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SUPPORTING INFORMATION

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PF-07059013: A non-covalent hemoglobin modulator favorably impacts disease state in a mouse model of sickle cell disease

To the Editor:

Sickle cell disease (SCD) is a severe genetic disorder that impacts approximately 20 million people worldwide.[1] The causative β6 Glu-Val substitution is a gain of function mutation; in the deoxygenated state, the mutant protein, Hb S, can form polymers, leading to red blood cell sickling and precipitating downstream consequences, including vaso-occlusion (pain crisis), hemolytic anemia, stroke and related pathophysiology.[2,3] Polymerization is exponentially dependent on deoxy Hb S concentration.[4] Thus, relatively small changes in deoxyHb S concentration will significantly impact polymerization, red blood cell sickling, and ultimately the clinical course of the disease. Pharmacologic evidence for the benefit of reducing the concentration of deoxyHb S arises from studies of covalent modification of Hb S, where stabilization of the oxygenated conformation increases Hb O_2 affinity, reduces RBC sickling, extends RBC half-life, and ultimately reduces the frequency of vaso-occlusive crisis (VOC).[5-8] In clinical trials, ex vivo carbamylation of patient blood led to improvements in hemolytic anemia; treated patients exhibited a 2.7 g/dl increase in hemoglobin, a 58% decrease in reticulocytes, a 65% decrease in irreversibly sickled cells, and an 80% decrease in frequency of painful crises.[8] Subsequent oxyHb S stabilizing molecules were developed based on the observation that benzaldehyde derivatives formed stable, covalent Schiff bases with hemoglobin. The most advanced of this class of molecules is the covalent compound Voxelotor (GBT 440, Oxbryta), which was approved by the FDA in 2019 for the treatment of SCD. In the pivotal study, 59% of patients in the higher dose group (1500 mg/day) had increases of 1 g/dl or greater in hemoglobin, with a mean hemoglobin modification of 26.5%.[9] While the impact of covalent Hb S modifiers on hemolytic anemia is well established, the rise in hemoglobin observed for voxelotor falls short of the effects observed by ex vivo carbamylation, and was not accompanied by a significant effect on the frequency of VOC. This suggests that the therapeutic potential of hemoglobin modification has not been fully realized.

PF-7059013 is a non-covalent modifier of hemoglobin that stabilizes the oxygenated state (see Gopalsamy et al.). Here we present the impact this molecule has on a well-established mouse model of sickle cell disease. Treatment with PF-7059013 demonstrated robust changes in key markers of hemolytic anemia in the Townes mouse model,[10] suggesting it has the potential to be a potent and efficacious therapy for SCD.

PF-07059013 was orally administered to Townes SCD model mice twice daily at a dose of 200 mg/kg for 15 days. This dose was selected as it was expected to result in approximately 25% hemoglobin coverage.[11] At 30 minutes post the initial dose, total blood concentrations for individual animals were 2–4 mM, consistent with the low total blood clearance observed in the single dose administration studies. These values translate to approximately 40%–60% hemoglobin coverage, based on measured hemoglobin concentrations. The high total blood concentrations observed following the initial dose were maintained for the duration the 15-day dose period (Supporting Information S1).

Animals treated with PF-07059013 show a significant stabilization of the oxygenated state. The average p50 decreased by 53.7% (±21.2%) in the treated group, relative to vehicle, and the average p20, a more sensitive marker of compound occupancy, decreased by

84.4% (±2.6%) in the treated group relative to vehicle (Supporting Information S1). As expected from the large shifts in oxygen affinity, blood from animals in the treated group showed significant reductions in RBC sickling. Under stringent hypoxic conditions, treatment with PF-07059013 resulted in a 37.8% (±9%) decrease in RBC sickling (Figure 1(A)).

Consistent with reduction of RBC sickling, following 15 days of dosing, mice treated with PF-07059013 showed significant improvement in markers of hemolytic anemia. PF-07059013 treated animals showed a 42.4% (±4.2%) increase in hemoglobin, with a mean increase in hemoglobin of 5 g/dl, as well as a 30.9% (±0.7%) increase in hematocrit, and a 39.2 (±9.3%) increase in red blood cells relative to vehicle. All of the changes were statistically significant, and the increases restored the hemoglobin, hematocrit, and RBC counts of the treated group to values similar to wild type (C7BL/6) mice (Figure 1(B)). In addition, treatment with PF-07059013 resulted in a

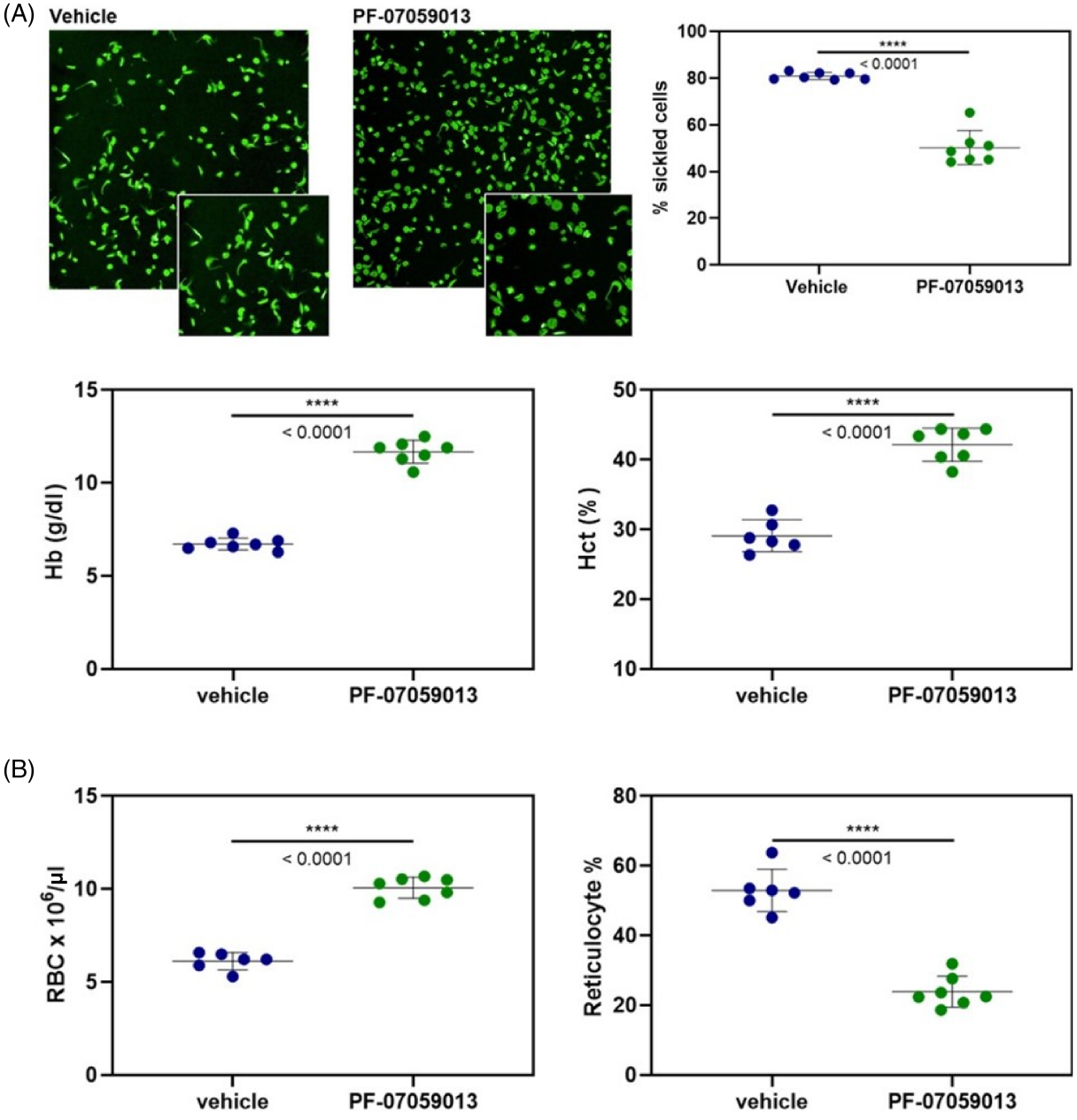

FIGURE 1 (A) Left: Representative images of red blood cells from vehicle treated and PFE-07059013 treated animals, following 4 h of hypoxic exposure. Inset: Expansion of section of depicted field to highlight differences in number of sickled cells. Right: Individual values for RBC sickling for vehicle treated and PF-07059013 treated mice. The treated animals showed a robust and statistically significant reduction in RBC sickling. (B) Markers of hemolytic anemia measured for vehicle treated (blue) and PF-07059013 treated (green) mice. Note, PF-07059013 treated animals showed increases in RBC count, hemoglobin (Hb), and hematocrit (Hct), indicating a substantial reduction in hemolysis. PF-07059013 treated animals also showed a significant decrease in reticulocytes, suggesting decreased hematopoiesis in the PF-07059013 animals, due to the decreased hemolysis

54.7% (±2.4%) decrease in reticulocytes (Figure 1(B)). Note, PF-07059013 achieves consistently high levels of hemoglobin occupancy in in vivo studies using the Townes SCD murine model, which were sustained for the duration of dosing. Taken together, these results indicate that a non-covalent molecule has the potential to be efficacious for the treatment of sickle cell disease, and that the presence of a reactive aldehyde is not a requirement for potency.

Comparing the activity of early covalent modifiers in the Townes model with PF-07059013 is not possible, as the development of many of those molecules predated the development of the transgenic mouse models. However, it is possible to compare the performance of PF-07059013 in the Townes SCD mouse with previously published Voxelotor pre-clinical data in the Townes model. Oksenberg et al. report that twice-daily oral dosing of Townes SCD mice with Voxelotor/GBT 440 at 100 or 150 mg/kg for 9–12 days resulted in hemoglobin occupancies ranging from 11%–39.7%.[12] The pharmacodynamic effects of Voxelotor were strongly correlated with the degree of hemoglobin occupancy, as only the animals attaining >30% occupancy (4/14) showed changes in reticulocyte count or red blood cell half-life relative to vehicle. Thus, PF-07059013 achieved high degrees of hemoglobin coverage upon twice-daily oral dosing at 200 mg/kg for 15 days. As PF-07059013 binds ditopically to Hb (two compound: one tetramer), the dose is comparable to the doses used in the Voxelotor animal studies,[12] as PF-07059013 requires twice as much compound to achieve the same hemoglobin occupancy percentages. In contrast with Voxelotor preclinical studies[12] all of the animals dosed with PF-07059013 ($n = 7$) achieved >40% hemoglobin occupancy. The consistently high occupancy level across all animals leads to a uniform improvement in markers of hemolytic anemia. Similarly, all PF-07059013 treated animals showed decreases in RBC sickling, ranging from 35.3%–45.5%. The observed decrease in RBC sickling with PF-07059013 treatment is consistent with the decrease observed for Voxelotor in Townes SCD mice that had high Hb occupancy.[12]

The role of increases in hemoglobin in the overall pathology of sickle cell disease, particularly as it relates to VOC, is not completely understood. In the Voxelotor pivotal trial, 59% of patients treated with 1500 mg/day experienced hemoglobin increases of 1 g/dl or greater (average = 1.1 g/dl), and showed reductions in reticulocytes and bilirubin, consistent with improvements in hemolytic anemia, following 24 weeks of dosing.[9] Post-hoc analysis of the pivotal trial results indicated that patients who achieved a hemoglobin level of 10 g/dl or greater had reduced incidence of VOC (50/179), with the greatest benefit observed in the small group of patients that had hemoglobin levels of 12 g/dl or greater (10/179).[13] These data are consistent with the observation that increased hemoglobin can lead to reductions in VOC, provided RBC sickling is sufficiently impeded; Diedrich et al. demonstrated a substantial reduction in VOC frequency following weekly extracorporeal carbamylation.[8] After 3 months of treatment, hemoglobin had increased by an average of 2.7 g/dl to an average of 8.8 g/dl and occurrences of VOC decreased by 80%.[8] An increase in hemoglobin alone is likely not sufficient, as SCD patients undergoing exchange transfusions still experience VOC.[14] Ex vivo carbamylation was most efficacious when hemoglobin occupancy was above 35%, suggesting achieving and maintaining high levels of hemoglobin occupancy may be crucial for making the maximum reduction in RBC sickling, reducing hemolysis, and increasing hemoglobin. These data indicate that it is possible to correlate the hemoglobin increase mediated by stabilization of the oxygenated state to resolution of VOC, and further suggest that the size of the increase in hemoglobin may be an important influencer of other clinical outcomes. Based in part on the magnitude and consistency of the response in the Townes SCD mouse model presented here, clinical studies of PF-07059013 are currently underway.

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CONFLICT OF INTEREST

All authors listed were employees of Pfizer Inc and declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Kelly M. Knee: designed research, performed research, analyzed data, wrote the paper. Reema Jasuja: designed research, performed research, analyzed data. Amey Barakat: performed research, analyzed data. Dharani Rao: performed research, analyzed data. Zane Wenzel: performed research, analyzed data. Jayasankar Jasti: performed research, analyzed data. Jonathan Novak: performed research, analyzed data. Kevin Beaumont: designed research, analyzed data. David W. Piotrowski: designed research, analyzed data. Phil Jeffery: designed research, analyzed data. Christine Bulawa: designed research, analyzed data. John E. Murphy: analyzed data, designed research. Jay M. Janz: performed research, designed research, analyzed data, wrote the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FLT3 inhibitor based induction and allogeneic stem cell transplant in complete remission 1 improve outcomes in patients with newly diagnosed Acute Myeloid Leukemia with very low FLT3 allelic burden

To the Editor:

Internal tandem duplication (ITD) mutations of the FMS-like tyrosine kinase (*FLT3*) gene are seen in approximately 25% of the patients with acute myeloid leukemia (AML) and are associated with a high risk of relapse and poor survival.[1] However, *FLT3*-ITD allelic ratio (AR), calculated as the ratio of mutated *FLT3*-ITD divided by wild type *FLT3* alleles (using DNA fragment analysis), less than 0.5, especially with concurrent *NPM1* mutations, have been categorized as a lower-risk disease by the European Leukemia Network (2017) guidelines.[2] The use of FLT3 inhibitors (FLT3i), such as sorafenib, midostaurin, gilteritinib, and quizartinib, has improved survival in patients with *FLT3*-mutated AML in the frontline, relapsed/refractory (R/R), and post-transplant maintenance settings. The FLT3i's appear to be effective irrespective of the *FLT3*-ITD ARs, but recent analysis have suggested a clearer benefit for second-generation FLT3i's compared with salvage chemotherapy in patients with R/R AML who had higher FLT3 ARs.[3] Furthermore, the phase III FLT3i trials, RATIFY and ADMIRAL, excluded patients with *FLT3* AR <0.05.[4,5] The role and impact of FLT3i's, allogeneic stem cell transplantation (ASCT) and *NPM1* co-mutation, in patients with very low allelic burden FLT3 mutations is unclear, often debated, and is the focus of this analysis.

In our institution, *FLT3* assays are reported as allelic frequency (AF) rather than AR. AF is calculated as the ratio of mutated *FLT3*-ITD divided by wild type *plus* mutated *FLT3*. In this study, baseline *FLT3*-ITD AFs ≤0.1 (equivalent to FLT3 ARs ≤0.11) were defined as very low level. We retrospectively reviewed patients who received therapy for newly diagnosed *FLT3*-mutated AML (excluding core-binding factor AML and acute promyelocytic leukemia) between 2012–2020 at our institution. We identified 50 patients with *FLT3*-ITD AF ≤0.1 (at diagnosis). A polymerase chain reaction (PCR) based DNA analysis followed by capillary