GTx011, A Novel Agent That Improves Rheological Properties Of Sickle Cell Blood By Increasing Oxygen Affinity For Hemoglobin.

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Introduction

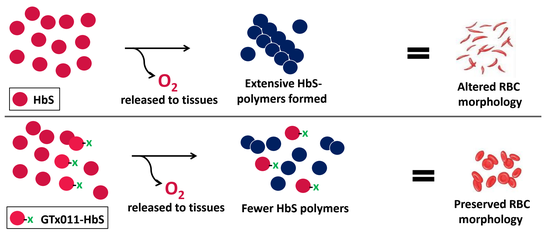
Sickle cell disorder (SCD) is an inherited hemoglobinopathy affecting a substantial portion of the African population and more than 90,000 African-Americans (1, 2). It is a genetic disorder characterized by the presence of sickled red blood cells (RBCs) that are the result of a single point mutation in Hemoglobin (Hb) in the β globin at the 6th position. The hydrophilic glutamic acid is replaced by a hydrophobic valine. This mutation does not alter the oxygen affinity or change the structure of Hb in the R (relaxed) state. However, in the T (tense) state, Hb from SS patients (HbS) forms polymers in low oxygen environments. When polymers within erythrocytes grow large enough, they can lead to the development of non-deformable RBCs. If the RBCs are further deoxygenated, they develop into the quintessential sickle shape. The non-deformable sickled cells increase the blood viscosity leading to a diminished flow through capillary beds. These non-deformable cells lead to the physiologic effects of SCD: anemia, ulcers, and major organ damage, including renal insufficiency and non-functional spleens (3,4).

Though the childhood death rate of individuals with SCD has drastically fallen due to disease management, transfusion therapy and hydroxyurea, there is no standard treatment for SCD (5). It has been shown that modifying O_2 affinity can alter the polymerization dynamics in a manner that may be beneficial in SCD (6, 7). However, to date, none of these modifiers are approved for use in patients.

In order to fulfill this unmet need, Global Blood Therapeutics has developed a novel series of antisickling compounds that increase the O_2 affinity of Hb, delay polymerization and improve the rheological properties of SS blood.

Mechanism of Action

Under physiologic conditions, HbS will form polymers upon deoxygenation. The formation of extensive polymers leads to sickling in the microcirculation. *In vitro* experiments show that GTx011-treated HbS delays polymerization and thereby should delay formation of sickled cells, allowing them to exit the microcirculation and get reoxygenated in the lungs.



Materials and Methods

Determination of oxygen equilibrium curves (OECs) in purified Hb, washed RBCs, and blood

Purified Hb samples (25μM) were incubated for 45 min at 37°C. Washed RBCs and blood samples (Hematocrit, Hct, adjusted to 20%) were incubated for 1 hour at 37°C in the absence or presence of compound. 100 µl of RBCs or blood was added to 5 mL of Hemox buffer at 37°C. Oxygen equilibrium curves (OECs) were then collected with a Hemox analyzer (8).

Oxygen Dissociation Assay

Determines the ability of GTx compounds to maintain the oxygenated state of hemoglobin (oxyHb) under deoxygenated conditions. Purified Hb (3 µM) was incubated for 1 hour at 37°C in the presence or absence of compound in 50 mM potassium phosphate buffer, pH=7.4 in 96-well half-area plates. Plates were then placed in a SPECTROstar Nano plate reader (BMG Labtech) at 37°C and N_2 (flow rate = 20 L/min) was blown over the plates for 2 hours. Visible spectra measurements were obtained every 6 min for 2 hours.

***In vitro* HbS Polymerization**

HbS polymerization was evaluated in an adapted version of the assay described in ref. 9. HbS was purified from SS RBC lysates through gel filtration and DE-52 anion exchange chromatography. Purified HbS was mixed with either HbA or GTx011-HbS (final [Hb]=50µM) in 1.8 M potassium phosphate for 1 hour at 37°C. The reaction mixture was then passively de-oxygenated (99.5 % N_2/ 0.5% O_2) at 4°C for 90 min and polymerization was induced via a temperature jump from 4°C to 37°C. Polymerization was quantitated by measuring turbidity of the HbS solution at 700 nm under continued hypoxia.

Viscosity

The viscosity of treated and control blood was measured in a cone plate viscometer (10). Blood Hct was adjusted to 30%, and then incubated for 30 min at room temperature with GTx011 or DMSO. The reaction mixture was deoxygenated for 2 hours in a gas permeable 24-well plate placed within a humidified hypoxic chamber (97.6% N_2/2.4% O_2). Room temperature equilibrated blood samples were run as oxygenated controls. Data was collected at sheer rates ranging from 60 s^{-1} to 415 s^{-1}.

Oxygen Affinity Studies

Hb modified by GTx011 maintains the oxyHb state in a low O_2 environment

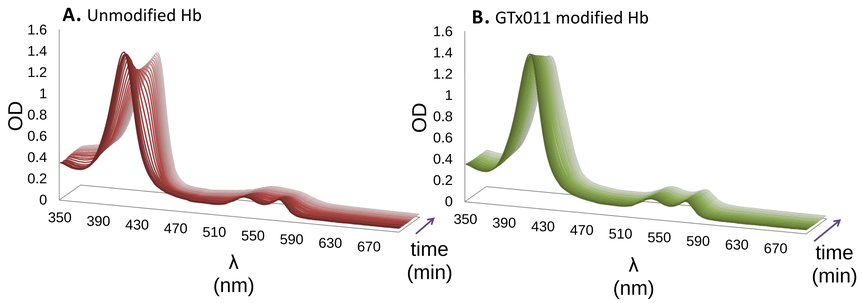


Figure 1. (A) Spectra of 3 µM Hb that is passively deoxygenated with N_2 in a 96-well plate reader over a 2 hour time period. The Soret bands (400-450nm) shows the Hb transitioning from an oxyHb state to a deoxyHb state. The same transition can be seen in the Q-band region (500-600nm). (B) Spectra of 3 µM Hb incubated with 4 µM GTx011 shows that GTx011-Hb maintains the oxyHb state after 2 hours of passive deoxygenation.

GTx compounds dose dependently maintain the oxyHb state

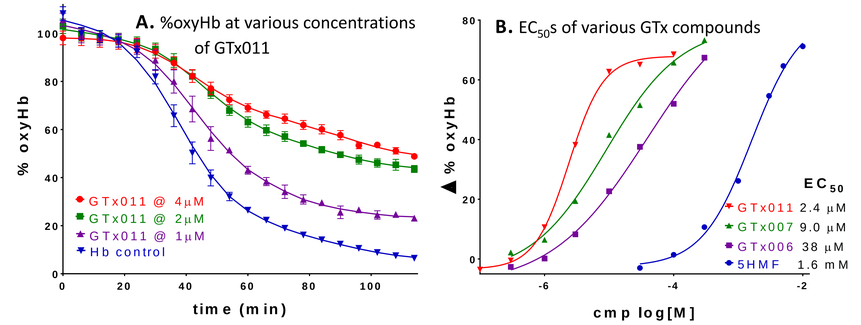
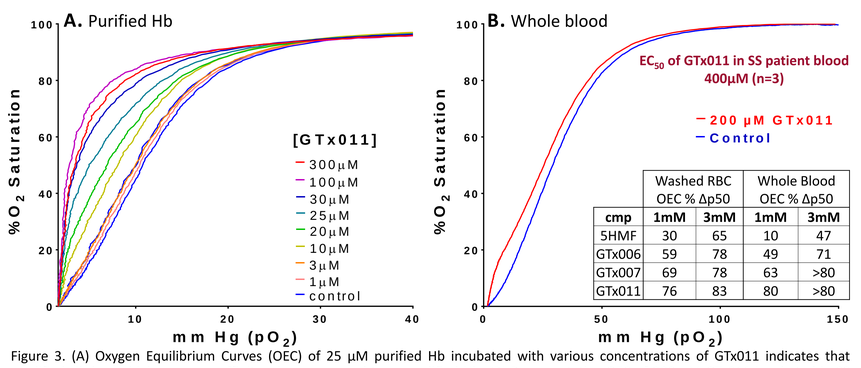


Figure 2. (A) %oxyHb at various time points during a 2 hour passive deoxygenation of Hb. GTx011 dose dependently delays the progression to a deoxy state ([Hb] = 3 µM). The %oxyHb is calculated based upon the AUC of the sample spectra compared to the AUC of deoxyHb and oxyHb spectra. (B) Dose response of GTx006, GTx007, GTx011 and 5HMF in the presence of 3µM Hb. GTx011 is the most potent compound in the Oxygen Dissociation Assay (ODA). The %oxyHb is calculated at 108 minutes after deoxygenation. Δ%oxyHb calculated by subtracting the hemoglobin control from the samples values.

GTx011 modulates O_2 affinity in purified Hb, washed RBCs and SS Blood



cmp	Washed RBC OEC % Δp50 1mM	Washed RBC OEC % Δp50 3mM	Whole Blood OEC % Δp50 1mM	Whole Blood OEC % Δp50 3mM
5HMF	30	65	10	47
GTx006	59	78	49	71
GTx007	69	78	63	>80
GTx011	76	83	80	>80

Figure 3. (A) Oxygen Equilibrium Curves (OEC) of 25 µM purified Hb incubated with various concentrations of GTx011 indicates that modified Hb has a higher oxygen affinity when compared to unmodified Hb. (B) Representative OEC of 200 µM GTx011 incubated in SS blood at 20% Hct displays a left shifted curve when compared to unmodified SS blood. Table summarizes the OECs of various GTx and control compounds in washed RBCs and in whole blood.

Polymerization and Rheological Studies

Hb modified by GTx011 recapitulates HbA at comparable fractions

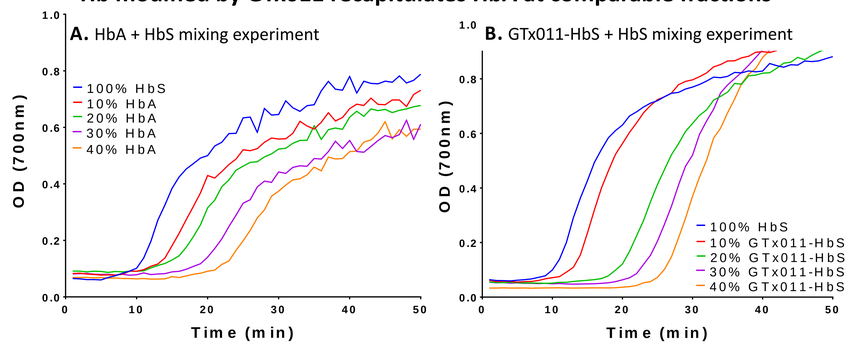
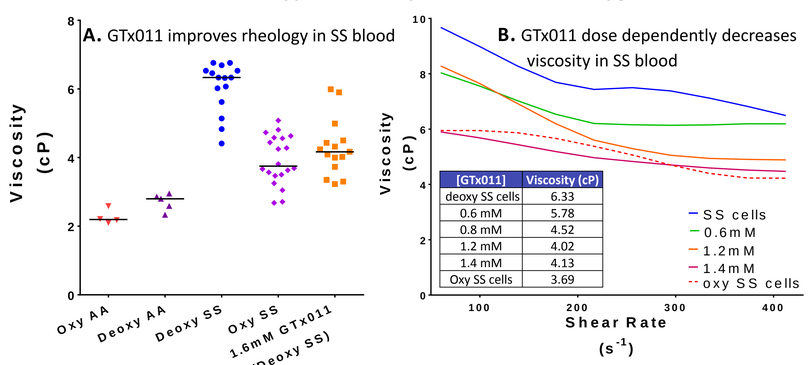


Figure 4. (A) HbA mixed at various ratios with HbS (final [Hb]=50 µM) in 1.8 M potassium phosphate buffer. The increasing concentrations of HbA delays the onset of polymerization. (B) GTx011 incubated with HbS was added to unmodified HbS at varying ratios. As the ratio of GTx011-HbS to HbS increases a delay in polymerization can be observed, similar to the delay observed with HbA.

GTx011 reduces the hyperviscosity observed in deoxygenated SS blood



[GTx011]	Viscosity (cP)
deoxy SS cells	6.33
0.6 mM	5.78
0.8 mM	4.52
1.2 mM	4.02
1.4 mM	4.13
Oxy SS cells	3.69

Figure 5. (A) Blood viscosity was determined for patient and donor samples under oxygenated and deoxygenated conditions. Hct was adjusted to 30%. GTx011 reduced the viscosity of the deoxygenated SS blood (n=15) when compared to untreated deoxygenated SS blood. (B) The viscosity of deoxygenated SS blood improves with increasing GTx011 concentrations; at higher doses GTx011 has a viscosity profile similar to oxygenated SS blood. (cP=centiPoise)

Conclusions

GTx011, an antisickling compound, has been shown to

- Left-shift the OEC curve, indicating a higher oxygen affinity than control Hb, RBCs and Blood.
- Maintain the oxyHb state under hypoxic conditions.
- Delay polymerization similar to HbA.
- Reduce the viscosity of deoxygenated SS blood.

GTx011 is anticipated to improve blood flow by increasing RBC deformability and reducing *in vivo* sickling events.

References

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Acknowledgements

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For more information on sickling and polymerization results of GTx011 please visit our Oral presentation tomorrow (#316)