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ORIGINAL PAPER

GBT021601 improves red blood cell health and the pathophysiology of sickle cell disease in a murine model

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Summary
The pathophysiologic mechanism of sickle cell disease (SCD) involves polymerization of deoxygenated haemoglobin S (HbS), leading to red blood cell (RBC) sickling, decreased RBC deformability, microvascular obstruction, haemolysis, anaemia and downstream clinical complications. Pharmacological increase in the concentration of oxygenated HbS in RBCs has been shown to be a novel approach to inhibit HbS polymerization and reduce RBC sickling and haemolysis. We report that GBT021601, a small molecule that increases HbS-oxygen affinity, inhibits HbS polymerization and prevents RBC sickling in blood from patients with SCD. Moreover, in a murine model of SCD (SS mice), GBT021601 reduces RBC sickling, improves RBC deformability, prolongs RBC half-life and restores haemoglobin levels to the normal range, while improving oxygen delivery and increasing tolerance to severe hypoxia. Notably, oral dosing of GBT021601 in animals results in higher levels of Hb occupancy than voxelotor and suggests the feasibility of once-daily dosing in humans. In summary, GBT021601 improves RBC health and normalizes haemoglobin in SS mice, suggesting that it may be useful for the treatment of SCD. These data are being used as a foundation for clinical research and development of GBT021601.

KEYWORDS
haemolytic anaemia, oxygen affinity, red blood cell health, sickle cell disease, sickle cell murine model

INTRODUCTION

The pathophysiologic mechanism of sickle cell disease (SCD) involves polymerization of sickle haemoglobin (HbS) following deoxygenation in the microvasculature, leading to red blood cell (RBC) sickling and decreased RBC health, deformability and survival.[1,2] These abnormalities drive haemolysis, anaemia, vascular inflammation and microvasculature occlusions, resulting in clinical complications, such as fatigue, painful vaso-occlusive crisis, reduced quality of life, considerable end-organ damage and premature death.[1,2]

Voxelotor is a first-in-class polymerization inhibitor approved by the US Food and Drug Administration for the treatment of SCD in patients aged ≥4 years and in Great Britain, the European Union, the United Arab Emirates, Kuwait, and Oman in patients aged ≥12 years. Voxelotor is a reversible covalent modifier of Hb that allosterically increases Hb-O_2 affinity, thereby increasing the proportion of oxygenated Hb in all RBCs.[3,4] GBT021601 is a potent second-generation HbS polymerization inhibitor with the same mechanism of action as voxelotor. Herein, we present the in vitro characterization of GBT021601 using blood

from patients with SCD and the pharmacodynamic (PD) effects of GBT021601 in a murine model of SCD (SS mice).[5] GBT021601 treatment demonstrated robust changes in key markers of RBC health and haemolytic anaemia in SS mice. Notably, the pharmacokinetic (PK) properties of GBT021601 in animal studies demonstrate its potential to achieve higher Hb occupancy than voxelotor with once-daily (QD) dosing. Additionally, the PD effects in SS mice are superior to those of any other molecule tested in this model system, indicating that GBT021601 has the potential to be an effective SCD treatment.

METHODS

Detailed methods are available as Supplemental Information.

GBT021601

(2-Hydroxy-6-[[(3S)-4-[2-(2-hydroxyethyl)pyridine-3-carbonyl]morpholin-3-yl]methoxy]benzaldehyde) was formulated in 0.5% methylcellulose/phosphate-buffered saline with 0.01% Tween 80, pH 7.4.

Blood source

SCD and healthy donor blood samples were obtained from the University of North Carolina (Chapel Hill, NC, USA) (IRB #88–0343 [GCRC-101]) and the Stanford Blood Center (Palo Alto, CA, USA) respectively.

Crystal structure of the GBT021601-HbS-CO complex

Haemoglobin A (HbA) and HbS were purified from blood as described previously.[4] Data collection and refinement statistics are shown in Table S1. X-ray crystallography coordinates have been deposited to the Protein Data Bank under PDB 7UVB. Details on and analysis of HbS modification using high-performance liquid chromatography (HPLC) are described in the Supplemental Information.

Modified oxygen dissociation assay

The ability of GBT021601-modified HbS to release O_2 was evaluated using a modified oxygen dissociation assay.[6]

In vitro HbS polymerization

The in vitro HbS polymerization reaction, described in the Supplemental Information, was modified from a previously described method.[7]

Oxygen equilibrium curves and sickling

Oxygen equilibrium curves (OECs) (for purified Hb and whole blood) were generated, and in vitro sickling of GBT021601-modified RBCs from patients with SCD and ex vivo sickling of RBCs from SS mice dosed with GBT021601 were conducted as described previously.[4]

PK measurements

PK analysis of GBT021601 was conducted in mice (C57BL/6 and SS), Sprague–Dawley rats, Beagle dogs and cynomolgus monkeys following intravenous (IV, 1 mg/kg) and oral (PO, 2 or 10 mg/kg) administration. Blood and plasma were serially collected from each animal up to 96–336 h post-dose and analysed for GBT021601 concentration using liquid chromatography with tandem mass spectrometry.

Murine model of SCD

Studies with ~8- to 12-week-old male knock-in Townes mice (B6; 129-*Hbb*$^{tm2(HBG1,HBB*)Tow}$/*Hbb*$^{tm3(HBG1,HBB)Tow}$ *Hba*$^{tm1(HBA)Tow}$/J) with an HbSS genotype (homozygous for *Hba*$^{tm1(HBA)Tow}$ and homozygous for *Hbb*$^{tm2(HBG1,HBB*)Tow}$) (Jackson Laboratory) were performed under the oversight of the Institutional Animal Care and Use Committee. GBT021601 (20, 40, 75 or 150 mg/kg QD) or vehicle only was administered via oral gavage to SS mice as a repeat dose for 21 days. At the end of the study, whole blood was collected (~3 h [C_{max}] or 24 h [C_{min}] after the last dose) either from the tail vein or by cardiac puncture under anaesthesia (using isoflurane in 100% O_2) for PK and PD analyses.

Tissue oxygenation and tolerance to hypoxia in SS mice

GBT021601 150 mg/kg QD was administered to SS mice via oral gavage for 21 days before subjecting the mice to any procedure. Blood gases and hypoxia tolerance measurements were taken ~2.5 to 3.5 h after the final dose of GBT021601. Cardiac output, blood gases, total Hb and lactate were measured in normoxia (21% fraction of inspired O_2 [FiO_2]) and hypoxia (10% FiO_2) as described previously.[8] O_2 delivery (DO_2) and O_2 consumption (VO_2) were calculated from the cardiac output, total Hb, and arterial (SaO_2) and venous O_2 saturation (SvO_2) as described previously.[8] Tolerance to hypoxia was determined as described previously.[8]

Data analysis

Hb occupancy was calculated by dividing the concentration of GBT021601 in blood by the concentration of Hb (based on measured haematocrit) in blood multiplied by 100.

Statistical analysis was performed using the following tests: Mann–Whitney or Student's *t*-test combined with Hochberg's method for multiple testing, or analysis of variance combined with the Holm–Šidák test. *p* values less than 0.05 were considered statistically significant.

RESULTS

GBT021601 increases Hb-O_2 affinity, inhibits HbS polymerization and reduces RBC sickling in the blood from patients with SCD in vitro

GBT021601 forms a reversible covalent bond with the N-terminal valine of the α-chain of Hb, binding to HbS in both 1:1 and 2:1 stoichiometries (Figure 1A,B). Confirmed by HPLC (Figure 1C), 1:1 binding is dominant when Hb is in excess, which contrasts with obligate 2:1 binding of allosteric Hb modifiers.[9,10] GBT021601 caused a dose-dependent left shift in OECs of purified human Hb (Figure 1D) and of blood from patients with SCD (Figure 1E) and healthy individuals (Figure 1F), leading to corresponding decreases in p50 (O_2 tension at which Hb is 50% saturated with O_2). This finding indicates that GBT021601 dose dependently increases Hb-O_2 affinity. Of note, the dose dependence of the p50 shift of GBT021601 is the same as that of voxelotor (Table 1). Because GBT021601 binds to the α-chain of Hb, it increases its Hb-O_2 affinity in blood of other animal species, including rat (Figure 1G) and monkey (Figure 1H). Moreover, GBT021601 dose dependently increased the proportion of oxyHbS during deoxygenation (Figure 1I), indicating that by stabilizing the oxyHbS state, GBT021601 delayed its transition to the deoxyHbS state in hypoxia. Importantly, while the rate of O_2 release from GBT021601-modified HbS seemed to be slower than that from unmodified HbS, the binding of GBT021601 to HbS did not prevent O_2 release (Figure 1I). Furthermore, by increasing the oxyHbS concentration, GBT021601 dose dependently inhibited HbS polymerization by delaying the onset of deoxyHbS polymer formation and reducing the polymer load (Figure 2A), consequently decreasing the number of sickled RBCs in hypoxia (Figure 2B–D). These results demonstrate that GBT021601 can potentially inhibit deoxyHbS polymer formation and prevent RBC sickling in vivo.

GBT021601 shows favourable PK and PD properties

The PK parameters of GBT021601 were determined after IV and PO administration in mice (C57BL/6 and SS), Sprague–Dawley rats, Beagle dogs and cynomolgus monkeys (Table S2; Figure S1). The mean blood-to-plasma ($AUC_{0-\infty}$) ratio ranged from approximately 31:1 in dogs to 286:1 in monkeys (Table S2), consistent with the preferential partitioning of GBT021601 into RBCs. In blood, the volume of distribution was small (0.039–0.326 L/kg), and systemic clearance was low (0.006–0.115 mL/min/kg), consistent with slow dissociation from Hb (Table S2). Terminal elimination half-life ($t_{1/2}$) of GBT021601 was similar in whole blood and plasma for each species (Figure S1), ranging from 27 h in SS mouse blood to 325 h in dog blood (Table S2). GBT021601 was well absorbed and had an absolute oral bioavailability ranging from 37% to 71% across the four species (Table S2). Of note, GBT021601 has a longer half-life than voxelotor and achieves greater exposure per dose in mice, rats, dogs and monkeys (Table S3). Additionally, the PD measurements in rats showed that GBT021601 blood concentration strongly correlated ($R^2 = 0.98$) with Hb-O_2 affinity, as determined by the changes in p50 (Δp50 [%]) (Figure S1). Together, these data indicate that GBT021601 has favourable PK and PD properties, and its oral absorption and sustained blood exposure in mice, rats, dogs and monkeys support dosing in humans.

GBT021601 improves RBC health and normalizes the pathophysiology of SCD in SS mice

To evaluate its effect on SCD pathophysiology, GBT021601 was administered orally at 20, 40, 75 and 150 mg/kg QD for 21 days in SS mice. Mean blood-to-plasma ratios ranged from 50:1 to 72:1 (Table 2), confirming the preferential partitioning of GBT021601 into RBCs. GBT021601-treated SS mice achieved dose-dependent mean Hb occupancies (C_{min}) of 6%, 12%, 22% and 31%, corresponding to 20, 40, 75 and 150 mg/kg doses respectively (Table 2). This resulted in dose-dependent increases in Hb-O_2 affinity, as demonstrated by left shifts in the OECs (Figure 3A) and corresponding decreases in p50 values of the SS mouse blood, from ~28 mm Hg (vehicle treated) to 11 mm Hg at the highest dose tested (Table 2). Consequently, GBT021601 reduced the percentage of irreversibly sickled RBCs circulating in vivo (Figure 3B) and the percentage of RBCs sickling in hypoxia ex vivo (Figure 3C). Notably, GBT021601 significantly reduced spleen weight (Figure 3D) compared with that observed in the bone marrow–transplanted SS mice expressing curative levels of HbA (>60% HbA),[11] consistent with an improvement in splenic blood flow and organ function.

GBT021601 dose dependently increased Hb in SS mice (Figure 4A). At 150 mg/kg dose, Hb increased from 7 g/dL in vehicle-treated SS mice to 13.7 g/dL in GBT021601-treated SS mice (Figure 4A and Table 3), thereby reaching the normal range (~13–16 g/dL)[12] for wild-type mice (C57BL/6) (Table 3). GBT021601 also dose dependently decreased reticulocytes in SS mice (Figure 4B), and treatment with GBT021601 150 mg/kg achieved the same level of reticulocytes as that observed in the bone marrow–transplanted SS mice expressing curative levels of HbA (>60% HbA).[11] Consistent with improving anaemia, GBT021601 caused a dose-dependent increase in RBC counts (Figure 4C), normalizing that and other haematological parameters at the 150 mg/kg dose (Table 3). Importantly, erythropoietin levels remained low (Figure 4D) with GBT021601 treatment.

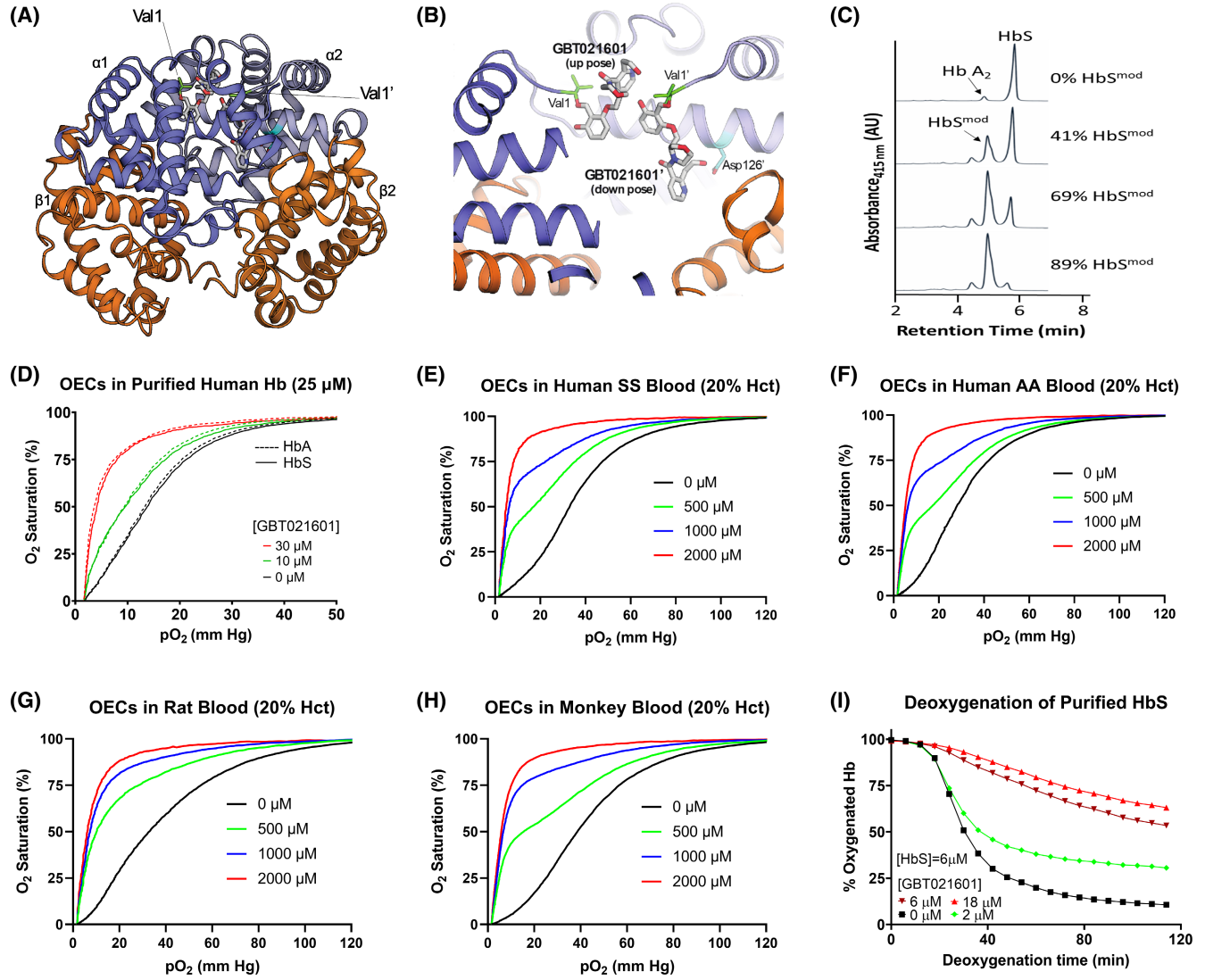

FIGURE 1 GBT021601 binds to the N-terminal valine of the alpha-chain of Hb, increases its affinity for O_2 and stabilizes the oxygenated Hb in hypoxia. (A) Crystal structure of GBT021601 bound to HbS. The alpha chains are coloured blue, and the beta chains are shown in orange. The N-terminal valine is coloured green. (B) Enlarged view of the GBT021601 binding site showing 2 GBT021601 molecules, each forming a reversible Schiff base with 1 N-terminal valine alpha-chain of the Hb tetramer. The crystal structure shows that 2 molecules of GBT021601 can fit into the binding pocket of HbS, and therefore GBT021601 can bind to HbS in both 1:1 and 2:1 stoichiometries. (C) Representative HPLC chromatograms ($n = 2$) showing the HbS-GBT021601 adduct (HbS^{mod}) formed with increasing HbS modification. $\%HbS^{mod}$ represents the percentage of the area of HbS^{mod} peak divided by the sum of the areas of the HbS^{mod} and HbS peaks shown in the chromatogram. (D) Representative OECs ($n = 2$, in duplicate) of purified Hb (HbA or HbS, each at 25 μM) incubated with various concentrations of GBT021601. (E) Representative OECs ($n = 2$, in duplicate) of whole blood (20% Hct, Hb ~1 mM) from a SCD patient incubated with various concentrations of GBT021601. (F) Representative OECs ($n = 2$, in duplicate) of whole blood (20% Hct, Hb ~1 mM) from healthy individuals incubated with various concentrations of GBT021601. (G) Representative OECs ($n = 1$, in duplicate) of whole blood (20% Hct, Hb ~1 mM) from Sprague–Dawley rats incubated with various concentrations of GBT021601. (H) Representative OECs ($n = 1$, in duplicate) of whole blood (20% Hct, Hb ~1 mM) from cynomolgus monkeys incubated with various concentrations of GBT021601. (I) Representative oxygen dissociation curves ($n = 2$, in duplicate) showing the effect of various concentrations of GBT021601 on the dissociation of O_2 from purified HbS (6 μM). Hb, haemoglobin; HbS, haemoglobin S; Hct, haematocrit; O_2, oxygen; OEC, oxygen equilibrium curve.

This finding, along with the increasing haematocrit and decreasing reticulocyte count, is consistent with an absence of a hypoxic response stemming from the increase in Hb-O_2 affinity.

In SCD, mitochondrial retention in immature RBCs causes oxidative stress and impairment of RBC health, shortening the RBC lifespan.[13–16] GBT021601 treatment reduced mitochondria-containing RBCs (Figure 4E) and improved RBC maturation (Figure 4F), indicating improved RBC health. Consistent with reduced haemolysis and improved RBC health, GBT021601 dose dependently increased the RBC half-life from 2.3 days in vehicle-treated SS mice to 8.4 days in SS mice treated with the 150 mg/kg dose (Figure 4G,H and Table 3). In comparison, the RBC

half-life in sickle trait (AS) mice is 10.6 days,[17] indicating that GBT021601 prolonged the RBC lifespan of SS mice to close to that of AS mice.

TABLE 1 Comparison of p50 in whole blood.

Compound concentration	p50 (mm Hg)		
	0 μM	500 μM	1000 μM
GBT021601	31.3 (3.4)	19.4 (2.3)	6.2 (0.1)
Voxelotor	29.3 (1.2)	17.9 (2.4)	5.8 (1)

Note: Voxelotor (GBT440) p50 values were previously reported.[4] $n = 130$, 30 and 100 replicates for the 0, 500 and 1000 μM voxelotor concentrations respectively. $n = 2$ replicates for all GBT021601 concentrations. Mean (standard deviation) values are shown.
Abbreviation: p50, partial pressure of O_2 at 50% Hb saturation with O_2.

In a separate study, SS mice were dosed with GBT021601 150 mg/kg for 21 days, and oxygen gradient ektacytometry was performed subsequently. Mean Hb occupancies achieved at C_{min} and C_{max} on day 21 were 43% and 72% respectively (Figure 5A). Oxygen gradient ektacytometry analysis using blood drawn at C_{min} from GBT021601-treated SS mice showed a dramatic improvement in the deformability index profile compared with that of the vehicle-dosed mice (Figure 5B). This corresponded to improvements in EI_{max}, EI_{min} and PoS (Figure 5B), demonstrating the potential of GBT021601 to favourably impact SCD pathophysiology in patients. These results indicate that GBT021601 improved RBC deformability in SS mice, consistent with the observed improvement in RBC health.

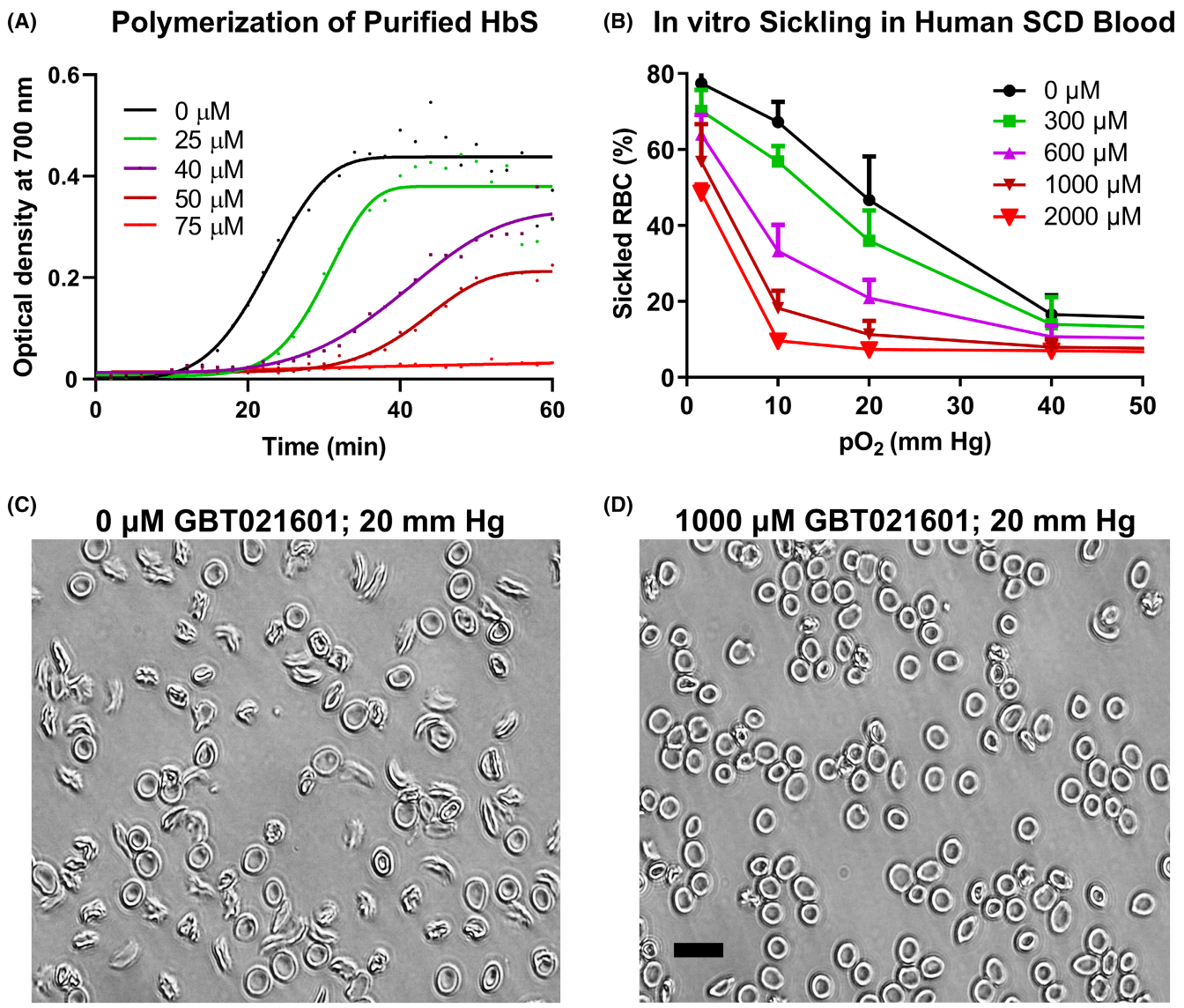

FIGURE 2 GBT021601 inhibits polymerization of purified HbS and reduces RBC sickling under hypoxic conditions in vitro. (A) Representative polymerization curves ($n = 3$) for purified HbS (50 μM) incubated with various concentrations of GBT021601. (B) Representative graphs ($n = 3$, in triplicate) showing sickling of whole blood from individuals with SCD (20% Hct ~1 mM of Hb) incubated with various concentrations of GBT021601 under varying oxygen tensions. Error bars represent standard deviation. (C, D) Representative images of blood from individuals with SCD incubated without GBT021601 (C) or with 1 mM GBT021601 at 20 mm Hg. Scale bar, 20 μm. HbS, haemoglobin S; Hct, haematocrit; RBC, red blood cell; SCD, sickle cell disease.

TABLE 2 Whole blood exposure and Hb occupancy of GBT021601 following repeated oral administration in SS mice.

Dose (mg/kg)	Duration (d)	Blood C_{min} (μM)	Plasma C_{min} (μM)	Blood: Plasma ratio	Hct (%)	Hb occupancy C_{min} (%)	p50 (mm Hg)
0	21	NA	NA	NA	33.6 (5.5)	NA	28
20	21	122 (25)	2 (1)	52 (12)	37.7 (5.6)	6 (1)	22
40	21	206 (22)	4 (1)	50 (15)	34.5 (4.4)	12 (2)	17
75	21	434 (57)	8 (1)	53 (9)	40.3 (4.9)	22 (1)	17
150	21	744 (72)	12 (5)	72 (37)	49.5 (11.3)	31 (6)	11

Note: Hb occupancy at C_{min} was calculated by dividing the minimum concentration of GBT021601 in blood by the concentration of Hb (based on measured haematocrit) in blood and expressed in %. $n = 6, 6, 5, 4$ and 6 mice for the 0, 20, 40, 75 and 150 mg/kg GBT021601-dosed groups for all parameters except p50, where $n = 1$ for each dosed group. Mean (standard deviation) values are shown.

Abbreviations: C_{min}, minimum concentration; d, days; Hb, haemoglobin; Hct, haematocrit; NA, not applicable; p50, O_2 tension at which Hb is 50% saturated with O_2; SS mice, sickle cell disease mice.

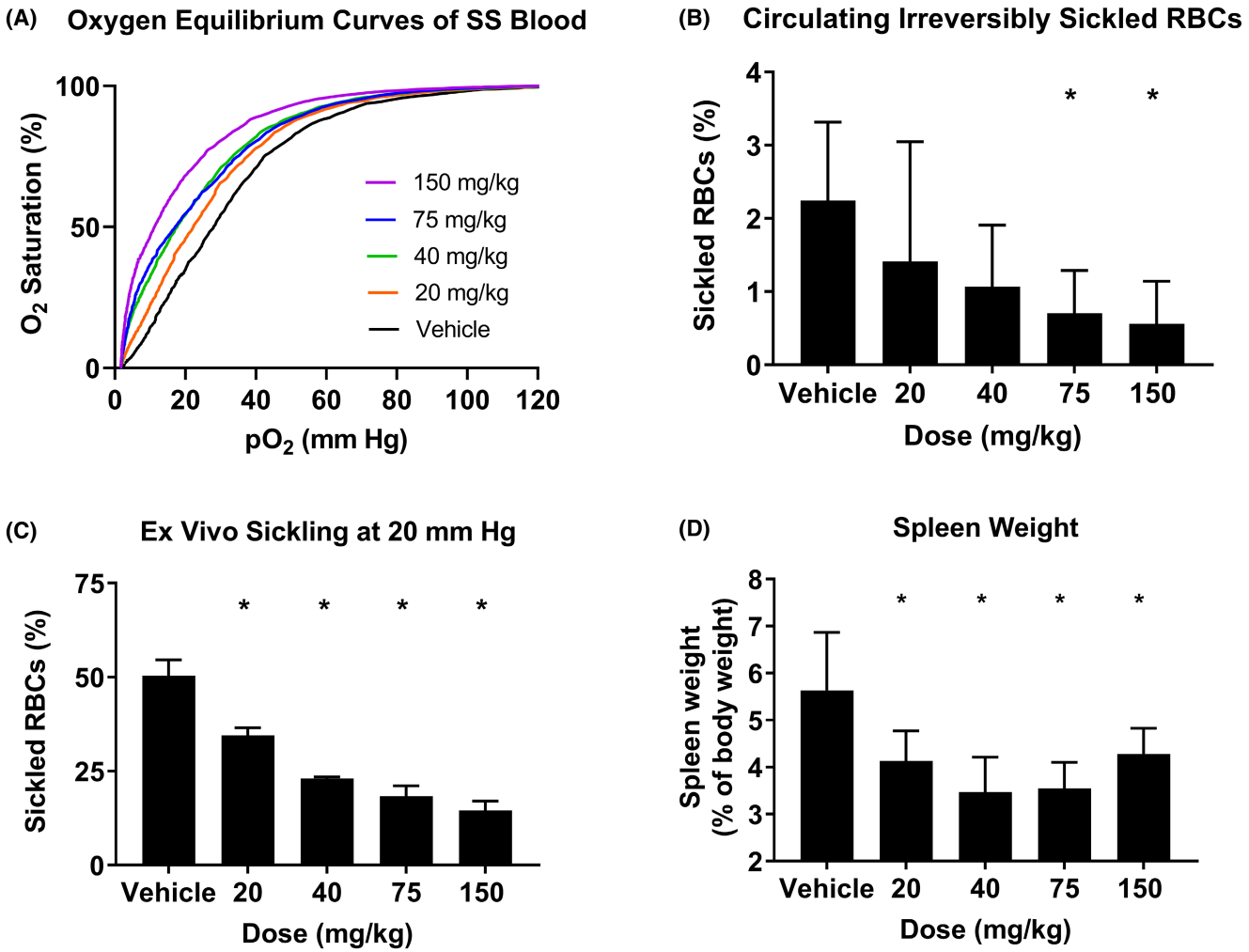

FIGURE 3 GBT021601 increases Hb-O_2 affinity and reduces RBC sickling and spleen weight in SS mice. (A) Representative OECs of blood from SS mice dosed with vehicle ($n = 6$) or GBT021601 at 20 mg/kg ($n = 6$), 40 mg/kg ($n = 6$), 75 mg/kg ($n = 4$) and 150 mg/kg ($n = 6$) for 21 days. (B) Percentage of irreversibly sickled RBCs quantified in blood drawn from SS mice treated with GBT021601 as described in (A). $n = 6$ for all dosed groups except for the 75 mg/kg dosed group where $n = 4$. (C) Percentage of sickled RBCs quantified in blood ($n = 4$ for all dosed groups) drawn from SS mice treated with GBT021601 as described in (A) and incubated under varying hypoxic conditions. (D) Spleen weights of SS mice dosed as described in (A). $n = 6$ for all dosed groups except for the 75 mg/kg dosed group where $n = 4$. Error bars represent standard deviation in all panels. p values were calculated using Student's t test and Hochberg's method for multiple testing. $^*p < 0.05$. Hb, haemoglobin; OEC, oxygen equilibrium curve; pO_2, partial pressure of oxygen; RBC, red blood cell; SS mice, sickle cell disease mice.

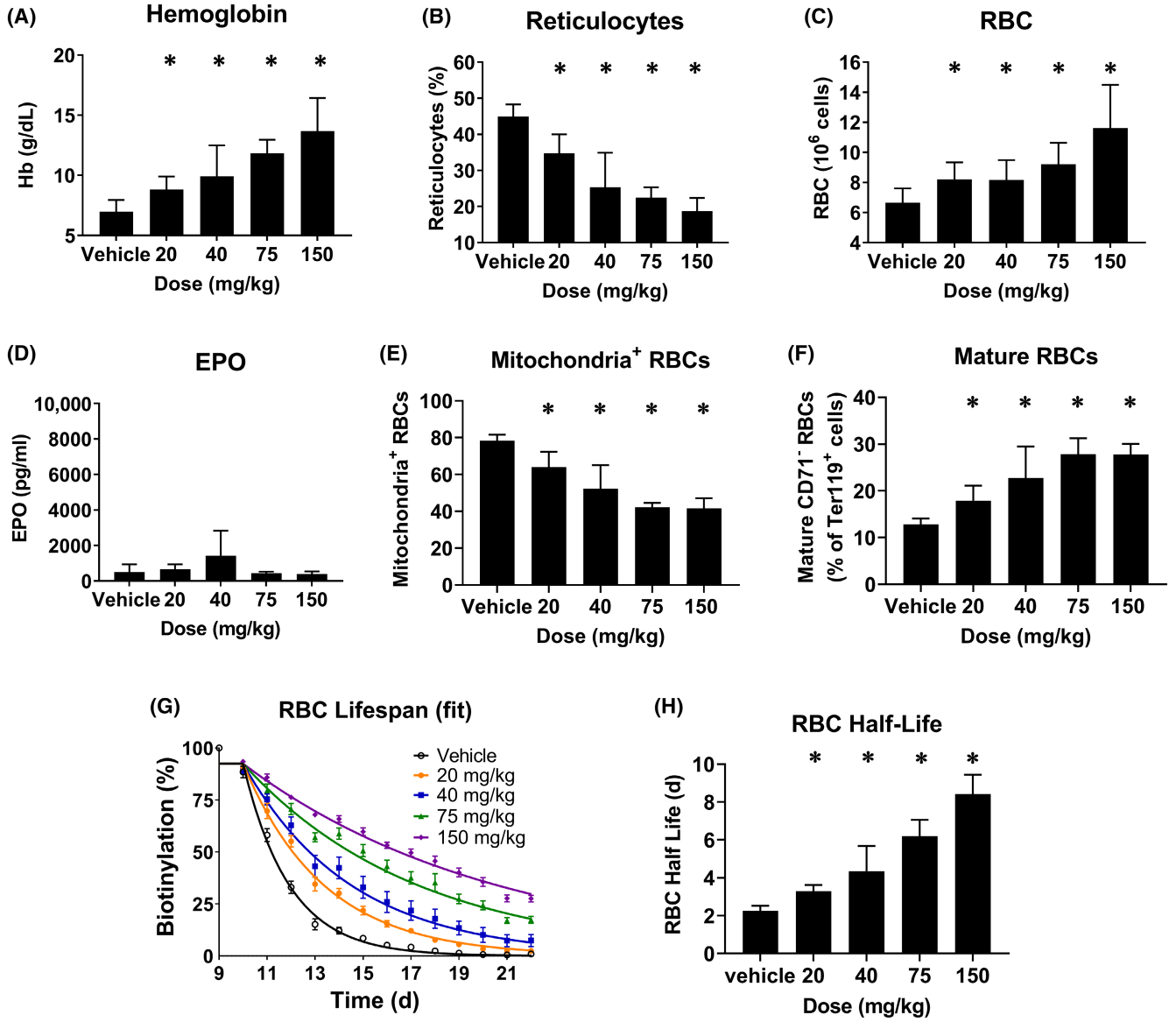

FIGURE 4 GBT021601 normalizes Hb, reduces reticulocytes and mitochondrial content, improves RBC maturation, and prolongs RBC lifespan in SS mice. (A) Hb levels from SS mice dosed with vehicle ($n = 6$) or GBT021601 at 20 mg/kg ($n = 6$), 40 mg/kg ($n = 6$), 75 mg/kg ($n = 4$) and 150 mg/kg ($n = 5$) for 21 days. (B) Percentage of reticulocytes in blood drawn from SS mice treated with GBT021601 as described in (A). $n = 6$ for all dosed groups except for the 75 mg/kg and 150 mg/kg dosed groups where $n = 4$ and $n = 5$ respectively. (C) RBC counts in blood drawn from SS mice treated with GBT021601 as described in (A). $n = 6$ for all dosed groups except for the 75 mg/kg and 150 mg/kg dosed groups where $n = 4$ and $n = 5$ respectively. (D) EPO levels in the blood from SS mice treated with GBT021601 as described in (A). $n = 6$ for all dosed groups except for the 75 mg/kg dosed group where $n = 4$. (E) Percentage of mitochondria$^+$ RBCs in the blood from SS mice treated with GBT021601 as described in (A). $n = 6$ for all dosed groups except for the 75 mg/kg dosed group where $n = 4$. (F) Percentage of mature CD71$^-$ RBCs in the blood from SS mice treated with GBT021601 as described in (A). $n = 6$ for all dosed groups except for the 75 mg/kg dosed group where $n = 4$. (G) Curve fit used to determine the half-life in (H) of biotinylated RBC lifespan in SS mice. The data show the percentage of biotinylated RBCs measured daily after biotin injection into SS mice on day 9. (H) Half-life of RBCs in blood from SS mice treated with GBT021601 as described in (A). $n = 6$ for all dosed groups in (G) and (H). Error bars represent standard deviation in all panels except (G), where error bars represent standard error of the mean. *P* values were calculated using Student's *t* test and Hochberg's method for multiple testing. $^*p < 0.05$. EPO, erythropoietin; Hb, haemoglobin; RBC, red blood cells; SS mice, sickle cell disease mice.

GBT021601 improves oxygen delivery and tolerance to hypoxia in SS mice

To determine the impact of the increased Hb-O_2 affinity on O_2 delivery, SS mice were dosed with GBT021601 150 mg/kg for 21 days and then exposed to hypoxia. Consistent with previous results, GBT021601 increased both Hb-O_2 affinity (p50 of 15 mm Hg; data not shown) and Hb (Figure 6A), and thereby increased the O_2-carrying capacity in SS mice. Due to the increase in Hb-O_2 affinity, GBT021601 increased arterial O_2 saturation (SaO_2) in SS mice exposed to normoxia (21% O_2) or hypoxia (10% O_2) (Figure 6B). The product of increased Hb and SaO_2 indicated an increase in blood O_2 content, which in combination with the GBT021601-mediated

TABLE 3 GBT021601 normalizes several haematological parameters in SS mice.

					GBT021601-dosed SS mice				
Parameters	**C57BL6**	**AA**	**AS**	**SS**	**0 mg/kg**	**20 mg/kg**	**40 mg/kg**	**75 mg/kg**	**150 mg/kg**
Hb (g/dL)	11.2–16.4	10–12.7	10–14.7	6.2–10.2	7.0 (1.0)	8.8 (1.1)	9.9 (2.6)	11.9 (1.1)	13.7 (2.7)
Retics (%)	3.8–4.2	4.8–10.6	4.1–16	54.2–79.3	44.9 (3.4)	34.8 (5.3)	25.4 (9.5)	22.4 (2.9)	18.7 (3.6)
Abs. retics (K/μL)	232–450	500–1219	423–1624	2970–5789	3003 (575)	2810 (270)	2000 (605)	2050 (304)	2126 (646)
RBC (M/μL)	6.1–10.7	10.5–11.5	10.3–12.4	5.5–7.3	6.6 (1.0)	8.2 (1.1)	8.2 (1.3)	9.2 (1.4)	11.5 (2.8)
Hct (%)	36–47.8	47.3–51.9	40.8–49.2	28.3–30.1	33.6 (5.5)	37.7 (5.6)	34.5 (4.4)	40.3 (4.9)	49.5 (11.3)
MCV (fL)	43.4–47.8	35.6–40.4	34.2–39.6	48–55.9	50.5 (2.3)	46.2 (1.2)	42.7 (4.9)	44.0 (2.9)	43.8 (3.3)
MCH (pg)	14.8–17.6	9–10.6	8.2–11	9.2–12	10.5 (0.2)	10.8 (0.2)	12.0 (1.3)	13.0 (1.3)	12.1 (1.0)
RBC $t_{1/2}$ (d)	16.8–23	15.7	10.6	2.4	2.3 (0.3)	3.3 (0.3)	4.4 (1.3)	6.2 (0.9)	8.4 (1.0)

Note: Reference interval values for all C57BL6 RBC parameters were previously reported.[19,20] Reference interval values for all AA, AS and SS RBC parameters were previously reported.[5,11,17] Absolute reticulocyte reference values were calculated as the product of % reticulocytes and RBC counts. n = 6, 6, 6, 4 and 5 mice for the 0, 20, 40, 75 and 150 mg/kg, respectively, GBT021601-dosed groups for all parameters. GBT021601 data are reported as mean (standard deviation).

Abbreviations: AA, Townes mice expressing homozygous human haemoglobin A; Abs. retics, absolute reticulocytes; AS, Townes mice expressing human haemoglobin A and S (sickle trait); C57BL6, wild-type mice; d, days; Hb, haemoglobin; Hct, haematocrit; K, thousand; M, million; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; RBC, red blood cell; RBC $t_{1/2}$, RBC half-life; retics, reticulocytes; SS mice, sickle cell disease mice (homozygous expression of human haemoglobin S).

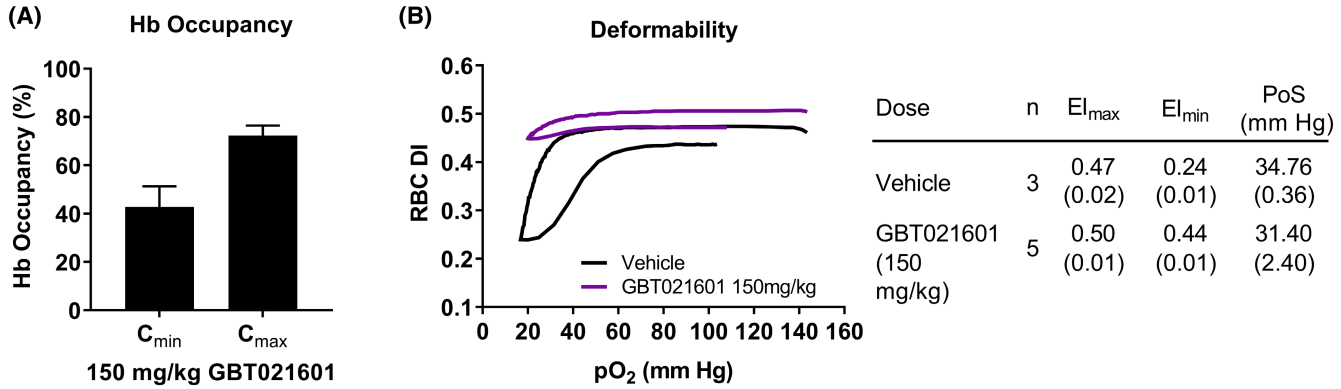

FIGURE 5 GBT021601 consistently achieves high Hb occupancy and improves RBC deformability in SS mice. (A) Percentage Hb occupancy of GBT021601 in SS mice dosed with 150 mg/kg GBT021601 (n = 4 and n = 3 for C_{min} and C_{max}, respectively) for 21 days. Hb occupancy is calculated by dividing the concentration of GBT021601 in blood by the concentration of Hb (based on measured haematocrit in multiplied by 100). Error bars represent standard deviation. (B) Oxygen gradient ektacytometry RBC deformability profile of blood from SS mice dosed with 150 mg/kg GBT021601 for 21 days. n = 3 and n = 5 for vehicle- and GBT021601-dosed SS mice respectively. The table shows the values for the elongation maximum (EI_{max}), elongation minimum (EI_{min}) and the point of sickling (PoS). Mean and standard deviation are shown in the table. DI, deformability index; Hb, haemoglobin; pO_2, partial pressure of oxygen; RBC, red blood cell; SS mice, sickle cell disease mice.

improvement in RBC health led to improved O_2 delivery (Figure 6C), O_2 consumption (Figure 6D) and decreased lactate (Figure 6E) in SS mice exposed to hypoxia. To evaluate tolerance to hypoxia, SS mice were exposed to progressive hypoxia from 15% O_2 to 10% O_2 and 5% O_2 (severe hypoxia). GBT021601 improved the tolerance of SS mice to severe hypoxia (Figure 6F). Taken together, these data suggest that the GBT021601-mediated increase in survival of SS mice was the result of improved delivery and extraction of O_2 in the context of increased Hb-O_2 affinity.

DISCUSSION

GBT021601 is a second-generation HbS polymerization inhibitor designed to optimize the potential for clinical benefit derived from stabilizing oxyHbS. Consistent with its potent polymerization inhibition activity, GBT021601 dose dependently reduced RBC sickling in blood from patients with SCD. Like voxelotor, GBT021601 exhibits favourable drug-like properties in PK studies in various animal species, with a high blood-to-plasma ratio, indicating its preferential partitioning into RBCs. However, as demonstrated here, key differentiating PK features of GBT021601 compared with voxelotor[4] include a longer half-life and the ability to achieve greater exposure per dose.

In this study, mean steady-state Hb occupancies of approximately 30%–40% at C_{min} and 70% at C_{max} and a significant decrease in RBC sickling, spleen weight and haemolysis were observed after oral QD administration of GBT021601 150 mg/kg in SS mice. Notably, GBT021601 increased Hb in SS mice compared with that observed in wild-type mice

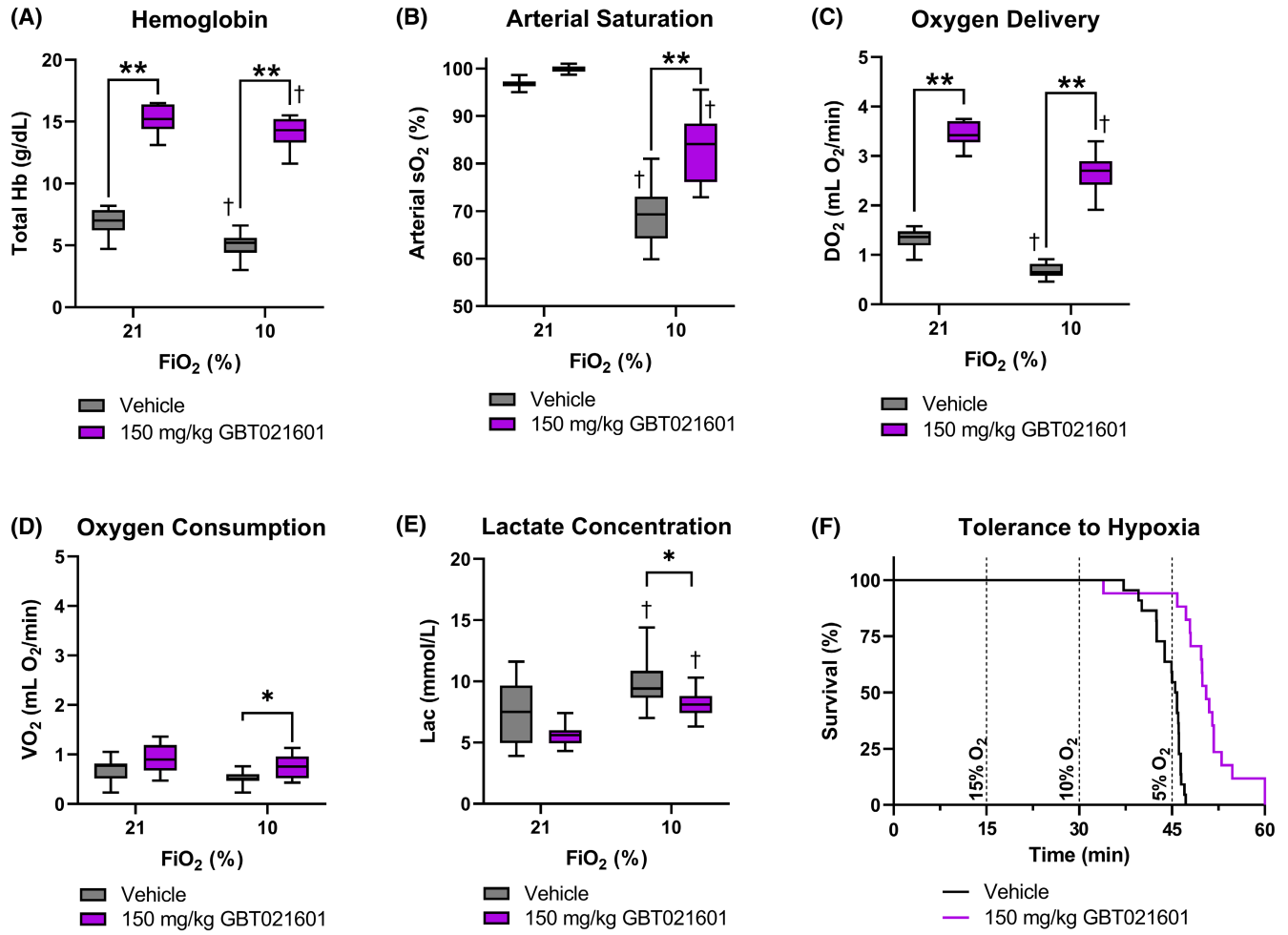

FIGURE 6 GBT021601 increases Hb and arterial saturation, reduces lactate, and improves oxygen delivery, oxygen consumption and tolerance to hypoxia in SS mice. (A) Hb levels under normoxia (21% FiO_2) and hypoxia (10% FiO_2) in SS mice dosed with vehicle ($n = 13$) or GBT021601 at 150 mg/kg ($n = 16$) for 21 days. (B) Arterial saturation of Hb (sO_2) under normoxia (21% FiO_2) and hypoxia (10% FiO_2) in SS mice dosed with GBT021601 as described in (A). The number of SS mice (N) is the same as described in (A) for both dosed groups. (C) Oxygen delivery (DO_2) under normoxia (21% FiO_2) and hypoxia (10% FiO_2) in SS mice dosed with GBT021601 as described in (A). The number of SS mice (n) is the same as described in (A) for both dosed groups. (D) Oxygen consumption (VO_2) under normoxia (21% FiO_2) and hypoxia (10% FiO_2) in SS mice dosed with GBT021601 as described in (A). The number of SS mice (n) is the same as described in (A) for both dosed groups. (E) Lactate concentration under normoxia (21% FiO_2) and hypoxia (10% FiO_2) in blood from SS mice dosed with GBT021601 as described in (A). The number of SS mice (n) is the same as described in (A) for both dosed groups. (F) Tolerance to continuously increasing hypoxia (from 15% to 10% to 5% FiO_2) of SS mice dosed with GBT021601 as described in (A). $n = 22$ for vehicle-dosed and $n = 17$ for 150 mg/kg GBT021601-dosed SS mice. $^*p < 0.05$, $^{**}p < 0.0001$ for two-way ANOVA with Tukey's multiple comparisons test between test groups; $\dagger p < 0.05$ versus 21% FiO_2 for two-way ANOVA with Šidák multiple comparisons test within each test group. ANOVA, analysis of variance; FiO_2, fraction of inspired O_2; Hb, haemoglobin; O_2, oxygen; SS mice, sickle cell mice.

(C57BL/6) and decreased reticulocytes to the same level as that observed in the bone marrow–transplanted SS mice expressing curative levels of HbA (>60% HbA).[11] Additionally, GBT021601 improved RBC deformability, maturation and half-life in SS mice. RBC half-life increased from 2.3 to 8.4 days in GBT021601-treated SS mice, comparing favourably with the reported RBC half-lives in AA and AS mice of 15.7 and 10.6 days respectively.[17] In comparison, repeated twice-daily oral administration of voxelotor at 100–150 mg/kg achieved a C_{max} Hb occupancy of approximately 36% at steady state, which significantly reduced reticulocyte counts and ex vivo sickling but only increased RBC half-life from 2.5 to 3.8 days in SS mice.[4] Based on these findings, GBT021601 is more potent than voxelotor and leads to a pronounced improvement in SCD pathophysiology, including normalization of multiple RBC health parameters in SS mice.

We previously reported that pharmacological increase in Hb-O_2 affinity improved tissue oxygenation and increased tolerance to severe hypoxia in mice.[8,18] In this study, chronic treatment of SS mice with GBT021601 increased Hb-O_2 affinity, reduced RBC sickling and improved RBC health and anaemia, which ultimately led to improved O_2 delivery and O_2 consumption. GBT021601 concurrently increased Hb and reduced reticulocytes without increasing erythropoietin, consistent with improved tissue oxygenation, not with tissue hypoxia. Furthermore, GBT021601 reduced lactate and increased the tolerance of SS mice to severe hypoxia (5% O_2). As SS mice were likely forced to tap into their O_2

delivery reserves during exposure to severe hypoxia, these results suggest that the increase in the O_2 content afforded by GBT021601 may have functionally contributed to tissue oxygenation. Although the increased Hb-O_2 affinity with GBT021601 could slow down O_2 release from Hb, the net effects may be positive in vivo, likely due to the improvements in RBC health, blood rheology, O_2 content, O_2 delivery and O_2 extraction.

In conclusion, GBT021601 robustly improves RBC health and normalizes several haematological parameters, including Hb in a murine model of SCD, thus demonstrating its potential to provide the optimal therapeutic benefits of effectively inhibiting HbS polymerization and reducing RBC sickling while maintaining O_2 delivery to peripheral tissues in patients with SCD. Ongoing and future clinical investigations are required to fully evaluate the potential benefit/risk of GBT021601 in patients with SCD.

AUTHOR CONTRIBUTIONS

Kobina Dufu, Donna Oksenberg, Pedro Cabrales and Brian E. Cathers conceived and designed the study; Kobina Dufu, Steven Strutt, James Partridge, Tzechiang Tang, Peter Rademacher, Vincent Siu, Mira Patel Pochron and Annie Nguyen Dang conducted the in vitro pharmacology experiments; Carsten Alt, Hilary Liao-Zou, Xin Geng, Mira Patel Pochron, Alexander T. Williams, Cynthia R. Muller and Pedro Cabrales conducted the in vivo pharmacology experiments; Kobina Dufu, Carsten Alt, Steven Strutt, James Partridge, Tzechiang Tang, Peter Rademacher, Vincent Siu, Hilary Liao-Zou, Xin Geng, Mira Patel Pochron, Annie Nguyen Dang, Alexander T. Williams, Cynthia R. Muller and Pedro Cabrales curated the data; Kobina Dufu, Carsten Alt, Steven Strutt, James Partridge, Tzechiang Tang, Peter Rademacher, Vincent Siu, Annie Nguyen Dang, Pedro Cabrales and Donna Oksenberg formally analysed the data; Peter Rademacher designed and interpreted the pharmacokinetic experiments; Zhe Li provided chemistry direction leading to the identification and synthesis of the compound; Kobina Dufu, Brian E. Cathers and Donna Oksenberg managed and provided oversight of the study; Kobina Dufu interpreted the data, prepared the figures and wrote the original draft of the manuscript; and Carsten Alt, Steven Strutt, Xin Geng, Mira Patel Pochron, Zhe Li, Pedro Cabrales, Donna Oksenberg and Brian E. Cathers edited the manuscript. All authors read and revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

Kobina Dufu, Carsten Alt, Steven Strutt, James Partridge, Tzechiang Tang, Peter Rademacher, Vincent Siu, Hilary Liao-Zou, Xin Geng, Mira Patel Pochron, Annie Nguyen Dang, Zhe Li, Donna Oksenberg and Brian E. Cathers are current employees of Pfizer Inc. or former employees and shareholders of Global Blood Therapeutics, Inc. Alexander T. Williams, Cynthia R. Muller and Pedro Cabrales were funded by Global Blood Therapeutics, Inc.

DATA AVAILABILITY STATEMENT

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

PATIENT CONSENT STATEMENT

Written informed consent was obtained from all healthy donors and patients with SCD who provided blood samples for analysis in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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