as appropriate. $P$ value of less than 0.05 was considered statistically significant for all tests. All preclinical experiments should be considered exploratory. The sample size for the phase 1 study was considered adequate to evaluate the safety, tolerability, and PK/PD of etavopivat; no formal power calculations were performed.

**Results**

**Etavopivat Activates PKR in Healthy Human RBCs In Vitro.** The activation of wild-type PKR by etavopivat in mature human RBCs ex vivo was evaluated in purified RBCs. Wild-type PKR was activated in a concentration-dependent manner. The calculated mean maximum activation was 89% and the half-maximal effective concentration ($EC_{50}$) was 113 nM.

**Etavopivat Modulates 2,3-DPG and ATP in Cynomolgus Monkeys.** After the administration of a single 50-mg/kg dose of etavopivat in NHPs, the etavopivat plasma concentration reached its maximal value ($C_{max}$) between 1 and 2 hours postdose, and 2,3-DPG declined to 47% of the baseline concentration between 12 and 24 hours postdose ($P = 0.04$).
Etavopivat for the Treatment of Sickle Cell Disease

Due to the temporal delay between etavopivat plasma concentration and 2,3-DPG modulation, direct plots between etavopivat PK and 2,3-DPG response are not meaningful. An indirect PK/PD model, previously applied to describe 2,3-DPG PK/PD relationships (Kha et al., 2015) and described in Supplemental Text 1, was used to quantitate the exposure-response relationship. This analysis estimates a calculated 2,3-DPG EC$_{50}$ of 18 ng/ml (34 nM) and a 50% 2,3-DPG reduction from baseline as the maximum pharmacodynamic effect in NHPs dosed with etavopivat. Primary PK/PD parameters in NHPs are summarized in Supplemental Table 1.

After a single 50-mg/kg dose, ATP was not significantly elevated from baseline up to 24 hours postdose. To understand the temporal behavior of ATP elevation upon etavopivat administration, ATP was measured in whole blood from NHPs dosed with 3, 8, or 22 mg/kg once daily for 5 consecutive days. Concentrations of ATP were not significantly altered at any dose on day 1 (Fig. 2C); however, by day 5, ATP concentrations were elevated at the 8- and 22-mg/kg doses, with day average increases of 18% ($P = 0.0004$) and 38% ($P = 0.004$), respectively, relative to average levels on day 1. As the dynamics of ATP formation and clearance were not adequately captured, this precluded a quantitative assessment of in vivo EC$_{50}$ for ATP. Etavopivat C$_{max}$ and area under the concentration time curve from time 0 to 24 hours after dosing (AUC$_{0-24}$) after single and repeated escalating doses in NHPs are presented in Supplemental Table 2. Day 5 percentage changes of 2,3-DPG and ATP, along with exposure (day 5 AUC$_{0-24}$) are summarized in Fig. 3.
Ex Vivo Etavopivat Treatment of RBCs from Patients with SCD Improved Hb-Oxygen Affinity and Reduced PoS. The ability of etavopivat to increase Hb-oxygen affinity in healthy subjects treated with a single dose (Fig. 4A) is indicative of the potential to mitigate the rate and extent of RBC sickling in patients with SCD. We tested this possibility ex vivo, using whole blood from donors with either HbSS or HbSC. The $P_{50}$ in the patients’ specimens before the in vitro incubation with vehicle control or etavopivat was in the range of 28–34 mm Hg. Despite optimization of the in vitro incubation media, especially of the buffer composition to maintain stable CO$_2$ and pH as far as possible, the $P_{50}$ values of the sickle red cells incubated under control conditions were relatively lower than baseline, as shown in Fig. 4, B and C. Nevertheless, etavopivat-incubated RBCs under the same conditions as vehicle control signiﬁcantly reduced $P_{50}$. The mean (S.D.) decrease in $P_{50}$ after treatment with etavopivat versus vehicle was 1.27 ± 0.76 mm Hg and 1.47 ± 0.63 mm Hg in HbSS and HbSC donors, respectively (Supplemental Table 3). In the HbSS group, $P_{50}$ (mean ± S.D.) was reduced to 24.8 ± 1.82 mm Hg after 4-hour incubation with etavopivat compared with 26.1 ± 1.99 mm Hg in the vehicle-treated group ($P = 0.0002$) (Fig. 4B). For the HbSC group, $P_{50}$ was reduced to 24.8 ± 1.58 mm Hg after incubation with etavopivat compared with 26.3 ± 1.24 mm Hg in the vehicle-treated group ($P = 0.0313$) (Fig. 4C). Etavopivat treatment induced a leftward shift of the Hb-oxygen dissociation curve in RBCs from donors with either HbSS or HbSC (Fig. 4, D and E), suggesting an improved oxygen-carrying capacity of Hb under conditions of decreased oxygen pressure.

We tested the deformability of RBCs across a pO$_2$ range, from 150 to <20 mm Hg, using oxygen gradient ektacytometry. PoS was calculated as the pO$_2$ at which RBC maximal deformability ($E_{\text{Lmax}}$) decreased by 5%. The mean (S.D.) decrease in PoS after incubation with etavopivat for 4 hours versus vehicle was 3.53 ± 4.29 mm Hg and 3.91 ± 2.40 mm Hg in HbSS and HbSC donors, respectively. Ex vivo incubation of RBCs from patients with HbSS with etavopivat decreased the PoS (mean ± S.D.) to 28.3 ± 8.09 mm Hg compared with 31.9 ± 7.93 mm Hg in the paired samples treated with vehicle control ($P = 0.0244$) (Fig. 5, A and C). PoS was also decreased in etavopivat-treated (19.3 ± 5.11 mm Hg) versus vehicle-treated (23.2 ± 5.61 mm Hg) RBCs from donors with HbSC ($P = 0.0313$) (Fig. 5, B and D).

Human PK of Etavopivat. After a single oral dose of 700 mg in healthy subjects (one female, five male), etavopivat was rapidly absorbed and median time to maximum concentration was approximately 0.5 hours. After reaching peak concentrations, mean concentrations of etavopivat declined in a multiexponential manner (Fig. 6). The estimated elimination half-life ($t_{1/2}$) was approximately 13 hours. Area under the concentration time curve from time 0 extrapolated to infinity (geometric mean [%CV]) was 6995 (29.4) ng·h/ml. Moderate intersubject variability in plasma etavopivat concentrations was observed.

Etavopivat Decreases 2,3-DPG and Increases Hb-Oxygen Affinity in Healthy Subjects. The PD profile after a single 700-mg dose of etavopivat in healthy subjects was comparable with a single 50-mg/kg dose in NHPs. The maximum inhibition of 2,3-DPG was observed 24 hours postdose, lagging behind the etavopivat C$_{\text{max}}$, which was observed approximately 0.5 hours postdose. In healthy subjects, concentrations of 2,3-DPG were collected for 7 days after a single 700-mg dose; maximal reductions of 2,3-DPG (mean 49%; $P = 0.0313$) were durable, lasting 48 hours postdose, and returned to baseline by day 7 (Fig. 6). Changes in whole blood concentrations of ATP after a single dose were not significant. After a single 700-mg dose in healthy subjects, etavopivat was also shown to increase Hb-oxygen affinity (or decrease $P_{50}$) by 4.85 mm Hg at 24 hours, when the greatest decrease in 2,3-DPG was observed (Supplemental Table 3). 2,3-DPG and $P_{50}$ were correlated positively, providing a PKR activation-mediated PD response to etavopivat that increased Hb-oxygen affinity (Fig. 7).

Discussion

Advancements in disease-modifying therapies are urgently needed for patients with SCD. Through methodical medicinal chemistry efforts, we have identified etavopivat, a potent, selective, orally bioavailable, and novel PKR activator. Here, we present data from preclinical NHP and early-stage clinical human studies to show that etavopivat decreased 2,3-DPG and increased ATP in NHPs after daily dosing. In RBCs from donors with SCD, etavopivat increased Hb-oxygen affinity of RBCs and delayed sickling upon deoxygenation. Decreasing the level of deoxygenation that RBCs can tolerate, before sickling, may reduce the incidence of VOC in patients with SCD.

Targeting PKR activation to reduce the concentration of 2,3-DPG is based on the rate-limiting role that PKR plays in regulating glycolysis in RBCs. A decrease in 2,3-DPG with PKR activation has been demonstrated in preclinical studies, in healthy subjects, and in patients with PKR deficiency after
treatment (Chubukov et al., 2016; Kung et al., 2017; Yang et al., 2019). Decreases in 2,3-DPG in both NHPs and humans were delayed relative to peak plasma concentrations of etavopivat. More specifically, in NHPs, maximum decreases in whole blood 2,3-DPG were observed between 12 and 24 hours postdose, sometime after the mean \( C_{\text{max}} \) was achieved, typically 1 hour postdose. A comparable observation was made after a single 700-mg dose of etavopivat was durable for 48 hours before slowly returning to baseline by day 7. After a single dose, maximum decreases in 2,3-DPG in NHPs dosed with 50 mg/kg and healthy human subjects treated with 700 mg were 47% and 49%, respectively, consistent with comparable unbound exposure at those dose levels (2342 and 2099 ng·h/ml for NHPs and humans, respectively). Mitigating 2,3-DPG has been proposed as a means to reduce HbS polymerization in patients with SCD (Eaton and Bunn, 2017). Basal concentrations of 2,3-DPG are higher in patients with SCD compared with healthy controls (Charache et al., 1970; Castro, 1980;
Ould Amar et al., 1996), and depletion of 2,3-DPG has been shown to reduce the tendency for RBC sickling in patients with SCD (Poillon et al., 1995).

2,3-DPG strongly influences the binding of oxygen to Hb; by binding within the central cavity of the Hb tetramer, 2,3-DPG causes allosteric changes and reduces the molecule's affinity to oxygen (MacDonald, 1977). Increased Hb-oxygen affinity (decreased P$_{50}$) with a mean delta P$_{50}$ of 4.85 mm Hg was observed in healthy subjects after the administration of a single 700-mg dose of etavopivat. P$_{50}$ and 2,3-DPG in blood were strongly correlated before and 24 hours after a single dose of etavopivat. The 2,3-DPG-mediated effect provides a PD-based rationale for the increased Hb-oxygen affinity after treatment with etavopivat. Furthermore, ex vivo experiments using RBCs from donors with HbSS or HbSC showed decreases in P$_{50}$, indicating increased Hb-oxygen affinity in the presence of etavopivat. Since higher concentrations of 2,3-DPG are associated with increases in deoxyhemoglobin, therapeutic

Fig. 5. Etavopivat reduced the dissolved oxygen pressure at which HbSS or HbSC RBCs initiate sickling. Isolated RBCs from donors with either HbSS or HbSC genotype were treated ex vivo with either 20 μM etavopivat or DMSO (vehicle) for 4 hours at 37°C. Curves for RBC deformability as a function of dissolved oxygen pressure (oxygenscan) were constructed using Lorcapa. The PoS at which the deformation of RBCs reached 5% from baseline was calculated and plotted. Treatment with etavopivat significantly decreased the PoS in RBCs from both HbSS (n = 11) (A) and HbSC (n = 6) (B) donors. Representative oxygenscans for RBCs from a HbSS donor (C) and an HbSC donor (D) are shown. Data were analyzed using the nonparametric Wilcoxon matched-pairs signed rank test. *P < 0.05.
approaches that increase Hb-oxygen affinity and consequently reduce deoxygenation of HbS are expected to decrease HbS polymerization, reduce sickling, and attenuate the clinical consequences of SCD.

In addition to the reduction in 2,3-DPG and associated improvement in Hb-oxygen affinity, the increased glycolytic flux induced by PKR activation is expected to increase RBC function and health by increasing ATP and reducing oxidative stress. Although no meaningful elevation in ATP was observed after a single dose in monkeys or healthy subjects, ATP concentrations were elevated by as much as 38% ($P = 0.004$) relative to day 1 in NHPs after 5 consecutive days of once-daily doses of etavopivat. In a recent study in Berkeley sickle cell mice, ATP levels were increased ($P < 0.01$) after 2 weeks of treatment with etavopivat, corresponding with etavopivat plasma levels of $7702 \pm 796$ ng/ml (Shrestha et al., 2021). ATP plays an essential role in maintenance of RBC membrane- cytoskeletal integrity (Betz et al., 2009), membrane repair via activation of flippase to reinternalize phosphatidylserine to the inner leaflet of the RBC membrane (Soupene and Kuypers, 2006; Weiss et al., 2012), cell hydration (Gallaher, 2017), maintenance of ionic (Mg$^{2+}$ and Ca$^{2+}$) gradients (Ortiz et al., 1990; Raftos et al., 1999; Rivera et al., 2005), RBC deformability (Weed et al., 1969; McMahon, 2019), and protection from oxidative damage (Banerjee and Kuypers, 2004). Therefore, increasing ATP concentrations is likely to have broad beneficial effects for patients with SCD. Moreover, in the current study, ex vivo treatment with etavopivat significantly improved the PoS in the RBCs from donors with the HbSS genotype, likely due to a reduction in 2,3-DPG. These dual effects of decreased 2,3-DPG and increased ATP suggest that etavopivat has the potential to reduce RBC sickling, hemolysis, anemia, and consequently VOC.

Pharmacologic interventions in clinical practice or development for the treatment of SCD include approaches to increase fetal Hb or Hb oxygenation to decrease Hb polymerization and to decrease vascular adhesion. The current standard of care for most patients with SCD is hydroxyurea (HU), which reduces sickling by increasing fetal Hb in RBCs and reduces painful episodes of VOC by ~50%; however, the effectiveness of HU can be confounded by poor patient compliance and discontinuation rates can be high (Brandow and Panepinto, 2010; Agrawal et al., 2014; Shah et al., 2019). In addition, although HU is broadly efficacious, response to treatment can be variable and a proportion of patients (10%–20%) are nonresponsive (Steinberg et al., 1997; Ma et al., 2007; Steinberg, 2008). Furthermore, HU is a myelosuppressive agent that may cause neutropenia and thrombocytopenia and thus requires routine blood monitoring for side effects (Agrawal et al., 2014). Although HU has been used to treat SCD for over 30 years, there remains a need for improved disease management, more pharmacological options, and the possibility of combination therapies to target different aspects of SCD pathology. Crizanlizumab was recently approved to reduce the frequency of VOC in adults and pediatric patients aged 16 years and older with SCD. However, no improvement in hemolysis, anemia, or markers of inflammation have been observed (Ataga et al., 2017; Blair, 2020a). These data suggest that although targeting endothelial adhesion may
decrease the incidence and severity of VOC, these agents are unlikely to address the anemia or chronic tissue damage associated with SCD. Drugs that increase Hb-oxygen affinity, such as the recently approved agent voxelotor, directly interfere with HbS polymerization by maintaining a higher proportion of Hb in an oxygenated state (Vichinsky et al., 2019; Blair, 2020b). Although the clinical safety profile for voxelotor to date has been positive, there is a recognized risk that excessive oxygen affinity could cause hypoxia by preventing release of oxygen into tissues, for which routine neurovascular monitoring has been recommended (Hebbel and Hedlund, 2018). Activation of PKR follows a physiologic pathway that increases Hb-oxygen affinity by decreasing 2,3-DPG and is likely to counteract the pathophysiology of SCD while enabling oxygen release in hypoxic tissues. As a result of the combination of decreased 2,3-DPG and increased ATP, it is proposed that targeting PKR activation may have a broad and significant impact on SCD, both in HbSS and HbSC, by improving the membrane integrity of RBCs.

In conclusion, by virtue of its ability to promote activity in the glycolytic pathway, etavopivat is hypothesized to have unique, beneficial, disease-modifying effects on SCD. As the enzyme that catalyzes the last step of glycolysis, PKR underpins reactions that directly impact the metabolic health and primary functions of RBCs. Etavopivat has two key effects: first, it decreases 2,3-DPG, which can reduce HbS polymerization and potentially attenuate clinical sequelae of vaso-occlusion in SCD; and second, it increases ATP, which provides metabolic resources to support membrane integrity and protect against the loss of RBC deformability and potentially to mitigate hemolysis in patients with SCD. The PD response to etavopivat in relation to PKR was demonstrated preclinically and confirmed in the first-in-human trial, providing valuable evidence of target modulation, which supports the rationale for subsequent clinical trials. Furthermore, the preliminary safety profile of etavopivat supports its advancement to additional clinical trials to determine the risk/benefit profile of PKR activation in SCD. Clinical trials of etavopivat in patients with SCD are currently in progress.

Authorship Contributions

Participated in research design: Schroeder, Fulzele, Forsyth, Ribadeneira, Guichard, Wilker, Marshall, Drake, Konstantinidis, Seu, Kalifa.
Conducted experiments: Ribadeneira, Fessler, Konstantinidis, Seu. Contributed new reagents or analytic tools: Konstantinidis, Seu. Performed data analysis: Schroeder, Fulzele, Forsyth, Ribadeneira, Guichard, Fessler, Konstantinidis, Seu, Kalifa. Wrote or contributed to the writing of the manuscript: Schroeder, Fulzele, Forsyth, Ribadeneira, Guichard, Wilker, Marshall, Drake, Fessler, Konstantinidis, Seu, Kalifa.

Acknowledgments

The authors would like to thank Dr. Frans Kuypers for helpful discussions and Dr. Eric Wu for his contribution to statistical analyses. Medical writing assistance was provided by Sue Reinwald and Katy Beck of Engage Scientific Solutions (Horsham, UK) and was funded by Forma Therapeutics, Inc.

References


SUPPLEMENTAL DATA

Etavopivat, a Pyruvate Kinase Activator in Red Blood Cells, for the Treatment of Sickle Cell Disease

Patricia Schroeder,1 Keertik Fulzele,1 Sanjeev Forsyth, Maria D. Ribadeneira, Sylvie Guichard, Erik Wilker, C. Gary Marshall, Adam Drake, Rose Fessler, Diamantis G. Konstantinidis, Katie G. Seu, and Theodosia A. Kalfa

1Co first authors

Journal of Pharmacology and Experimental Therapeutics

JPET-AR-2021-000743
NHP PK and 2,3-DPG data were modeled using a simultaneous PK/PD approach utilizing Phoenix® NLME software (Certara, Princeton, NJ). The PK data were fit using a two-compartment model with first-order absorption. The 2,3-DPG data were fit using a basic indirect-response model (Dayneka et al., 1993; Sharma and Jusko, 1998) with a stimulation of loss function for 2,3-DPG shown in equation 1, where 2,3DPG \(_0\) is baseline 2,3-DPG levels, \(K_{out}\) is a first-order loss rate constant, \(S_{max}\) is the maximum ability of etavopivat to affect \(K_{out}\), and \(SC_{50}\) is the etavopivat concentration resulting in 50% of the maximum stimulation achieved at the effect site.

\[
\frac{d2,3DPG}{dt} = 2,3DPG_0 \cdot K_{out} - K_{out} \cdot (1 + \frac{S_{max} \cdot C}{C + SC_{50}}) \cdot 2,3DPG
\]

The maximum 2,3-DPG suppression (2,3DPG\(_{max}\)) was estimated from equation 2.

\[
2,3DPG_{max} = \left(\frac{2,3DPG_0}{1 + S_{max}}\right)
\]

Half of the maximal 2,3-DPG response (2,3DPG\(_{50}\)) was estimated from equation 3

\[
2,3DPG_{50} = 2,3DPG_0 - \frac{2,3DPG_0 - 2,3DPG_{max}}{2}
\]

The plasma concentration resulting in half of the maximum decrease in 2,3-DPG (EC\(_{50}\)) was calculated using equation 4.

\[
EC_{50} = \frac{SC_{50} \cdot (2,3DPG_0 - 2,3DPG_{50})}{2,3DPG_{50} \cdot (1 + S_{max}) - 2,3DPG_0}
\]
Table S1. Etavopivat primary PK/PD parameters in non-human primates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3DPG₀ (µg/mL)</td>
<td>841</td>
<td>9.79</td>
</tr>
<tr>
<td>Kᵅout (h⁻¹)</td>
<td>0.100</td>
<td>0.019</td>
</tr>
<tr>
<td>Sₘₐₓ</td>
<td>0.870</td>
<td>0.159</td>
</tr>
<tr>
<td>SC₅₀ (ng/mL)</td>
<td>33.5</td>
<td>12.9</td>
</tr>
</tbody>
</table>

2,3DPG₀, baseline 2,3-DPG levels; Kᵅout, first-order loss rate constant; PD, pharmacodynamic; PK, pharmacokinetic; Sₘₐₓ, maximum ability of etavopivat to affect Kᵅout; SC₅₀, etavopivat concentration resulting in 50% of the maximum stimulation achieved at the effect site; S.D, standard deviation.
**Table S2.** Etavopivat exposure in non-human primates after single and repeated escalating doses

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>AUC&lt;sub&gt;0-24&lt;/sub&gt; (h·ng/ml)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>AUC&lt;sub&gt;0-24&lt;/sub&gt; (h·ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>94 (44)</td>
<td>608 (292)</td>
<td>83 (45)</td>
<td>295 (52)</td>
</tr>
<tr>
<td>8</td>
<td>352 (95)</td>
<td>1116 (163)</td>
<td>725 (198)</td>
<td>1274 (86)</td>
</tr>
<tr>
<td>22</td>
<td>424 (320)</td>
<td>1728 (658)</td>
<td>689 (200)</td>
<td>2568 (803)</td>
</tr>
<tr>
<td>50</td>
<td>1823 (489)</td>
<td>8877 (1749)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*n = 4 animals per dose*

AUC<sub>0-24</sub>, area under the concentration time curve from time 0–24 hours after dosing; C<sub>max</sub>, maximum plasma concentration; ND, not determined; PK, pharmacokinetic; S.D, standard deviation.
Table S3. Change in $P_{50}$ following etavopivat treatment in healthy subjects and ex vivo RBC from donors with SCD

<table>
<thead>
<tr>
<th></th>
<th>$P_{50}$ Pre-dose (mmHg)</th>
<th>$P_{50}$ 24 hours post-dose (mmHg)</th>
<th>Change in $P_{50}$ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy subjects:</strong> single 700-mg dose ($n = 6$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.5 (1.53)</td>
<td>21.6 (2.05)</td>
<td>4.85 (0.68)</td>
</tr>
<tr>
<td><strong>P$_{50}$ - vehicle (DMSO) Treatment (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ex vivo SCD RBC (HbSC disease)</strong> ($n = 6$)</td>
<td>26.3 (1.24)</td>
<td>24.8 (1.58)</td>
<td>1.47 (0.63)</td>
</tr>
<tr>
<td><strong>Ex vivo SCD RBC (HbSS disease)</strong> ($n = 13$)</td>
<td>26.1 (1.99)</td>
<td>24.8 (1.82)</td>
<td>1.27 (0.76)</td>
</tr>
</tbody>
</table>

Data are mean (S.D.)

DMSO, dimethyl sulfoxide; $P_{50}$, the partial pressure of dissolved oxygen at which Hb is 50% saturated with oxygen; RBC, red blood cell; SCD, sickle cell disease; S.D., standard deviation.