Central Physiologic Mechanisms Which Augment O_2 Release (Bohr Effect and 2,3-DPG Binding) Are Preserved in the Presence of Voxelotor at the Therapeutic Target of 30% Hb Modification

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INTRODUCTION

- Voxelotor (formerly GBT440) is a small-molecule sickle hemoglobin (HbS) polymerization inhibitor being developed by GBT for the treatment of sickle cell disease (SCD).[1,2]
- The mechanism of action specifically targets the underlying mechanism of SCD.
  - Voxelotor increases hemoglobin-oxygen (Hb-O_2) affinity and stabilizes HbS in the oxygenated hemoglobin state (oxyHb), thereby reducing red blood cell sickling, leading to reduction in hemolysis and improvement in anemia.
- Key physiologic mechanisms that drive O_2 off-loading to tissues *in vivo* include the Bohr effect and 2,3-diphosphoglycerate (DPG) binding.[3]
  - The Bohr effect describes the capacity of hemoglobin (Hb) to off-load more O_2 in hypoxic peripheral tissues in response to increased H^+ concentration (acidosis).
  - H^+ ions bind to Hb and allosterically modulate the Hb-O_2 affinity by stabilizing the "T" structural state. This modulation of Hb affinity is critical to transporting and providing O_2 to tissues and organs.
  - Similarly, the binding of 2,3-DPG to Hb stabilizes the deoxygenated Hb state (deoxyHb), reduces Hb-O_2 affinity, and allosterically facilitates tissue O_2 release.
- Voxelotor dosing aims to reduce polymerization sufficiently to provide clinical benefit while allowing Hb to continue delivering O_2.

OBJECTIVE

- To investigate O_2 off-loading dynamics of voxelotor-modified Hb *in vitro* under increased acidity or increased DPG content

METHODS

Kinetics of O_2 Release in the Oxygen Dissociation Assay With Purified Hb

- The oxygen dissociation assay (ODA) takes advantage of the spectrophotometric difference between oxyHb and deoxyHb and can therefore be used to evaluate the oxyHb-to-deoxyHb transition under deoxygenated conditions.
  - The ODA quantitates the spectral shift from 415 nm (oxyHb) to 430 nm (deoxyHb) during deoxygenation and therefore gives information on the rate of Hb O_2 release.[4]
- Purified Hb (6 µM) was incubated for 1 hour at 37 °C with voxelotor (0, 2, or 6 µM) in 50 mM potassium phosphate buffer in 96-well half-area plates exposed to N_2 at 20 L/min for 2 hours.
  - Plates were analyzed with a SPECTROstar® Nano plate reader (BMG LABTECH), and visible spectra measurements were obtained every 6 min for 2 hours.
  - In experiments containing 2,3-DPG, the concentration of 2,3-DPG used was 1.2 mM.
- Area above the curve was determined by subtracting the area under the curve from the total graph area. These values were then normalized to the Hb control at pH 7.4.

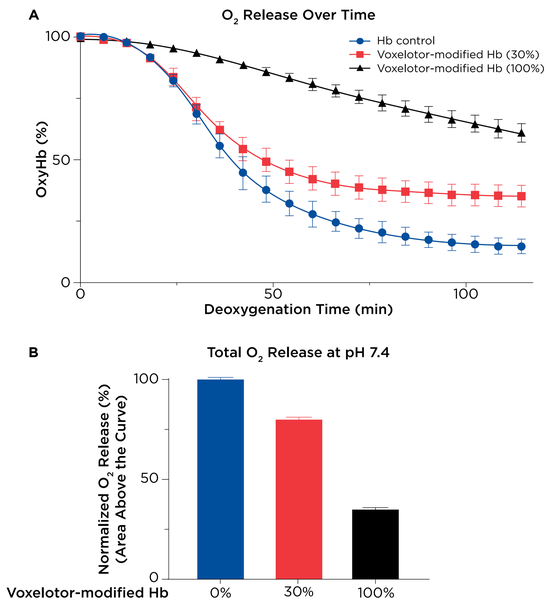
Measurements of Hb-O_2 Affinity in Purified Hb and Blood

- Oxygen equilibrium curves (OECs) were collected using a HEMOX analyzer (TCS Scientific).
- Whole blood samples (hematocrit, adjusted to 20%, ~1 mM Hb) were incubated for 1 hour at 37 °C in the absence or presence of voxelotor (300 µM) prior to analysis.[2]
  - After incubation, samples were then diluted into either pH 7.4 or pH 6.8 TES/saline buffer for OEC determination.

RESULTS

Voxelotor-Modified Hb Releases O_2

Figure 1

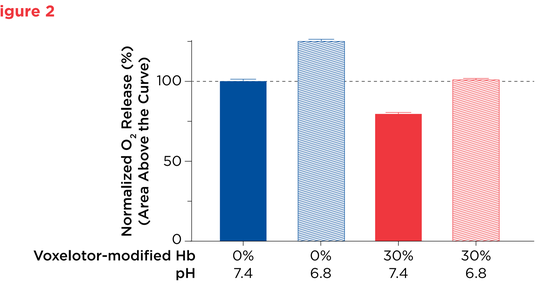


Hb, hemoglobin; oxyHb, oxygenated hemoglobin.

- The ODA was used as a model to study the kinetics of the oxyHb-to-deoxyHb transition.
- **Figure 1A** shows the percentage of oxyHb remaining as voxelotor-modified Hb is deoxygenated over time.
- The area above the curve from **Figure 1A** (Methods) is shown in **Figure 1B** and was used to calculate O_2 release.
  - These data demonstrate that at 30% Hb modification, there was a 20% reduction in O_2 release compared with unmodified Hb.
  - At 100% Hb modification, voxelotor-modified Hb was still able to release O_2.

Voxelotor-Modified Hb Responds to Increasing Acidity

Figure 2

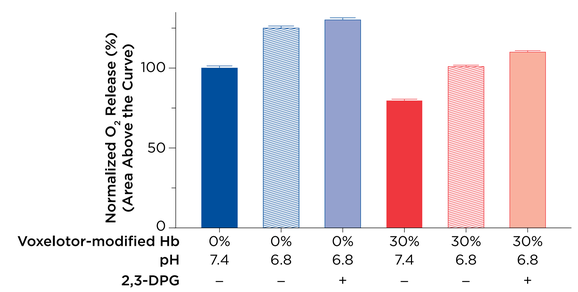


Hb, hemoglobin.

- Similar to unmodified Hb, voxelotor-modified Hb was able to respond to increasing acidity by releasing relatively more O_2 (**Figure 2**).
- Acidification at low pH normalized the O_2 release to be equivalent to that of unmodified Hb at pH 7.4 (dashed line).

Voxelotor-Modified Hb Responds to Multiple Allosteric Modulators

Figure 3

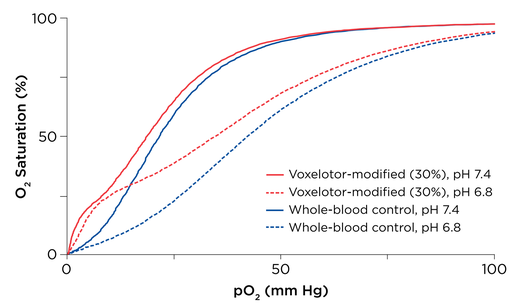


DPG, diphosphoglycerate; Hb, hemoglobin.

- As 2,3-DPG was added into a pH 6.8 buffer, further increase in O_2 release was observed (**Figure 3**).
- These data illustrate the additive effect of multiple allosteric modulators on both voxelotor-modified Hb and unmodified Hb.

Voxelotor-Modified Whole Blood Retains the Bohr Effect

Figure 4



pH	Whole-Blood Control (p50, mm Hg)	Voxelotor-Modified Blood (30%) (p50, mm Hg)
7.4	26	22
6.8	49	40

p50, oxygen tension to achieve 50% Hb saturation; pO_2, partial pressure of oxygen.

- Whole blood (20% hematocrit, ~1 mM Hb, and includes DPG) was incubated with 300 µM voxelotor at pH 6.8 and 7.4. Voxelotor-modified blood caused a leftward shift of the OECs relative to the control (**Figure 4**).
- In whole blood, there was a decrease in Hb-O_2 affinity with increasing acidity in the presence of voxelotor.[5]

DISCUSSION

Individuals With Hb Variants of High Hb-O_2 Affinity Are Asymptomatic

Table 1

Variant	% of Total Hb	Hb Level (g/dL)	% Bohr[a]	DPG Sensitivity
Cubujuqui	29	14.7	40	Some
Dallas	23	14.5	100	Normal
Kawachi	26	15.4	20	Some
HPFH	23	12.0	180	Minimal
Hb + voxelotor	38[b]	15.5[b]	50	Some

Table adapted from Bunn & Forget 1986.
[a]% Bohr is calculated as the ratio: [Δlog p50]/[ΔpH for Hb variant]/[Δlog p50]/[ΔpH for adult Hb].
[b]Data from phase 1 study in healthy volunteers.
DPG, diphosphoglycerate; Hb, hemoglobin; HPFH, hereditary persistence of fetal hemoglobin; p50, oxygen tension to achieve 50% Hb saturation.

- Individuals with heterozygous Hb (containing both adult Hb and high-affinity Hb mutations, expressing <50% of a high-affinity stable Hb variant) often do not have clinical symptoms, despite showing a diminished response to the Bohr effect or decreased 2,3-DPG binding capacity.[6–8]
- Fractional expression of stable high-affinity Hb mutants, with properties similar to those of voxelotor-modified Hb, showed no observable clinical manifestation (**Table 1**).
  - In the phase 1 voxelotor study (NCT02285088), healthy volunteers did not show changes in reticulocyte counts, erythropoietin levels, or hemodynamic responses at submaximal exercise.[1]

CONCLUSIONS

- **Voxelotor-modified Hb…**
  - Releases O_2
  - Releases more O_2 with increasing acidity (Bohr effect)
  - Responds to multiple allosteric modulators (H^+, 2,3-DPG).
- Voxelotor-modified blood shows a progressive decrease in Hb-O_2 affinity in conditions of increasing acidity.
- Heterogenous mixtures of high Hb-O_2 affinity variants can be clinically asymptomatic.
  - At therapeutic doses, the effect of voxelotor mimics that of heterogenous Hb mixtures.
- Sickle cell blood rapidly transitions to the T state, due to the abnormally high concentrations of 2,3-DPG and an exaggerated Bohr effect, further aggravating polymerization dynamics.[5] Therefore, voxelotor may provide additional benefit by blunting the effect of the excess 2,3-DPG and reducing the exaggerated Bohr effect.

Disclosures

- **Mira Pochron, Vincent Siu, Donna Oksenberg, Kobina Dufu**: Global Blood Therapeutics, employee and stockholder.
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- This study was sponsored by Global Blood Therapeutics.

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