Development of Potential Agents for the Treatment of Sickle Cell Anemia

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I. Introduction

Sickle cell anemia or sickle cell disease (SCD) is an inherited blood disorder that affects between 70,000 and 100,000 Americans.[1] This disease causes red blood cells (RBC) to become misshapen and therefore unable to function properly, due to a single point mutation in one of the subunits of the protein Hemoglobin (Hb) (structure of Hb in figure 1)[14] Hb is a quaternary allosteric protein consisting of four subunits-- α1, β1, α3, and β2, with a prosthetic heme group (containing iron) bound to each subunit.[2] In figure 1, the alpha subunits are labeled in green and cyan, while the beta subunits are labeled in dark blue and purple. The heme group is highlighted in grey. The normal function of Hb is to deliver oxygen via the heme group, from the lungs to the tissues. Hemoglobin accomplishes this by having multiple conformations that have either a high oxygen affinity (the relaxed, or R-state) or a low oxygen affinity (the tense, or T-state).[10] In normal patients, these two states exist in an equilibrium; Hb in the high-affinity R-state binds to oxygen in the lungs (oxygenated Hb), carries it to the tissues, then changes confirmation to the low-affinity T-state to release the oxygen to cells (deoxygenated Hb).[2] This equilibrium is maintained by allosteric effectors that regulate the transition from one state to another.[2]

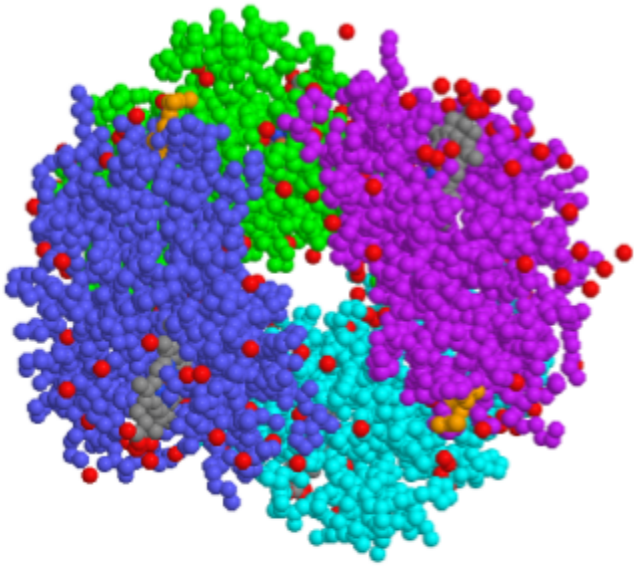


Figure 1: Structure of hemoglobin. Orange residues are the location of the sickle cell mutation. Heme groups are in grey. Alpha subunits in green and cyan, Beta subunits in dark blue and purple.
Figure from JMOL Protein Explorer, (PDB ID #1BZ0)

Sickle cell anemia is caused by a point mutation in one of the β subunits of Hb, turning the 6th amino acid in the β chain from a hydrophilic β-Glutamine to a non-polar β-Valine to form a sickle Hb (Hb S) (see figure 1, location of mutation highlighted in orange).[14] The mutations do not have an effect on the R-state of Hb S; however, in the T-state the exposed β-Val6 of Hb S makes hydrophobic contacts with adjacent Hb S molecules leading to polymerization, and eventually causing sickling of red blood cells.[2] Making the polymerization process worse is the fact that Hb S has a significantly low affinity for oxygen, and therefore Hb S easily and rapidly lose bound oxygen resulting in high concentration of deoxygenated Hb, which subsequently polymerize.[2] Sickle cell anemia can result in the obstruction of blood vessels by sickled red blood cells (vaso-occlusive crisis), stroke, chronic organ dysfunction, splenic sequestration (the trapping of sickled RBC in the spleen), and other complications.[14]

Current treatments for SCD include Hydroxyurea therapy, blood transfusions, and bone marrow transfusions.[14] As clinical symptoms of SCD are known to arise after levels of fetal Hb (HbF) drop, hydroxyurea is known to resynthesize HbF in adults.[14] Furthermore, hydroxyurea is also known to cause a reduction in the expression of cell adhesion molecules that promote vaso-occlusive crisis.[14] However, hydroxyurea has been reported to be toxic, especially toward younger patents, and the Food & Drug Administration (FDA) has only approved the treatment for adult patients.[14] Blood transfusions have been proven to alleviate clinical complications associated with SCD.[14] However, this form of therapy is dangerous as the patients may not react positively toward the donated blood.[14] Furthermore, RBC received through transfusion are ultimately metabolized at the end of their life cycle, necessitating a new transfusion.[14] Blood marrow transfusion (BMT) appears to be the only potential cure for SCD.[14] However, this treatment is still being researched, and donors for SCD patients are very hard to match.[14] It is obvious that a different approaches must be explored to treat SCD.

To prevent the rapid and premature release of oxygen and consequent Hb S polymerization of RBC, a drug can be designed to increase the oxygen affinity of sickle Hb. There are natural allosteric inhibitors of Hb, such as 2,3-bisphosphoglycerate (2,3-BPG), which bind to the β-cleft of Hb (see Figure 2); and ties together the two β-subunits, to stabilize the T-state, and consequently lowering the oxygen affinity of Hb.[2] There have been multiple studies that propose the use of allosteric effectors to increase the oxygen affinity of Hb to prevent RBC sickling.[4] Aromatic aldehydes are the main antisickling candidates, as they can form a Schiff-base interaction with the free N-terminal αVal1 amine to further stabilize the R-state Hb and increase the Hb affinity for oxygen.[9]

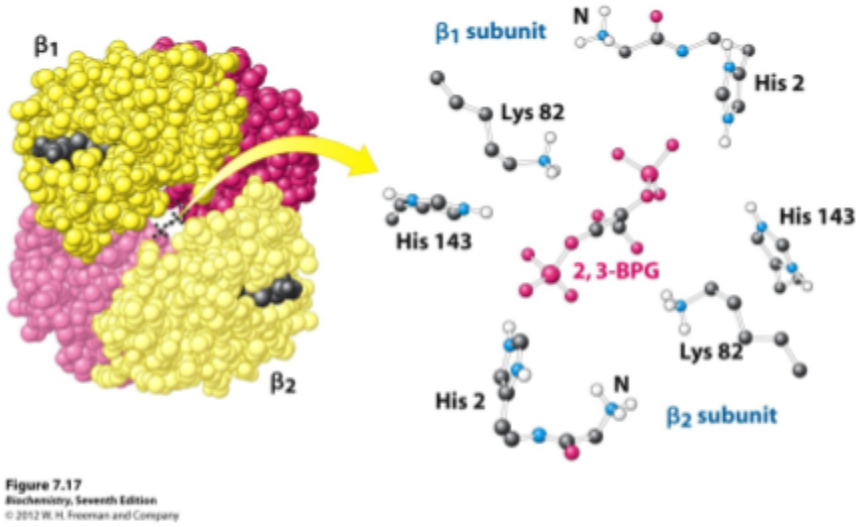


Figure 2: Binding of 2,3-BPG to Hemoglobin
Image from: http://oregonstate.edu/instruct/bb450/fall14/stryer7/7/figure_07_17.jpg

Vanillin was one of the first allosteric effectors studied as an antisickling agent for the treatment of SCD.[3] Although vanillin was tested and had the ability to elicit *in vitro* antisickling effects, it was non-bioavailable *in vivo* and therefore non-clinically viable.[2,3] Since then there have been more allosteric binding compounds that bind to Hb and stabilize the R-state to increase Hb affinity for oxygen, including 5-hydroxymethyl-2-furfural (5-HMF).[5] This compound is

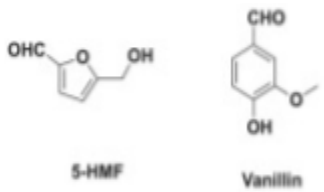


Figure 3: Structures of 5-HMF and Vanillin
(Image from Pegare PP, et al. (2018))

one of several that has undergone clinical studies as it has a high antisickling potency; however 5-HMF undergoes extensive metabolism of the aldehyde moiety, scheme shown in figure 4, which leads to a short half-life and a low bioavailability.[5] Therefore, the pharmacologic effect of 5-HMF is reduced and phase II clinical trials were ended.[4,5]

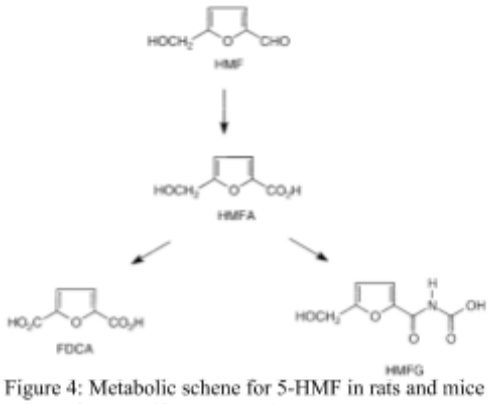
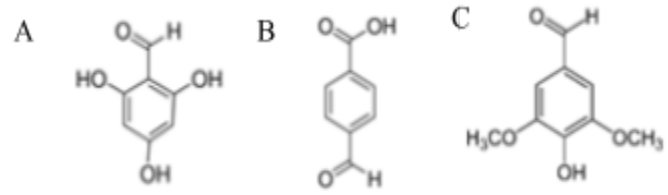


Figure 4: Metabolic schene for 5-HMF in rats and mice (Figure from Godfrey VB, et al. (2010)

This study seeks to investigate the possible antisickling properties of five aromatic aldehydes (pictured in figure 5).



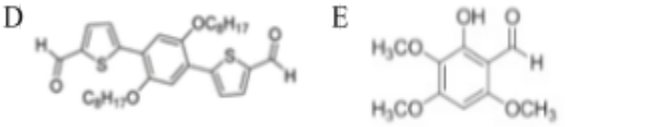


Figure 5: Proposted Allosteric Effectors
(A) Figure from Maton A, et al. (2016)
(B) Figure from Cheng B, et al. (2017)
(C) Figure from Kamimura N, et al. (2017)
(D) Figure from Geng Y, et al. (2015)
(E) Figure from Salae AW, et al. (2017)

II. Oxygen Equilibrium Study

The aim of this experiment is to study the P_{50} (partial pressure at which 50% of the Hb is bound to oxygen in the blood. In figure 6, the normal oxygen dissociation curve is denoted in red; the normal P_{50} values are denoted by dotted lines. The blue lines in figure 6 show the left or right-shift of the oxygen dissociation curve, usually influenced by the binding of effectors. If the

effector binds to Hb and promotes its affinity to oxygen (shifting the oxygen equilibrium to the left), the P_{50} will increase; if the effector decreases the affinity to oxygen (shifting the oxygen equilibrium to the right), the P_{50} will decrease.

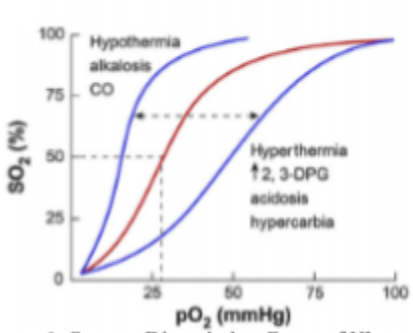


Figure 6: Oxygen Dissociation Curve of Hb. The normal P50 value is indicated by dashed lines. The normal oxygen equilibrium curve is denoted in red, the left and right shift curves are denoted in blue.
(Figure from Safo M, Kato G (2014))

Part A: Formation of Hb-effector complexes in normal whole blood

To measure the P_{50} of the proposed aromatic aldehydes (structures in figure 5), first the Hb-aldehyde adducts need to be formed. Reaction of the aromatic aldehyde compounds with Hb A (normal Hb) will follow the procedures of Boyiri T, et al. (1995).

The Hb-aldehyde adducts will be formed by first dissolving the proposed aromatic aldehyde effectors and Hb A in buffer with a pH of 7.4 (blood pH). The Hb (in a 5.3 mM solution with buffer) and proposed effectors (in a 20 mM solution with buffer) will be mixed in a 1:1 ratio to give a final concentrations of 2.7 and 10 mM respectively. Therefore there will be about a molar ratio of effector to Hb of approximately 4:1. As Randad RS, et al. (1991) explains, this ratio of effector to Hb is designed to optimize the P_{50}, as the shift in the P_{50} is dependent on the effector/Hb ratio. The Hb molarity used in the experiment is intended simulate the RBC Hb concentration. A control solution will also be set up, with equal parts Hb and buffer. These mixtures will then be incubated at room temperature for 30 minutes.

Part B: Determination of P_{50}

Oxygen dissociation curves will be run and recorded on an Aminco Hem-O-Scan oxygen dissociation analyzer from Travenol Laboratories, as described in Randad RS, et al. (1991).

III. Discussion

The P_{50} value of an effector bound to Hb can tell us its potential antisickling activity. An effector bound to Hb high antisickling activity will have a high oxygen affinity (i.e. promote transition into the R-state of Hb), and therefore will produce a large left shift in the P_{50} value. We can express the activity of the compound by calculating the ratio of P_{50} of the Hb-effector complex divided by the P_{50} of the control solution.[13] Furthermore, the percent of change in the P_{50} can be calculated using the following equation:[6]

$$\Delta P_{50}\,(\%) = \frac{P_{50}\ of\ lysates\ from\ untreated\ cells - P_{50}\ of\ lysates\ from\ treated\ cells}{P_{50}\ of\ lysates\ from\ untreated\ cells} x\ 100$$

The proposed aromatic aldehyde effectors (in figure 5) are hypothesized to have antisickling properties. If these effectors can produce a strong right-shift in the P_{50}, further studies must be done to test the potency of the effectors. A measurement of time and concentration dependent adduct formation can help study the potency of the effectors i.e. the amount of time they form Hb-effector complexes before metabolism and the concentration at which Hb-effector complex formation is optimal. Furthermore, to study the binding of the Hb-effector complex, X-ray crystallographic studies must be done. If these effector compounds are concluded to be viable as potential agents for use as antisickling drugs, these compounds can go onto *in vivo* pharmacokinetic/pharmacodynamic (PK/PD) studies to observe their *in vivo* antisickling properties.

The development of a new drug for SCD is crucial, as the population suffering from the disease is high, and no proper treatment exists yet. Although it is possible the aromatic aldehyde effectors proposed in this paper may have low antisickling properties, low potency *in vivo*, or high rates of metabolism; this school of thought-- development of antisickling aromatic aldehyde effectors to treat SCD-- is extremely important in treating sickle cell disease patients all over the world.

IV. References

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