Supporting Information

Interactions of an Anti-sickling drug with Hemoglobin in Red Blood Cells from a Patient with Sickle Cell Anemia

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Experimental procedures

Proteins and chemicals

The GBT440 incubated blood samples were centrifuged at 1000 g for 10 minutes. The supernatant was discarded, and the RBC was washed with 3-fold volumes of PBS by centrifuging at 1000 rpm for 10 minutes in order to remove excess GBT400. The washing step proceeded 3 times. Then 50 μL of RBC was added 200 uL of water and then well mixed and incubated at room for 20 minutes to lyse the RBC. The 250 μL of Hb lysate solution was added 25 μL of condensed PBS (x10, Fisher Sci), and then centrifuged at 13000 g for 20 minutes to remove the debris. For the $NaBH_3CN$ stabilized Hb samples, the Hb lysates (4mM) were incubated with $NaBH_3CN$ (50 mM) at room temperature for 20 minutes before any analyses. Hb concentration was measured based on the absorbencies at λ = 541, 576, 630, and 700 nm using recently published extinction coefficients [1]. All the Hb concentrations in this study are reported based on heme concentration.

Reverse phase high performance liquid chromatograph (RP-HPLC)

RP-HPLC was performed with a Zorbax 300 SB C3 column (4.6x250 mm) coupled to a Waters HPLC system consisting of a Waters 626 pumps, 2487 dual-wavelength detector and a 600s controller installed with Empower 2 (Waters Corp, Milford, MA) [2]. 20 μg of Hb in 25 μL of water was loaded on C3 column equilibrated with 35% acetonitrile containing 0.1% TFA. Globin chains were eluted with a gradient of 35-50% acetonitrile within 100 min at a flow rate of 1 mL/min. The eluent was monitored at 280nm for globin chains and at 405 nm for the heme components.

LC/MS/MS Analysis

Hb samples from patient hemolysate (with and without GBT440) were digested with trypsin, desalted and analyzed by mass spectrometry using our previously described method [3]. Briefly, tryptic peptides were analyzed by reverse phase liquid chromatography mass spectrometry (RP LC/MS/MS) using an Easy nLC II Proxeon nanoflow HPLC system coupled online to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Data were acquired using a top10 method (for 60 minutes), dynamically choosing the most abundant precursors (scanned at 400-2000 m/z) from the survey scans for HCD fragmentation.

Mass spectrometry was used to confirm the presence of GBT440 attached peptides for each patient hemolysate same and by searching data against the Swiss-Prot Human database (release 2014_03, containing 542782 sequence entries) using the Mascot (version 2.4) search engine (Matrix Sciences, London, UK) as described previously[3]. Variable modifications for all MS/MS data including the following masses: methionine oxidation (+16) and GBT440 binding to N-terminal peptides and lysine residues (+337). Mascot output files were analyzed using the software Scaffold 4.2.0 (Proteome Software Inc.). Hb peptide identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides. Probabilities were assigned by the Protein Prophet algorithm [4].

Intact Mass Analysis

Subunits of intact HbS from patient hemolysate (incubated with GBT440 (1;0.3 and 1:1 ratios) were isolated using RP-HPLC prior to LC-MS analysis. Samples were analyzed using a Q-Exactive as described above with the instrument configured to acquire data in Full-MS mode. Isotopically resolved charge state envelopes (multiply charged ions of intact monomers) representing mass spectra of intact Hb subunits were deconvoluted using the protein deconvolution software Xtract (Thermo Scientific). The deconvoluted mass spectrum yielded a measured molecular mass that was within 0.025 Da (3 ppm) of the calculated value.

Figure S1

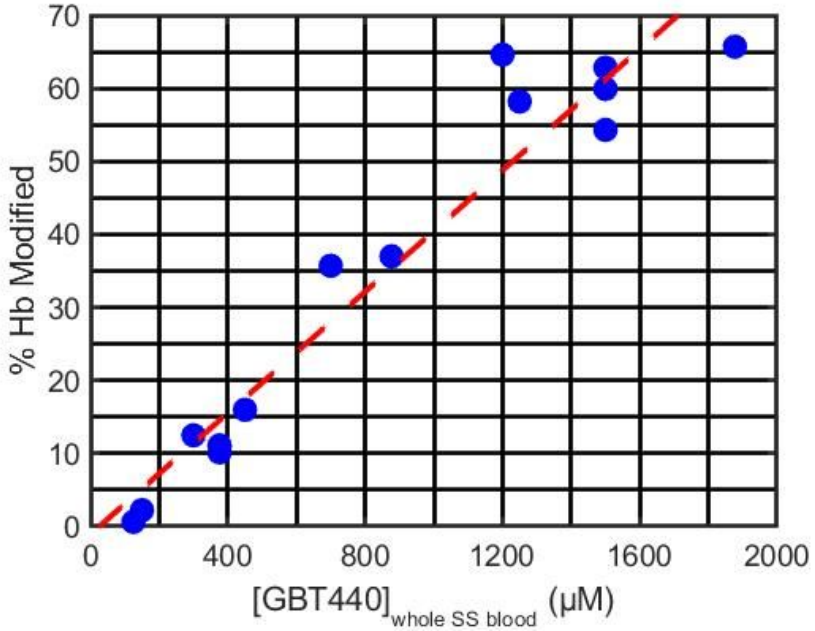

Figure S1. Percent hemoglobin modified for whole SS blood samples treated with varying concentrations of GBT440. Blood was drawn from four SS patients with hematocrits between 21% and 28%. The percent hemoglobin modified for each sample was determined by the linear combination analysis in Figure 1 of the main text. Briefly, the best linear least squares fit was found between the oxygen affinity curve of each sample treated with GBT440 and an untreated sample and a 1:50 erythrocyte suspension treated with 200 μM GBT440, a concentration found by Li *et al* to modify all hemoglobin, from the same donor. From a linear regression analysis, the following relationship was found with an R^2 of 0.93: $\%\ \mathrm{Hb\ Modified} = 0.0415 \times [\mathrm{GBT440}]_{\mathrm{whole\ SS\ blood}} - 1.0391$. From this, it was concluded that GBT440 shifts the hemoglobin quaternary equilibrium to the R conformation in SS erythrocytes in a linearly dose dependent manner throughout the range of modification desirable for therapeutic intervention.

References:

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