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Original Article

GBT1118, a voxelotor analog, protects red blood cells from damage during severe hypoxia

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Abstract: A lack of objective metrics in Sickle Cell Disease (SCD) makes it difficult to assess individual patient therapy options or assess the effects of therapy. This is further complicated by mechanisms of action involving multiple interconnected effects, that combine to relieve SCD symptoms. In 2019, based on the increase in hemoglobin concentration observed in the HOPE trial, the Food and Drug Administration approved voxelotor (Oxbryta®, Global Blood Therapeutics) for SCD patients 12 years and older. The main mechanism of action for voxelotor was increased hemoglobin-oxygen affinity, but other mechanisms may apply. In this study, we assessed the effect of GBT1118, an Oxbryta analog, on hypoxia-induced lethal and sub-hemolytic red blood cell (RBC) membrane damage using RBC Mechanical Fragility (MF), a metric of existing membrane damage and prospective hemolysis. RBC MF was measured non-invasively using a proprietary system comprising an electromagnetic bead mill and fiberoptic spectrophotometry detection. Three cycles of severe hypoxia (<5% oxygenated hemoglobin) with follow-up reoxygenation resulted in a significant increase in RBC MF for all SCD (Hb-S >60%) samples. Supplementation with GBT1118 caused no significant changes in pre-hypoxia RBC MF. However, following GBT1118 treatment, cell stability showed significantly less degradation, as evidenced by a significantly smaller RBC MF increase after three cycles of hypoxia-reoxygenation. These findings indicate that GBT1118 prevents hypoxia-induced membrane damage in sickled RBC, in part by alternative mechanisms not associated with induced changes in hemoglobin-oxygen affinity.

Keywords: Sickle cell disease, erythrocyte, mechanical fragility, voxelotor, hypoxia, polymerization

Introduction

Sickle Cell Disease (SCD) is one of the most common hereditary blood disorders, affecting up to 100,000 people in the U.S. alone, with up to 2 million people carrying the sickle cell trait. It is particularly common among people whose ancestors come from sub-Saharan Africa, India, Saudi Arabia, and Mediterranean countries. SCD is a monogenetic disease, but it is has a wide variation of phenotypic expressions. Due to the high heterogeneity of SCD, its severity can vary wildly among patients [1, 2]. Symptoms include chronic anemia, pain crises, pulmonary hypertension, acute chest syndrome, ischemic and hemorrhagic stroke, splenic and renal dysfunction. Treatment is focused on symptomatic relief with analgesics and preventing complications, with blood transfusions often used in severe anemia cases.

Sickling of red blood cells (RBC), the singular feature of SCD that gives the disease its name, is clinically manifested in hemostatic imbalance, vaso-occlusive events, and characteristic anemia. RBC in SCD are characterized by disruptions in erythrocyte membrane lipid bilayer and proteins, leading to cell dehydration, abnormal interactions with other blood cells and endothelium, and hemolysis. In particular, sickling-induced exposure of phosphatidylserine on the membrane surface plays a role in increased hemolysis, cell-cell interactions, and endothelial activation [3].

Cell sickling and the resultant destabilization of the RBC membrane also contribute to premature RBC destruction and release of Hb. This limits nitric oxide bioavailability, and promotes oxidative damage with heme release; free Hb oxidation contributes to proinflammatory and

vaso-occlusive events, and together with hemolysis-associated ATP release, promotes further endothelial activation [4].

Elevated hemolysis was suggested as the cause of end organ dysfunction [5] and the possible proximate cause of multiple SCD complications such as pulmonary hypertension, priapism, ulcers, stroke [6] and further linked to disease-associated mortality [7]. RBC lysis represents the end result of progressively accumulating membrane sub-lethal damage. Such sub-hemolytic damage to red cell membranes was previously implicated in shortened cell life span [8], increased trapping in spleen [9], and poor cell mechanical properties [10] with RBC hemolytic propensity shown to be significantly donor-dependent [11]. Similarly, in SCD, genetically predisposed severity of the disease can be modulated by membrane alterations of sickled RBC [12].

In 2019, the FDA approved Oxbryta® (also known as voxelotor and GBT440; Global Blood Therapeutics) for treatment of SCD in patients 12 years of age and older, which was shown to increase patient Hb levels in a pivotal phase 3 trial [13]. Voxelotor is a Hb modulator that increases hemoglobin-oxygen affinity, thereby reducing Hb-S polymerization and RBC sickling under hypoxic conditions [14, 15]. These changes result in lower blood viscosity *in vitro* [16] and decreased hemolysis [15], cumulatively improving and stabilizing oxygen delivery, probably through reduction of hypoxia-induced membrane perturbations and damage.

RBC mechanical fragility (MF) is defined as a cell's propensity to hemolyze under applied mechanical stress. Conversely, RBC (mechanical) stability represents a cell's ability to withstand such stress without lysing. Notably, mechanical fragility and deformability reflect related, but significantly different properties of cell membranes, which are regulated through different interactions of skeletal proteins. Decreased mechanical fragility, or RBC stability under mechanical stress, is associated with the spectrin-actin-protein 4.1 complex and spectrin self-association [17]. Deformability, on the other hand, depends on rearrangement of the skeletal network occurring at constant membrane surface area. Specifically, deoxy-Hb displacement of ankyrin from band 3 was shown to act as an oxygen-dependent switch leading to increased deformability, likely allowing more rapid clearance of deoxygenated RBCs from the tissues [18].

Various approaches are used to measure RBC MF [19], with the most common being by bead(s) moving through the sample. We previously used bead milling to evaluate the MF of both sickled and non-sickled cells, demonstrating large variability in RBC MF properties of normal donors and sickle subject samples. The results also indicated that both normal donors and SCD patients can be sub-divided into groups based on the ability of their RBC to withstand mechanical stress [20].

In the present work, we assessed the effect of GBT1118, a voxelotor-analog, on hypoxia-induced RBC membrane damage (lethal and sublethal) using a standardized mechanical fragility (MF) assay.

Materials and methods

Patients

Whole blood (WB) was obtained from sickle cell patients (n=4; Hb-S fraction, mean ± SD of 72%±11%) and from normal Hb-A donors (n=4). The sickle cell patients presenting at steady state for outpatient visits at the Children's Hospital of Michigan Sickle Cell Center were recruited. Informed consent was obtained, and blood samples were collected according to a protocol approved by the Wayne State University Institutional Review Board (IRB #041718MP4E).

Hypoxia cycles

Deoxygenation was induced by protocatechuate/protocatechuate dioxygenase (PCA/PCD) oxygen scavenging system and re-oxygenation was by gentle agitation under normoxic conditions for 15 minutes. Spectrophotometric controls verified a low level of oxygenated-Hb (less than 10%) during hypoxic conditions and recovery of RBC oxygenation to pre-hypoxic levels after 15 minutes of incubation under room air (**Figure 1**). Microscopic examination confirmed RBC sickling under hypoxia with predominant cell morphology recovery after reoxygenation. An aliquot for MF was collected before hypoxia (at normoxia) and another also at normoxia, but

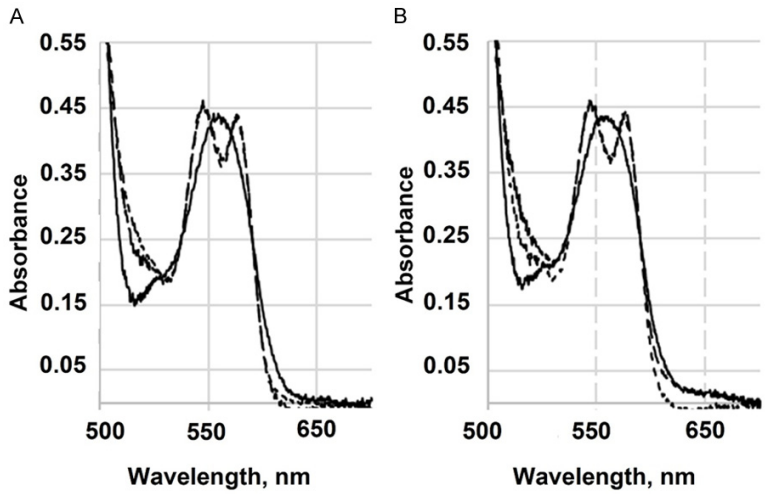

Figure 1. Absorption spectra of a blood sample normoxic before hypoxia (---), deoxygenated (—), and at normoxia after sample reoxygenation (––) not supplemented (A) and supplemented (B) with GBT1118. Note that pre-deoxygenation and reoxygenated spectra, (observed absorption maxima at 543 and 577 nm), are essentially overlapping, and clearly different from deoxygenated Hb spectrum (with the observed maximum at 555 nm).

after 3 cycles of hypoxia (3 cycle deoxygenation +3 cycle reoxygenation).

RBC mechanical fragility (MF), a metric indicative of existing RBC membrane damage and prospective hemolysis, was measured with mechanical shear stress applied by oscillating a ferromagnetic cylindrical bead (18 mm × 3.5 mm, L × ∅) within a sample contained in flexible Tygon™ tube (34 mm × 4.8 mm, L × ∅). Oscillations (6 Hz) were induced using a proprietary electromagnetic bead mill. This approach was shown to generate a combination of qualitatively different stresses resulting from flows at the bead end and through the bead annulus [21]. Hemolysis (Hem) was expressed as a fraction of cell-free hemoglobin (Hb^{F}) relative to total hemoglobin concentration (Hb^{T}) according to Eq. 1, which included the correction for sample hematocrit as detailed by Sowemino-Coker [22].

$$Hem = \frac{Hb^{F}_{576} - Hb^{F}_{700}}{Hb^{T}_{576} - Hb^{T}_{700}} * (1 - Hematocrit) \quad \text{(Equation 1)}$$

Total hemoglobin concentration for each diluted RBC sample was determined by subjecting a small (150-200 µL) aliquot to ultrasound for 40 seconds (0.1 second pulses, with 0.1 second intervals between pulses, at 4°C) delivered by a Branson Digital Sonifier 450 (Danbury, CT), at 15% intensity (from the manufacturer specified 400 Watt). Spectroscopic measurements were performed with a NanoDrop N100 spectrophotometer (Thermo Scientific, Waltham, MA). Cell-free Hb fraction was determined non-invasively within the tube at each applied stress duration using a proprietary spectral analysis approach as detailed previously [21].

RBC fragility profiles are defined as the incremental hemolysis resulting from applied stress of variable duration. Unlike single point measurements that use single stress duration at a single stress intensity as implemented e.g. by Raval et al. [23], MF profiles allow quantification of sample RBC propensity to hemolyze over the range of applied stress magnitudes, from those resulting in minimal to those resulting in nearly total RBC lysis in the tested sample. Hemolysis observed after 10 minutes of stress application (maximum stress duration) is reported as Hem_{10}. Mechanical fragility-based indices (MFI) represent the area under the curve (AUC) of the profile up to a given applied stress duration, obtained from best fit regression to the experimental data. MFI was determined for the most labile RBC fraction (average induced hemolysis about 20%, MFI_1) and for total induced hemolysis over 10 minutes stress application time (average induced hemolysis about 85%, MFI_10). The percentage increase in hemolysis (FR-MFI), an additional metric used in this study, represents cell fractions contributing to the incremental increase in hemolysis over a given time interval (e.g., over 30 seconds or over 1 minute) of stress application at different cumulative durations of applied stress. The comparison between the FR-MFI values at different stress durations offers a more detailed view of changes in size of RBC fractions, different in stability, under mechanical stress in response to pathology, conditions, or treatment. FR-MFI values were calculated for each one minute of stress application up to the maximum stress duration of 10 minutes.

Table 1. Baseline Red Blood Cell Mechanical Fragility*

	Hb-S	Control (not supplemented)			Supplemented with GBT1118		
		MFI_1	MFI_10	Hem_{10}	MFI_1	MFI_10	Hem_{10}
SCD patients (N=4)	72%±11%	0.11±0.03	5.2±1.0	77±12	0.10±0.01	5.2±0.5	73±7
Normal Donors (N=4)	0%	0.09±0.01	5.2±0.3	82±2	0.11±0.03	5.8±0.6	88±4

*Data are presented as mean ± SD, with SD representing subject-to-subject variability.

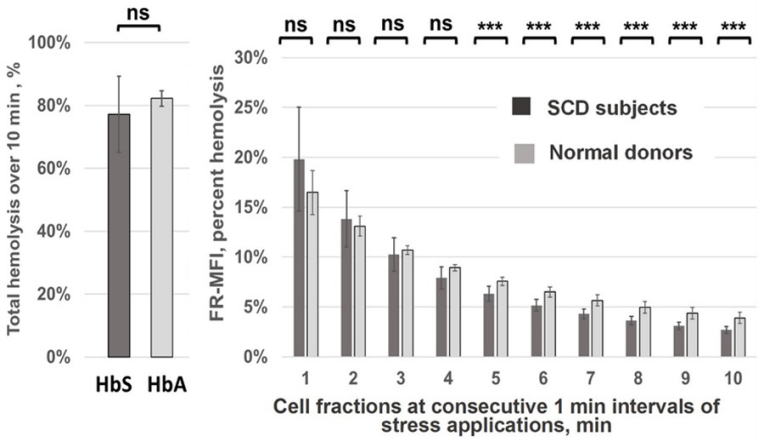

Figure 2. Contribution of cell fractions with different MF to RBC lysis for SCD Hb-S subjects (v) and normal (Hb-A) donors (v). Fractions are represented by incremental increase in hemolysis over 1 minute of consecutive stress application (FR_MFI). Significance is shown between the fractions from recruited Hb-S subjects and normal donors with P<0.05 (***), ns, no significance. Error bars are ±1 SD.

Results

Average baseline RBC MF for SCD subjects and normal (Hb-A) donors, as represented by the hemolysis observed at 10 minutes of stress application (Hem_{10}), MF indices for the labile fraction (MFI_1) and for the total hemolysis over the 10 minutes of stress application (MFI_10) are presented in **Table 1**. In SCD and normal donor normoxic samples, not exposed to hypoxia, there was no significant change in RBC stability due to treatment with GBT1118 as represented by MFI_1 and MFI_10 (paired t-test, P>>0.05).

When appropriate, samples were incubated for 1 hour at 22°C with GBT1118 in 0.9% final concentration DMSO (treated sample) or with an equal volume of DMSO-containing buffer (control sample). GBT1118 concentration was calculated based on sample Hb content and was 1.1±0.1 mM for SCD donors and 1.83± 0.06 mM for normal donors. In all cases, GBT1118 treatment concentration was 25% of sample hemoglobin concentration, corresponding to the clinically relevant level of Hb modification [14]. GBT1118 was from Global Blood Therapeutics; PCD was a generous donation by Dr. Palfy, University of Michigan; all other reagents were from Sigma Aldrich.

Statistical analysis

Data are presented in the form of mean ± standard deviation, range and coefficients of variability (CV) where appropriate. Student t-test paired or non-paired as appropriate, with a two-tailed *P*-value of 0.05, was used to test for significance.

Note that for normal donors the same assessment done using hemolysis at 10 minutes of stress application (Hem_{10}) shows statistical significance (paired t-test, P=0.04). Unlike MFI_10, which is a metric describing cumulative hemolysis over 10 minutes of stress application, Hem_{10}, is a single point metric showing the final, non-cumulative hemolysis magnitude, and thus it does not reflect the rate of change in hemolysis over the full time of stress application. The observation highlights the difference in assessments when done by a single-point measurement (as Hem_{10}) compared to the profile integration metrics (as MFI_10).

Analysis of increase in RBC lysis over 10 minutes of stress application reveals an unequal contribution of cell fractions differing in their hemolytic propensity to the cumulative hemolysis. Contribution to the total sample hemolysis of RBC fractions differing in their hemolytic propensity decreased with increased cell resistance to hemolysis (1-minute fraction shown, **Figure 2**). The contribution of more labile frac-

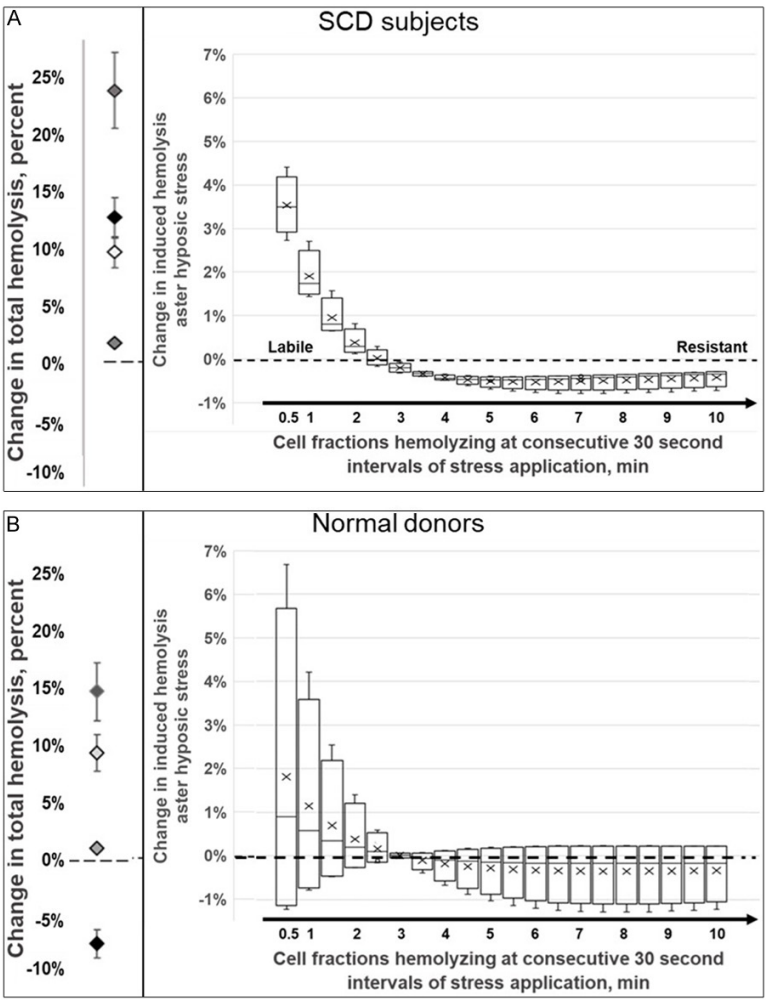

Figure 3. Changes in RBC induce hemolysis resulting from 3 cycles of hypoxia as compared to the control sample without GBT1118 supplementation for SCD subjects (A) and for normal donors (B). Shown are changes in total induced hemolysis (Hem_{10}) after 10 minutes of stress application with error bars are ± SD on the four measurements, and relative contribution of induced hemolysis fractions as a percent of total induced hemolysis in each sample. The difference for each of *i* RBC hemolyzed fraction is expressed in terms of the magnitude of induced hemolysis over the consecutive 30 second durations normalized to the total hemolysis in the sample, and was calculated according to the formula 2:

$$Change_i = \left(\frac{HemC_i^3}{HemC_{10}^3} - \frac{HemC_i^0}{Hem_{C10}^0} \right) \quad \text{Formula 2}$$

Where $HemC_i^0$ and $HemC_i^3$ are incremental increases in hemolysis associated with *i* fraction in a sample before and after 3 cycles of hypoxia. $HemC_{10}^0$ and $HemC_{10}^3$ are the values of total hemolysis in the sample achieved over the 10 minutes of stress application before and after the hypoxia.

tions (FR-MFI_1 and FR_MFI_2) was elevated in SCD (Hb-S) subjects as compared to normal (Hb-A) donors. However, the contribution of more resistant fractions to the total sample hemolysis in normal donors was higher than that in SCD donors. Despite the disparity, the difference in magnitude of more labile fractions between SCD patients and normal donors failed to reach statistical significance due to high subject-to-subject variability within each group. Such variability declined for more stress-resistant fractions with the differences between the groups becoming significant ($P<0.05$).

Hypoxia cycles induced a larger increase in RBC MF of SCD subjects compared to normal donors. In all cases, the increase in cumulative hemolysis was due to an increased contribution (magnitude) of more labile RBC fractions which was offset to a small degree by decreased relative contribution of less labile (more resistant to hemolysis) cell fractions (**Figure 3**). While extremely consistent for SCD samples (**Figure 3A**), in normal donors this effect was much less uniform (**Figure 3B**). When assessed by integrated indices, hypoxic cycles induced a marked and statistically significant increase in RBC MFI, for both MFI_1 and MFI_10 in SCD subjects (**Figure 4A**, ± GBT1118), while the increase of MFI in normal donors lacked significance (**Figure 4B**, ± GBT1118). For both groups, changes in the labile fraction were more pronounced than those for cumulative 10 minute hemolysis. Hypoxic cycles did not result in any significant changes in pre-existing (pre-stress application) hemolysis.

When incubated with GBT1118, samples from SCD subjects exhibited a significantly smaller increase in RBC MF after three cycles of hypoxia with follow-up reoxygenation (**Figure 4A**, +GBT1118). Resultant RBC MFI_1 and MFI_10 in such, GBT1118 incubated samples, were not

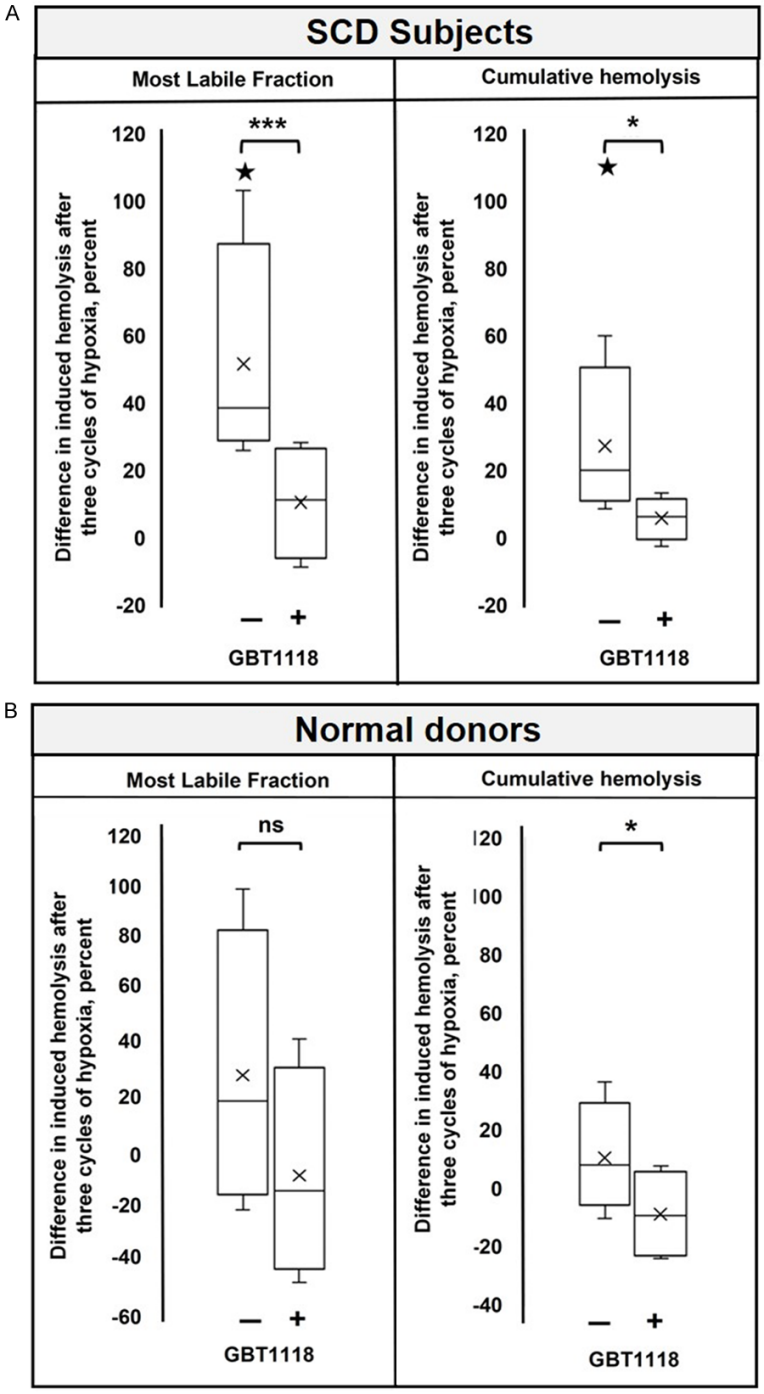

Figure 4. Changes in RBC mechanical fragility for SCD subjects (A) and normal donors (B) as shown by changes in the most labile fraction (MFI_1), and in the cumulative induced RBC MF index for 10 minutes of stress application (MFI_10). Shown is the difference between averaged MFI values before and after 3 cycles of hypoxia with (+) and without (-) supplementation with GBT1118. ↔ denotes statistical significance of hypoxia-induced changes compared to the pre-hypoxia normoxic baseline, and ***P<0.05) and *0.05<P<0.1 denote statistical significance of the differences of hypoxia-induced MF changes in samples with and without GBT1118. ns, no significance.

significantly different from those in control samples pre-hypoxia, while the values obtained after hypoxia without the compound were significantly elevated. Specifically, upon incubation with GBT1118, for the 4 samples, percent change from the pre-hypoxic condition after the 3 cycles of hypoxia with reoxygenation to normoxia between the cycles was reduced on average by about 40% for the labile fraction (MFI_1) and by 20% for the total hemolysis (MFI_10). Supplementation with GBT1118 resulted in variability in responses between the samples from normal donors. However, such treatment-induced difference lacked significance (**Figure 4B**, +GBT1118).

More detailed analysis of RBC fractions that differed in their resistance to induced hemolysis reveals that in SCD patients, hypoxia induced a shift in relative fraction sizes with increased contribution from more labile fractions (FR-MFI_1 and FR-MFI_2) and decreased contribution from the more hemolysis-resistant fractions (FR-MFI_4 to FR-MFI_10) (**Figure 5A**). This effect was significantly arrested in the presence of GBT-1118. The effect was similar in normal donors, even if the average changes in hemolysis as assessed by MFI_1, MFI_10, and RBC fractions (FR-MFI) lacked statistical significance (**Figure 5B**).

Discussion

Sickle cell membrane properties play an important role in both adhesive and obstruc-

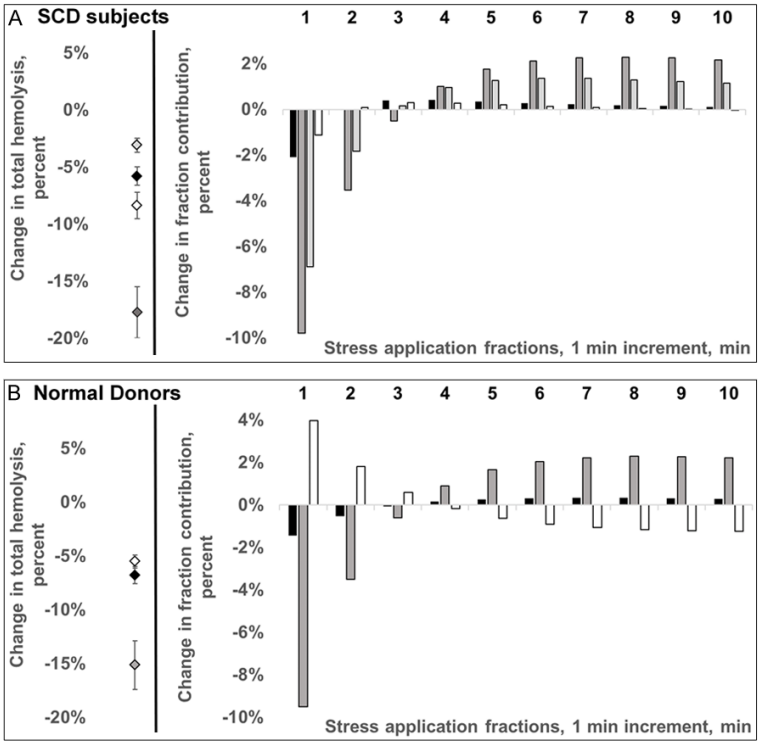

Figure 5. Changes in RBC-induced hemolysis as a result of incubation with GBT1118 after 3 cycles of hypoxia as compared to control sample without GBT1118 supplementation for SCD subjects (A) and for normal donors (B) with error bars representing ± SD on the three measurements. Shown are changes in total induced hemolysis after 10 minutes of stress application. Also shown are relative contributions of induced hemolysis fractions as a percent of total induced hemolysis in the four individual SCD and four normal donor samples. The difference for each of *i* RBC hemolyzed fraction is expressed in terms of the magnitude of induced hemolysis over the consecutive 1-minute durations normalized to the total hemolysis in the sample, and was calculated according to the formula 3:

$$Change_i = \left(\frac{HemT_i^3}{HemT_{10}^3} - \frac{HemT_i^0}{HemT_{10}^0}\right) - \left(\frac{HemC_i^3}{HemC_{10}^3} - \frac{HemC_i^0}{HemC_{10}^0}\right) \quad \text{Formula 3}$$

Where $HemT_i^0$ and $HemT_i^3$ are incremental increases in hemolysis associated with *i* fraction in a sample supplemented (Treated) with GBT1118 before and after 3 cycles of hypoxia. $HemT_{10}^0$ and $HemT_{10}^3$ are the values of total hemolysis in treated samples achieved over the 10 minutes of stress application. Other values as in **Figure 3**.

tive events, contributing to the pathophysiology of SCD [24], with sickling shown to correlate with severity of major SCD complications [25]. The percentage of sickled cells after deoxygenation is the basis of the standard "sickling assay" (where sickling can be induced by either low oxygen tension or change in pH) and had been used previously as a predictor of disease outcomes (e.g. [26, 27]). In addition to vaso-occlusion, destabilization of the RBC membrane and cell sickling also result in premature RBC destruction and release of hemoglobin; such hemolysis is suggested as the possible proximate cause of multiple SCD complications.

Hypoxia is an integral part of normal cell physiology; in normal cells reversible binding of Band 3 anion exchange protein (cdb3) and deoxygenated (deoxy-Hb), but not oxygenated (oxy-Hb) hemoglobin results in transient rupture of the cdb3-ankryn bridge and increased cdb3 diffusion, providing an oxygen tension-dependent switch to membrane properties [18, 28]. Mechanistically, less-tight association of ankyrin with the cell membrane, and thus dissociation between the phospholipid bilayer and underlying spectrin-actin cytoskeleton, can be expected to weaken the membrane upon cell deoxygenation, making it less elastic and at the same time less resistant to mechanical stress. This process is then reversed upon hemoglobin re-oxygenation. Such physiologically important rearrangements are likely to be disrupted in RBC with a sufficiently high content of Hb-S e.g. through the disruption of junctions in ankyrin-cdb3 and spectrin-actin-protein 4.1 complexes that could lead to membrane fragmentation under sufficiently high shear stress [17]. It was shown that cycles of Hb-S polymerization and re-oxygenation critically impact membrane structural proteins, like Band-3 complex resulting in membrane microvesiculation [29], with hypoxia-induced polymerization causing plasma membrane damage [20, 30].

While mechanics behind RBC stability are not yet fully understood, it was shown that depletion or disruption of Band 3 results in a significant increase in RBC fragility (reduced stability) and severe anemia [31, 32]. Interestingly,

integrity of the Band 3-ankyrin linkage was shown to be essential specifically for cell's ability to withstand mechanical stress with a minimal impact on cellular integrity when such stress is absent [33].

Additionally, the interaction of deoxy-Hb-S with cdb3 is much stronger than that of deoxy-Hb-A, with a portion of deoxy-Hb-S being irreversibly bound to the complex. Even the reversibly bound fraction of deoxy-Hb-S was shown to dissociate much more slowly than deoxy Hb-A, impacting sickle RBC membrane properties [34]. Moreover, production of superoxide, facilitated under hypoxic conditions, is exacerbated in SCD, contributing to elevated oxidative damage to membrane structures involved in regulation of membrane stability [35]. ROS-induced damage was linked with irreversible micro-rheologic abnormalities of sickle RBC membranes, abnormal Hb-S-membrane interactions, and was suggested as a key factor in accelerated sickle RBC senescence [36]. With normal RBC having a life span of about 120 days, in SCD, due to accumulating damage from sickling, cells exhibit a much shorter survival. This is highly variable between patients, with a range of 14-54 days range reported [37].

Decreasing hypoxia-induced Hb-S polymerization is one of the most promising approaches in SCD treatment with many an avenue to achieve this goal. One such avenue is through promoting of synthesis of fetal hemoglobin (Hb-F), commonly achieved using the maintenance drug hydroxyurea, which does not polymerize and was shown to relieve symptoms in some patients. Longer RBC survival was reported for SCD patients with high Hb-F, presumably due to inhibition of hemoglobin polymerization and thus reduction in cell membrane damage [38]. Polymerization is also retarded with decreased intracellular Hb and Hb-S concentrations, e.g., through reduction of RBC dehydration that is often observed in SCD [39]. Hb deoxygenation-reoxygenation cycles and Hb-S polymerization are also associated with elevated oxidative stress, which in turn is associated with increased RBC membrane stiffness. Some therapies, e.g., Endari (L-glutamine), offer mechanisms to protect Hb and cell membrane from oxidative damage [40].

An alternative treatment pathway is through increasing Hb affinity to oxygen to decrease Hb-S deoxygenation and thus to reduce Hb-S polymerization. One approach is through modulation of erythrocytic 2,3-diphosphglycerate (2,3-DPG) that reduces oxygen to Hb affinity and is elevated in SCD patients. It can also be accumulated under hypoxic conditions in tissues, contributing to Hb-S polymerization and sickling. Treatments are being developed to reduce 2,3-DPG e.g., by activating RBC pyruvate kinase [41].

Another approach utilizes Hb-modifying compounds. Some vanillin derivatives have been shown to weaken stabilizing interactions in the polymer's structure, by forming high oxygen affinity Schiff-base adducts with Hb, shifting the oxygen equilibrium curve to the left [42, 43]. A similar anti-sickling effect is achieved by voxelotor, which binds to the N-terminal of the α chain of Hb, delaying Hb-S polymerization and preventing RBC sickling [15]. By delaying and reducing hypoxia-induced sickling, Hb-modifying compounds would be expected to protect sickle cells from the detrimental effects of hypoxia, and through elevation of Hb-oxygen affinity reduce sickling, thus possibly reducing the risks of VOC.

GBT1118, a voxelotor analog, is another allosteric modifier of Hb affinity to oxygen that was shown to increase tolerance to severe hypoxia in mice [44]. Consistent with reduction of Hb-S polymerization, GBT1118 was reported to protect RBC hydration under both deoxygenated and oxygenated conditions, as well as to reduce hypoxia-induced hemolysis [45]. Increased affinity to oxygen resulting from shifting the T-R quaternary equilibrium toward the R state and associated reduction in Hb-S polymerization, is often considered to be the main mechanism of action for both GBT1118 and voxelotor. The results of this study, however, cannot be explained through the changes in oxygen affinity as under the severe hypoxia utilized here essentially all Hb was deoxygenated and no difference in deoxygenation was observed between non-treated samples and samples treated with GBT1118 (**Figure 1**). Considering the 15 minutes' duration of deoxygenation, the rate of polymerization as well as the delay in polymerization [46] are not likely to be factors. Also note that both samples before and after hypoxia cycles were tested at normoxia, at the same Hb oxygenation.

It was previously suggested that there are additional inhibitory effects on intracellular Hb-S polymerization other than that resulting from shifting the T-R equilibrium and oxygen affinity

[47]. Among the four Hb-modifying compounds discussed were (i) blockage of sites of intermolecular contacts on Hb-S fiber, (ii) effect on cell hydration, and (iii) weakening of fiber contacts by altering intracellular conditions [48]. However, voxelotor reduction in SCD blood viscosity was also observed under normoxia, with no deoxygenation [49], suggesting a contribution from a mechanism(s) unrelated to inhibition of Hb-S polymerization.

It was previously suggested that an interaction of GBT1118 with the cation transport systems in the cell may be able to increase RBC hydration and reduce cytoplasmic viscosity [50]. It should be noted, that increased hydration would be expected to reduce polymerization due to a decrease in cell Hb-S concentration. For voxelotor, inhibition of polymerization due to changes in RBC volume was observed as a result of 24-hour but not of 4-hour incubation [48]. The cumulative time of exposure to GBT1118 used in our work was well under 3 hours, including both one-hour incubation and the duration of 3 hypoxia cycles, and thus, assuming similar impact regardless of the exact compound to Hb ratio, was not likely to result in significant changes in RBC hydration.

While RBC MF on average is elevated in SCD, as compared to normal donors, significant variability with the overlapping MFI ranges for the two groups was previously reported [20]. Analysis of RBC FR-MFI fractions reveals that compared to normal Hb-A donors, RBC from SCD patients exhibit progressively increasing contributions from a more labile fraction with decreased contributions from more stress-and-lysis resistant RBC fractions. This observation is aligned with the understanding that *in-vivo* sickling is a significant contributing factor to degradation of RBC membrane. Our present work highlights the importance of viewing polymerization-associated phenomena in terms of membrane damage occurring over Hb-S deoxygenation and reoxygenation cycles.

Further evaluation of changes in RBC fractions indicated that cycles of severe hypoxia induce damage to RBC membrane "transitioning" cells from more stress-resistant to more labile populations, with the effect markedly exacerbated with Hb-S present. Incubation with GBT1118 while not affecting RBC MF at normoxia, resulted in normalization, relative to normal (Hb-A) donors, of RBC response to severe hypoxic cycles (**Figure 4**). Under these conditions, there was a decreased amount of lysis from the more labile RBC fractions, likely due to decreased fraction sizes. This was accompanied by an increased amount of lysis from the more resistant RBC fraction: again, presumably due to an increase in these cell fraction sizes. The net effect was a decrease in cumulative sample hemolysis representing, on average, increased cell resistance to stress-induced hemolysis. Interestingly, reduction in hypoxia-induced membrane damage in the presence of GBT1118, as assessed by changes in RBC MF, was observed not only for SCD patients, but for normal Hb-A donors as well. That observation indicates that the reduction of hypoxia-induced membrane damage effected by GBT1118 is likely not limited only to that associated with Hb-S polymerization.

Conclusions

FDA recently approved Oxbryta (voxelotor; Global Blood Therapeutics) for treatment of SCD in patients 12 years of age and older based on the results of a pivotal phase 3 trial shown to increase patient Hb levels after the treatment. Accelerated approval granted to Oxbryta is a mechanism that enables the FDA to approve drugs to fill an unmet medical need based on a result that is reasonably likely to predict a clinical benefit to patients. Further trials are required by the FDA to verify and better define voxelotor's clinical benefit.

Our studies suggest that GBT1118, a voxelotor analog, preserves sickle RBC health at least in part through prevention of RBC membrane hypoxia-induced damage by mechanisms not associated with compound-induced changes in hemoglobin-oxygen affinity. It also shows, at least in part, that such elevated resistance to mechanical stress and hemolysis is due to effects not necessarily related to Hb-S polymerization and likely not associated with changes in cell hydration. Further studies are required to differentiate membrane perturbations arising (and as shown here arrested by GBT1118) from direct damage due to polymerization from those due to increased oxygen radical formation, as well as to differentiate the inhibition of cell damage due to changes in the extent of polymerization from that due to changes in polymerization rates.

Disclosure of conflict of interest

M. Ferranti, A. Herppich are employees, and P. Hines and M. Tarasev are employees and shareholders of Functional Fluidics Incorporated, a company developing and commercializing assays for blood cell assessment. Financial support was received from Global Blood Therapeutics Incorporated for the completion of the study. Global Blood Therapeutics Incorporated did not participate in the conductance of the study, data analysis or interpretation of the experimental study results.

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References

[1] Steinberg MH. Predicting clinical severity in sickle cell anaemia. Br J Haematol 2005; 129: 465-481.

[2] Alli NA, Patel M, Alli HD, Bassa F, Coetzee MJ, Davidson A, Essop MR, Lakha A, Louw VJ, Novitzky N, Philip V, Poole JE and Wainwright RD. Recommendations for the management of sickle cell disease in South Africa. S Afr Med J 2014; 104: 743-751.

[3] Kuypers FA. Red cell membrane lipids in hemoglobinopathies. Curr Mol Med 2008; 8: 633-638.

[4] Kato GJ, Steinberg MH and Gladwin MT. Intravascular hemolysis and the pathophysiology of sickle cell disease. J Clin Invest 2017; 127: 750-760.

[5] Vercaemst L. Hemolysis in cardiac surgery patients undergoing cardiopulmonary bypass: a review in search of a treatment algorithm. J Extra Corpor Technol 2008; 40: 257-267.

[6] Kato GJ, Gladwin MT and Steinberg MH. Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev 2007; 21: 37-47.

[7] Nouraie M, Lee JS, Zhang Y, Kanias T, Zhao X, Xiong Z, Oriss TB, Zeng Q, Kato GJ, Gibbs JS, Hildesheim ME, Sachdev V, Barst RJ, Machado RF, Hassell KL, Little JA, Schraufnagel DE, Krishnamurti L, Novelli E, Girgis RE, Morris CR, Rosenzweig EB, Badesch DB, Lanzkron S, Castro OL, Goldsmith JC, Gordeuk VR and Gladwin MT; Walk-PHASST Investigators and Patients. The relationship between the severity of hemolysis, clinical manifestations and risk of death in 415 patients with sickle cell anemia in the US and Europe. Haematologica 2013; 98: 464-472.

[8] Brinsfield DE, Hopf MA, Geering RB and Galletti PM. Hematological changes in long-term perfusion. J Appl Physiol 1962; 17: 531-534.

[9] Sandza JG Jr, Clark RE, Weldon CS and Sutera SP. Subhemolytic trauma of erythrocytes: recognition and sequestration by the spleen as a function of shear. Trans Am Soc Artif Intern Organs 1974; 20 B: 457-462.

[10] Baskurt OK, Uyuklu M and Meiselman HJ. Protection of erythrocytes from sub-hemolytic mechanical damage by nitric oxide mediated inhibition of potassium leakage. Biorheology 2004; 41: 79-89.

[11] Kanias T, Stone M, Page GP, Guo Y, Endres-Dighe SM, Lanteri MC, Spencer BR, Cable RG, Triulzi DJ, Kiss JE, Murphy EL, Kleinman S, Gladwin MT, Busch MP and Mast AE; NHLBI Recipient Epidemiology Donor Evaluation Study (REDS)-III Program. Frequent blood donations alter susceptibility of red blood cells to storage- and stress-induced hemolysis. Transfusion 2019; 59: 67-78.

[12] Hebbel RP. Beyond hemoglobin polymerization: the red blood cell membrane and sickle disease pathophysiology. Blood 1991; 77: 214-237.

[13] Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, Hassab H, Achebe MM, AlkiPE ndi S, Brown RC, Diuguid DL, Telfer P, Tsitsikas DA, Elghandour A, Gordeuk VR, Kanter J, Abboud MR, Lehrer-Graiwer J, Tonda M, Intondi A, Tong B and Howard J; HOTrial Investigators. A phase 3 randomized trial of voxelotor in sickle cell disease. N Engl J Med 2019; 381: 509-519.

[14] Hutchaleelaha A, Patel M, Washington C, Siu V, Allen E, Oksenberg D, Gretler DD, Mant T and Lehrer-Graiwer J. Pharmacokinetics and pharmacodynamics of voxelotor (GBT440) in healthy adults and patients with sickle cell disease. Br J Clin Pharmacol 2019; 85: 1290-1302.

[15] Oksenberg D, Dufu K, Patel MP, Chuang C, Li Z, Xu Q, Silva-Garcia A, Zhou C, Hutchaleelaha A, Patskovska L, Patskovsky Y, Almo SC, Sinha U, Metcalf BW and Archer DR. 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: 141-153.

[16] Patel M, Cabrales P, Dufu K, Metcalf B and Sinha U. GTx011, an anti-sickling compound, improves SS blood rheology by reduction of HbS polymerization via allosteric modulation of O2 affinity. Blood 2014; 124: 1370.

[17] Chasis JA and Mohandas N. Erythrocyte membrane deformability and stability: two distinct

membrane properties that are independently regulated by skeletal protein associations. J Cell Biol 1986; 103: 343-350.
[18] Chu H, McKenna MM, Krump NA, Zheng S, Mendelsohn L, Thein SL, Garrett LJ, Bodine DM and Low PS. Reversible binding of hemoglobin to band 3 constitutes the molecular switch that mediates 02 regulation of erythrocyte properties. Blood 2016; 128: 2708-2716.
[19] Gu L, Smith WA and Chatzimavroudis GP. Mechanical fragility calibration of red blood cells. ASAIO J 2005; 51: 194-201.
[20] Tarasev M, Muchnik M, Light L, Alfano K and Chakraborty S. Individual variability in response to a single sickling event for normal, sickle cell, and sickle trait erythrocytes. Transl Res 2017; 181: 96-107.
[21] Alfano KM, Tarasev M, Meines S and Parunak G. An approach to measuring RBC haemolysis and profiling RBC mechanical fragility. J Med Eng Technol 2016; 40: 162-171.
[22] Sowemimo-Coker SO. Red blood cell hemolysis during processing. Transfus Med Rev 2002; 16: 46-60.
[23] Raval JS, Waters JH, Seltsam A, Scharberg EA, Richter E, Daly AR, Kameneva MV and Yazer MH. The use of the mechanical fragility test in evaluating sublethal RBC injury during storage. Vox Sang 2010; 99: 325-331.
[24] Ballas SK and Connes P. The paradox of the serrated sickle erythrocyte: the importance of the red blood cell membrane topography. Clin Hemorheol Microcirc 2015; 63: 149-152.
[25] Alvarez O, Montague NS, Marin M, O'Brien R and Rodriguez MM. Quantification of sickle cells in the peripheral smear as a marker of disease severity. Fetal Pediatr Pathol 2015; 34: 149-154.
[26] Ikuta T, Thatte HS, Tang JX, Mukerji I, Knee K, Bridges KR, Wang S, Montero-Huerta P, Joshi RM and Head CA. Nitric oxide reduces sickle hemoglobin polymerization: potential role of nitric oxide-induced charge alteration in depolymerization. Arch Biochem Biophys 2011; 510: 53-61.
[27] Abdulmalik O, Safo MK, Chen Q, Yang J, Brugnara C, Ohene-Frempong K, Abraham DJ and Asakura T. 5-hydroxymethyl-2-furfural modifies intracellular sickle haemoglobin and inhibits sickling of red blood cells. Br J Haematol 2005; 128: 552-561.
[28] Stefanovic M, Puchulu-Campanella E, Kodippili G and Low PS. Oxygen regulates the band 3-ankyrin bridge in the human erythrocyte membrane. Biochem J 2013; 449: 143-150.
[29] Westerman M and Porter JB. Red blood cell-derived microparticles: an overview. Blood Cells Mol Dis 2016; 59: 134-139.
[30] Presley TD, Perlegas AS, Bain LE, Ballas SK, Nichols JS, Sabio H, Gladwin MT, Kato GJ and Kim-Shapiro DB. Effects of a single sickling event on the mechanical fragility of sickle cell trait erythrocytes. Hemoglobin 2010; 34: 24-36.
[31] Peters LL, Shivdasani RA, Liu SC, Hanspal M, John KM, Gonzalez JM, Brugnara C, Gwynn B, Mohandas N, Alper SL, Orkin SH and Lux SE. Anion exchanger 1 (band 3) is required to prevent erythrocyte membrane surface loss but not to form the membrane skeleton. Cell 1996; 86: 917-927.
[32] Southgate CD, Chishti AH, Mitchell B, Yi SJ and Palek J. Targeted disruption of the murine erythroid band 3 gene results in spherocytosis and severe haemolytic anaemia despite a normal membrane skeleton. Nat Genet 1996; 14: 227-230.
[33] Low PS, Willardson BM, Mohandas N, Rossi M and Shohet S. Contribution of the band 3-ankyrin interaction to erythrocyte membrane mechanical stability. Blood 1991; 77: 1581-1586.
[34] Shaklai N, Sharma VS and Ranney HM. Interaction of sickle cell hemoglobin with erythrocyte membranes. Proc Natl Acad Sci U S A 1981; 78: 65-68.
[35] Rifkind JM, Ramasamy S, Manoharan PT, Nagababu E and Mohanty JG. Redox reactions of hemoglobin. Antioxid Redox Signal 2004; 6: 657-666.
[36] Hebbel RP, Eaton JW, Balasingam M and Steinberg MH. Spontaneous oxygen radical generation by sickle erythrocytes. J Clin Invest 1982; 70: 1253-1259.
[37] Quinn CT, Smith EP, Arbabi S, Khera PK, Lindsell CJ, Niss O, Joiner CH, Franco RS and Cohen RM. Biochemical surrogate markers of hemolysis do not correlate with directly measured erythrocyte survival in sickle cell anemia. Am J Hematol 2016; 91: 1195-1201.
[38] Franco RS, Yasin Z, Palascak MB, Ciraolo P, Joiner CH and Rucknagel DL. The effect of fetal hemoglobin on the survival characteristics of sickle cells. Blood 2006; 108: 1073-1076.
[39] Nagalla S and Ballas SK. Drugs for preventing red blood cell dehydration in people with sickle cell disease. Cochrane Database Syst Rev 2018; 10: CD003426.
[40] Kassa T, Wood F, Strader MB and Alayash AI. Antisickling drugs targeting βCys93 reduce iron oxidation and oxidative changes in sickle cell hemoglobin. Front Physiol 2019; 10: 931.
[41] Yang H, Merica E, Chen Y, Cohen M, Goldwater R, Hill C, Kim H, Kosinski P, Kung C, Silver B, Utley L and Agresta S. Phase I single (SAD) and multiple ascending dose (MAD) studies of the safety, tolerability, pharmacokinetics (PK) and pharmacodynamics (PD) of AG-348, a first-in-class allosteric activator of pyruvate kinase-R, in healthy subjects. Blood 2014; 124: 4007.

[42] Pagare PP, Ghatge MS, Musayev FN, Deshpande TM, Chen Q, Braxton C, Kim S, Venitz J, Zhang Y, Abdulmalik O and Safo MK. Rational design of pyridyl derivatives of vanillin for the treatment of sickle cell disease. Bioorg Med Chem 2018; 26: 2530-2538.

[43] Nnamani IN, Joshi GS, Danso-Danquah R, Abdulmalik O, Asakura T, Abraham DJ and Safo MK. Pyridyl derivatives of benzaldehyde as potential antisickling agents. Chem Biodivers 2008; 5: 1762-1769.

[44] Dufu K, Yalcin O, Ao-leong ESY, Hutchaleelala A, Xu Q, Li Z, Vlahakis N, Oksenberg D, Lehrer-Graiwer J and Cabrales P. GBT1118, a potent allosteric modifier of hemoglobin O(2) affinity, increases tolerance to severe hypoxia in mice. Am J Physiol Heart Circ Physiol 2017; 313: H381-h391.

[45] Al Balushi H, Dufu K, Rees DC, Brewin JN, Hannemann A, Oksenberg D, Lu DC and Gibson JS. The effect of the antisickling compound GBT1118 on the permeability of red blood cells from patients with sickle cell anemia. Physiol Rep 2019; 7: e14027.

[46] Coletta M, Hofrichter J, Ferrone FA and Eaton WA. Kinetics of sickle haemoglobin polymerization in single red cells. Nature 1982; 300: 194-197.

[47] Safo MK and Kato GJ. Therapeutic strategies to alter the oxygen affinity of sickle hemoglobin. Hematol Oncol Clin North Am 2014; 28: 217-231.

[48] Li Q, Henry ER, Hofrichter J, Smith JF, Cellmer T, Dunkelberger EB, Metaferia BB, Jones-Straehle S, Boutom S, Christoph GW, Wakefield TH, Link ME, Staton D, Vass ER, Miller JL, Hsieh MM, Tisdale JF and Eaton WA. Kinetic assay shows that increasing red cell volume could be a treatment for sickle cell disease. Proc Natl Acad Sci U S A 2017; 114: E689-E696.

[49] Dufu K and Oksenberg D. GBT440 reverses sickling of sickled red blood cells under hypoxic conditions in vitro. Hematol Rep 2018; 10: 7419.

[50] Dufu K, Patel M, Oksenberg D and Cabrales P. GBT440 improves red blood cell deformability and reduces viscosity of sickle cell blood under deoxygenated conditions. Clin Hemorheol Microcirc 2018; 70: 95-105.