GBT440 reverses sickling of sickled red blood cells under hypoxic conditions *in vitro*

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Abstract

Sickle cell disease is characterized by hemolytic anemia, vasoocclusion and early mortality. Polymerization of hemoglobin S followed by red blood cell sickling and subsequent vascular injury are key events in the pathogenesis of sickle cell disease. Sickled red blood cells are major contributors to the abnormal blood rheology, poor microvascular blood flow and endothelial injury in sickle cell disease. Therefore, an agent that can prevent and or reverse sickling of red blood cells, may provide therapeutic benefit for the treatment of sickle cell disease. We report here that GBT440, an anti-polymerization agent being developed for the chronic treatment of sickle cell disease, increases hemoglobin oxygen affinity and reverses *in vitro* sickling of previously sickled red blood cells under hypoxic conditions. Our results suggest that besides preventing sickling of red blood cells, GBT440 may mitigate vasoocclusion and microvascular dysfunction by reversing sickling of circulating sickled red blood cells *in vivo.*

Introduction

Sickle cell disease (SCD) is caused by a point mutation in the β-globin gene leading to the formation of hemoglobin S (HbS). The pathophysiological features of SCD include hemolytic anemia, vasoocclusion, end-organ damage and early death.[1] Multiple factors contribute to the complex pathophysiology of SCD; however vasoocclusion and microvascular dysfunction accounts for a major part of the pathophysiology and contributes to the morbidity and mortality associated with this disease.[2,3] The key initiating event of the molecular pathogenesis of SCD is the polymerization of deoxygenated HbS (deoxy-HbS).[4] Polymerization of deoxy-HbS leads to formation of non-deformable and membrane-damaged sickled red blood cells (RBC).[1] RBC deformability is a key determinant of blood viscosity and an important requirement of blood flow, which in turn influences the efficiency of oxygen delivery to tissues.[2] Under hypoxic conditions, RBC deformability is largely dependent on cytoplasmic viscosity determined by HbS polymer content within RBCs of patients suffering from SCD.[5,6] Due to the inability of HbS polymer containing sickled RBCs to deform, they often physically obstruct the flow of blood while traveling through the microvasculature of tissues via a process referred to as vasoocclusion.[7] In addition, adhesion of sickled RBC to postcapillary venule endothelium is postulated to initiate and propagate the painful episodes of vasoocclusive crisis.[8] Several processes including inflammation, activation of endothelial cells and local hypoxia may further promote vasoocclusion leading to microvascular dysfunction. However, even though the precise mechanism of vasoocclusive crisis is not completely understood, non-deformable sickled RBCs remain important contributors to vasoocclusion biology.[9-12] Therefore, an agent that can prevent and or reverse sickling of RBCs, may potentially provide therapeutic benefit in treating the pathophysiological features of SCD including vasoocclusion.

We previously reported that GBT440, an anti-polymerization agent currently being developed for the chronic treatment of SCD and in phase 3 clinical trials (NCT03036813, http://clinicaltrials.gov/), increases hemoglobin oxygen affinity and prevents sickling of RBCs.[13] In this work, we further characterized the anti-sickling activity of GBT440 and showed that GBT440 reverses sickling of sickled RBCs under hypoxic conditions *in vitro*. Our results suggest that besides preventing sickling of red blood cells, GBT440 may mitigate vasoocclusion and microvascular dysfunction by reversing sickling of circulating sickled red blood cells *in vivo.*

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Contributions: KD conceived of the study, conducted experiments, interpreted and discussed data, prepared the figures and wrote the manuscript; DO provided data interpretation, discussion and study oversight.

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Materials and Methods

Blood source

SCD blood was obtained from homozygous sickle cell patients from the University of North Carolina [UNC, Chapel Hill, NC (IRB #88-034)].

Whole blood hemoximetry

Oxygen dissociation and association curves were determined using a Hemox Analyzer TCS Scientific.[14] Whole blood samples were diluted 50-fold into Hemox buffer (30 mM TES, 130 mM NaCl, 5 mM KCl) and transferred to the Hemox Analyzer. The blood samples were first saturated with compressed air to a partial pressure of O_2 (pO_2) of 150 mmHg and then flushed with pure nitrogen to deoxygenate to a pO_2 1.6 mmHg. To ensure complete deoxygenation, the deoxygenated blood sample was maintained at a pO_2 of 1.6 mm Hg for 30 minutes. To re-oxygenate, the deoxygenated blood samples were flushed with compressed air to increase the pO_2 from 1.6 to 150 mmHg. The absorbance at wavelengths that correspond to the isosbestic point (570 nm) and deoxy-Hb (560 nm) were recorded as a function of the sample pO_2 during deoxygenation and or oxygenation. Data for Hb-oxygen dissociation or association curves were collected using TCS software. For experiments in which a deoxygenated blood sample was to be analyzed under continued hypoxic conditions, a dummy blood sample was used to set the pO_2 of the Hemox Analyzer to 1.6 mmHg. Subsequently, the dummy blood sample was replaced with the deoxygenated exper-

imental blood sample (without exposure to air) and the sample was oxygenated from a pO_2 of 1.6 to 150 mmHg to measure oxygen association curves.

In vitro sickling assay

To isolate RBCs from sickle patient blood (SS RBCs), 500 µL of PBS (pH 7.4; isotonic) was added to SS blood (500 µL) and centrifuged (Eppendorf 5430 R centrifuge) at 250 g for 5 min. After centrifugation, the supernatant was discarded. This process was repeated twice after which the resulting packed SS RBCs (100% Hct) were used for sickling experiments. Twenty µL of packed SS RBCs was added to 30 µL of PBS (final Hct of 40%) in a 96-well gas permeable plate (cat # 8602001, Coy Laboratory Products) and incubated for 0.5 hours at 37ºC in a humidified hypoxic chamber (4% O_2, 96% N_2). GBT440 (in 100% DMSO) was added to 50 µL of deoxygenated-PBS at varying concentrations. DMSO alone was used as a no compound-control. Next, sickled SS RBCs (50 µL) was added to 50 µL of GBT440 solution and mixed. The final GBT440 concentrations were, 1 mM, 2 mM or 5 mM and the final DMSO concentration was 2.5% per reaction. The reaction mixture was incubated under continued hypoxia for an additional 2 hours. Next sickled SS RBCs were imaged using a Bright-field microscope fitted with an Infinity Lite camera (Lumenera Corp., Nepean, ON, Canada) in the hypoxic chamber. Images (40× magnification) were quantitated by manually counting round cells (un-sickled cells) *versus* ill-shaped cells (sickled cells) or by using the CellVigene software (Vigene-Tech Inc., Carlisle, MA, USA) designed to quantify sickling based on circularity (the ratio of the longest axis to the shortest axis of each RBC).

Results

GBT440/hemoglobin S interaction during oxygenation and deoxygenation

We previously reported that GBT440 prevents sickling of SS RBCs.[13] In this study, we evaluated whether GBT440 could reverse sickling of already sickled SS RBCs under hypoxic conditions. By measuring Hb-oxygen dissociation curves (ODCs), we initially evaluated the stability of the GBT440-HbS interaction during two cycles of oxygenation and deoxygenation (Figure 1A). In this experiment, Hb-oxygen association curves (OACs) were not measured. The duration from the first to the second deoxygenation step was 1.5 hours (Figure 1A). The ability of GBT440 to left-shift the ODC (Figure 1B) of blood was used to inform the interaction between GBT440 and HbS within SS RBCs. As previously reported, stoichiometric modification of Hb with GBT440 results in a hyperbolic left-shifted ODC relative to the sigmoidal ODC of unmodified blood indicating an increase in Hb-O_2 affinity steming from the ability of GBT440 to delay the transition from the oxygenated to deoxygenated Hb states.[13] The oxygen saturation of GBT440-modified SS blood at a PO_2 of 1.6 mmHg was essentially zero (Figure 1B; insert), indicating that at this oxygen tension complete deoxygenation of HbS was achieved and thus, SS RBCs are expected to contain HbS polymers. Importantly, GBT440-modified SS blood maintained the left-shift of the ODC relative to control during the second deoxygenation cycle. These data indicate that GBT440/HbS interaction is maintained during two cycles of oxygenation and deoxygenation of GBT440 modified-SS blood. Moreover, our data suggests that the GBT440/HbS interaction is stable in the presence or absence of HbS polymers within SS RBCs.

GBT440 modifies polymerized hemoglobin S in sickled SS red blood cells

We next determined whether GBT440 could modify HbS in already sickled SS RBCs containing HbS polymers under hypoxic conditions. Accordingly, SS blood was incubated in a hypoxic chamber (0.6% O_2 and 99.4% N_2) for 0.5 hours to induce sickling. As shown in Figure 2A, ~80% of the SS RBCs sickled indicating the presence of HbS polymers. GBT440 was then added to the sickled SS blood in the hypoxic chamber and incubated for an additional 2 hours. The GBT440-modified SS blood was subsequently oxygenated in a Hemox Analyzer and the OAC was measured. In this case, the left-shift of the OAC was used to inform the interaction between GBT440 and HbS polymers within the sickled SS

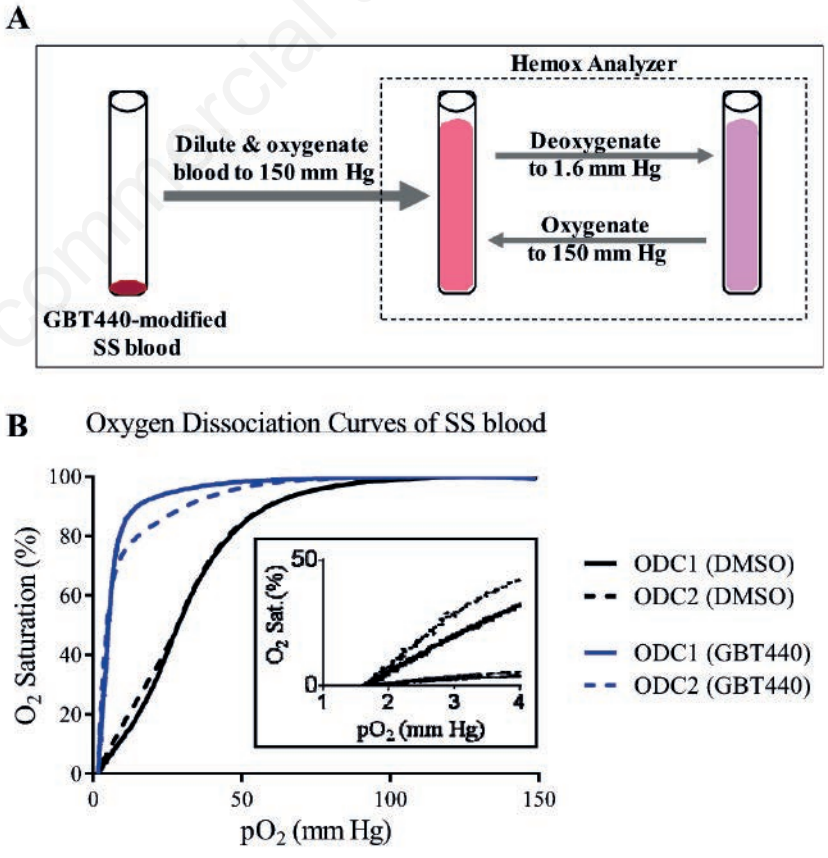

Figure 1. The GBT440/HbS interaction is maintained during cycles of oxygenation and deoxygenation. (A) Schematic representation of deoxygenation and oxygenation cycles of GBT440-modified sickle cell blood in Hemox Analyzer. (B) Oxygen dissociation curves of sickle cell blood (at 20% hematocrit containing~ 1 mM hemoglobin) modified with GBT440 (1 mM).

RBCs. As depicted in Figure 2B, GBT440 dose-dependently caused a left shift of the OAC relative to control indicating that GBT440 can interact with polymerized HbS within sickled SS RBCs.

GBT440 reverses sickling of sickled SS red blood cells

We next determined whether GBT440 could reverse sickling of already sickled SS RBCs at a pO_2 of ~32 mm Hg (or 4% O_2), an O_2 tension mimicking typical hypoxic conditions in tissue capillaries. SS RBCs were first allowed to sickle (~80% sickled; Figure 3A) in a hypoxic chamber (4% O_2, 96% N_2) for 0.5 hours and subsequently treated with GBT440 for an additional 2 hours. As shown in Figure 3B, without GBT440, the percentage of sickled cells increased from 80% to 99% after 2 hours of continued incubation under hypoxia (Figure 3B, D). In contrast, the percentage of sickled cells decreased from 80% to 57%, 35% and 33% in the presence of 1 mM, 2 mM and 5 mM GBT440, respectively (Figure 3C, D). These results indicate that GBT440 reversed sickling of already sickled SS RBCs under hypoxic conditions.

Discussion

In this study, we demonstrate using ODCs that the interaction between GBT440 and HbS remains intact in SS RBCs during two cycles of deoxygenation with intermittent oxygenation. Furthermore, we show that GBT440 dose-dependently left-shifts the OAC of sickled SS RBCs, indicating that GBT440 is able to modify polymerized HbS and consequently reverse sickling of previously sickled cells under hypoxic conditions. Our findings have multiple implications for the development and potential use of GBT440 for the treatment of SCD. The ability of GBT440 to maintain its interaction with HbS during cycles of oxygenation and deoxygenation supports a potentially prolonged GBT440 on-target effect *in vivo*. Furthermore, the ability of GBT440 to modify sickled SS RBCs containing polymerized HbS and consequently reverse sickling suggests that GBT440 may have the ability to reduce the number of circulating sickled SS RBCs *in vivo* by preventing sickling of un-sickled SS RBCs and by reversing sickling of sickled SS RBCs. Relative to the substoichiometric target concentration of GBT440 to Hb being evaluated in clinic trials (~30% modification of total Hb in blood), greater than stoichiometric concentrations of GBT440 to Hb (based on 1:1 binding) were required in our *in vitro* assays to demonstrate a reversal of sickling most likely due to the more strenuous hypoxic conditions used herein. Of note, the effect of GBT440 on irreversibly sickled cells (ISCs) was not evaluated in this study. Nevertheless, GBT440 is not expected to reverse sickling of ISCs since they are irreversibly sickled primarily due to defects in cell membrane/cytoskeleton and independent of HbS polymer load.[15] However, by preventing and reversing sickling of younger SS RBCs, GBT440 is expected to reduce the number of circulating ISCs overtime and thus maintain the health of SS RBCs *in vivo*. In support of this, GBT440 demonstrated a marked reduction in circulating ISCs during the Phase 1/2 clinical trial in SCD patients (NCT02285088).[16]

Lastly, the ability of GBT440 to modify HbS in sickled SS RBCs and reverse sickling under hypoxic conditions also implies that GBT440 may have the ability to structurally promote a Tense (T) state (deoxyHb) to Relaxed (R) state (oxyHb) transition independent of oxygen binding. Though, the later remains to be demonstrated experimentally, GBT440 was recently found to inhibit sickling in the absence of oxygen *in vitro*.[17] Since SS RBCs were sickled prior to addition of GBT440, our results suggests that in addition to having the ability to extend the HbS polymerization delay time as previously reported, GBT440 can reverse sickling by decreasing the intracellular HbS polymer load.

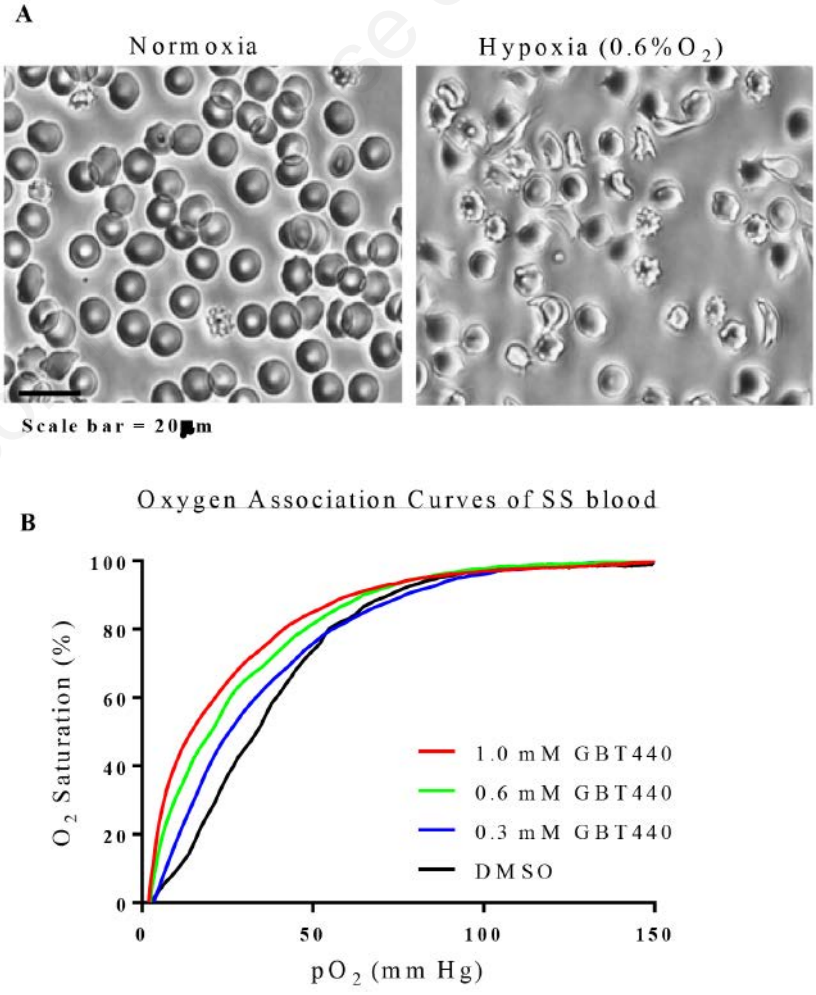

Figure 2. GBT440 modifies polymerized HbS in sickled SS RBCs. (A) Representative images of SS RBCs at room air (normoxia; left panel) and at 0.6% O_2 (hypoxia; right panel), respectively. Solid black line represents the scale of the images (B) Oxygen association curves of sickled blood (containing SS RBCs with polymerized HbS) treated with GBT440 in (A).

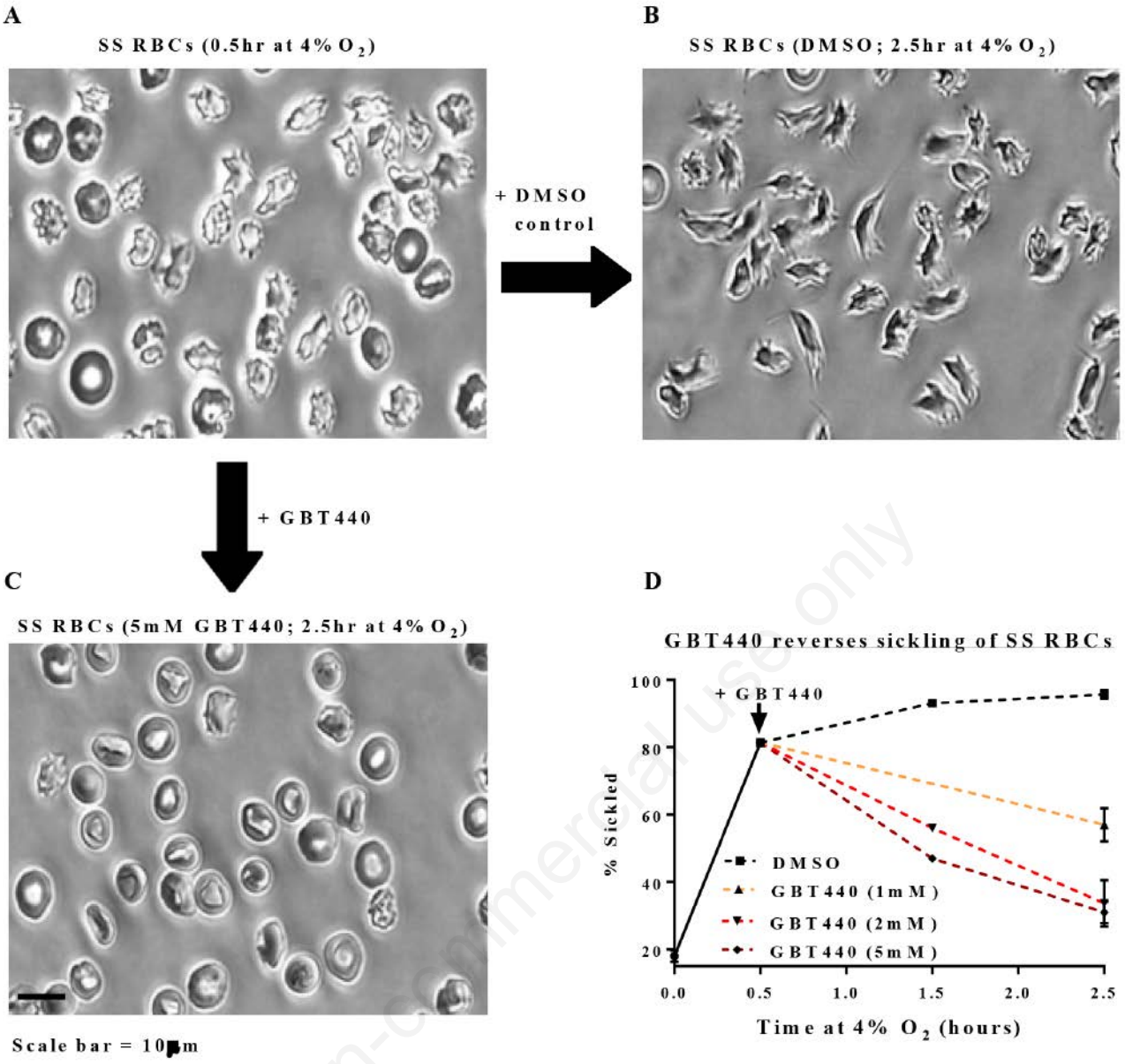

Figure 3. GBT440 reverses sickling of sickled SS RBCs. (A) Representative image of sickled SS RBCs at 4% O2 for 0.5 hours. (B) Representative image of sickled SS RBCs in (A) treated with DMSO (control) at 4% O2 for an additional 2 hours. (C) Representative image of sickled SS RBCs in (A) treated with GBT440 (5 mM) at 4% O2 for an additional 2 hours. Solid black line represents the scale of the images in (A), (B) and (C). (D) Graph showing quantification of sickled SS RBCs treated with GBT440 or control in (A), (B) and (C).

Conclusions

In summary, GBT440's ability to inhibit polymerization and consequently prevent SS RBC sickling implies that chronic application of GBT440 at therapeutic doses should potentially improve all downstream effects of polymerization including the overall health of circulating SS RBCs, SS RBC lifespan and anemia. The ability of GBT440 to reverse sickling may have implications in mitigating both acute and chronic vasoocclusion-related complications in SCD.

References

1. Bunn HF. Pathogenesis and treatment of sickle cell disease. N Engl J Med 1997;337:762-9.
2. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. Lancet 2010;376: 2018-31.
3. Kurantsin-Mills J, Klug PP, Lessin LS. Vaso-occlusion in sickle cell disease: pathophysiology of the microvascular circulation. Am J Pediatr Hematol Oncol 1988;10:357-72.
4. Eaton WA, Hofrichter J. Sickle Cell Hemoglobin Polymerization. Adv Prot Chem 1990;40:263-79.
5. Musielak M. Red blood cell-deformability measurement: review of tech-

niques. Clin Hemorheol Microcircul 2009;42:47-64.
6. Dong C, Chadwick RS, Schechter AN. Influence of sickle hemoglobin polymerization and membrane properties on deformability of sickle erythrocytes in the microcirculation. Biophys J 1992;63:774-83.
7. Clark MR, Mohandas N, Shohet SB. Deformability of oxygenated irreversibly sickled cells. J Clin Invest 1980;65:189-96.
8. Wagner MC, Eckman JR, Wick TM. Sickle cell adhesion depends on hemodynamics and endothelial activation. J Lab Clin Med.2004;144:260-7.
9. Kault DK, Fabry ME, Nagel RL. Microvascular sites and characteristics of sickle cell adhesion to vascular endothelium in shear flow conditions: Pathophysiological implications. Med. Sci 1989;86:3356-60.
10. Embury SH. The not-so-simple process of sickle cell vasoocclusion. Microcirculation 2004;11:101-13.
11. Higgins JM, Eddington DT, Bhatia SN, Mahadevan L. Sickle cell vasoocclusion and rescue in a microfluidic device. Proc Natl Acad Sci USA 2007;104:20496–500.
12. Bartolucci P, Brugnara C, Teixeira-Pinto, A. Erythrocyte density in sickle cell syndromes is associated with specific clinical manifestations and hemolysis. Blood 2012;120:3136-4.
13. Oksenberg D, Dufu K, Patel PM, et al. GBT440 increases haemoglobin oxygen affinity, reduces sickling and prolongs RBC half-life in a murine model of sickle cell disease. Br J Haematol 2016;175:141-53.
14. Guarnone R, Centenara E, Barosi G. Performance characteristics of hemoxanalyzer for assessment of the hemoglobin dissociation curve. Haematologica 1995;80:426-30.
15. Huang Z, Hearne L, Irby EC, et al. Kinetics of increased deformability of deoxygenated sickle cells upon oxygenation. Biophys J 2003;85:2374-83.