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Shifts in affinity and enthalpy of oxygen binding to human hemoglobin A induced by pyridoxal and pyridoxal 5′-phosphate

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A therapeutic agent capable of inhibiting the polymerization of hemoglobin S would relieve the symptoms of sickle cell disease. The P_{50} of oxygen binding to hemoglobin A (HbA) in the presence of an effector has been used for the initial screening of potential antisickling agents. We evaluated the thermodynamic parameters, P_{50}, ΔG, ΔH, and ΔS and Hill coefficient for oxygen binding to human HbA_o (HbA stripped of all endogenous effectors) in the presence of two antisickling agents, pyridoxal (PL) and pyridoxal 5′-phosphate (PLP), and two sickling-enhancing agents, 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate (IHP). The P_{50} of oxygen binding to HbA_o in phosphate/EDTA buffer, pH 7.6, was found to be 560 Pa. The P_{50} was increased to 653 Pa and 1746 Pa by DPG and IHP, respectively. The antisickling agents, PLP, and PL, had opposite effects on the P_{50}, shifting it to 840 Pa and 187 Pa, respectively, indicating that a decrease in P_{50} may not be the best predictor of an antisickling agent. The Hill coefficient was 2.9 for both HbA_o and HbA_o + DPG and 2.6 for HbA_o + IHP. The Hill coefficient was reduced by both PLP to 2.3 and by PL to 2.1. The enthalpies for oxygen binding to HbA_o, HbA_o + DPG, and HbA_o + IHP were −48.1 kcal/mol Hb, −48.0 kcal/mol Hb, and −42.2 kcal/mol Hb, respectively. In contrast, the enthalpies for HbA_o + PLP and HbA_o + PL were −57.0 kcal/mol Hb and −51.1 kcal/mol Hb. Our study suggests that decreases in cooperativity and enthalpy, alone or in combination, may be useful indices of potential antisickling agents. © Elsevier Science Inc. 1997 (J. Nutr. Biochem. 8:19–24, 1997.)

Keywords: antisickling agents; pyridoxal; pyridoxal 5′-phosphate; oxygen-binding to HbA_o; sickling enhancing agents

Introduction

The binding of oxygen to hemoglobin (Hb) is a complex process that is influenced by the genetically determined form of the Hb molecule and the concentration of allosteric effectors in the erythrocyte.[1,2] The normally occurring adult form of Hb, HbA, is a tetramer composed of two identical α-globin chains and two identical β-globin chains. Each globin chain contains one heme molecule that is capable of binding one oxygen molecule. Binding of oxygen to Hb is a cooperative process in which the binding of one oxygen molecule facilitates the binding of the others. In patients wtih sickle cell disease (SCD), HbS (in which valine is substituted for glutamic acid in the sixth position of the β chains) replaces HbA in the erythrocyte.[1] Hemoglobin S has less affinity for oxygen than HbA, and in the deoxy form, HbS is much less soluble than HbA. The low solubility of deoxyHbS and high concentration of Hb in the eythrocyte allows for progressive polymerization and formation of deoxyHbS fibers, which ultimately cause the erythrocytes to take on the characteristic sickled shape.[3] In the fetus, HbF (in which two identical γ chains replace the β chains) is the predominant form of Hb. Fetal Hb has a greater affinity for oxygen than HbA, facilitating the transfer of oxygen from mother to fetus.

Several naturally occurring allosteric effectors of oxygen binding to Hb are found in erythrocytes.[4] These include

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2,3-diphosphoglycerate (DPG), inositol hexaphosphate (IHP), and two major forms of vitamin B-6; pyridoxal (PL) and pyridoxal 5′-phosphate (PLP). Both DPG and IHP bind to a positively charged cavity in deoxyHb, resulting in stabilization of the deoxy form of Hb and a decrease in the affinity of HbA for oxygen. In a manner similar to DPG, PLP binds to the deoxyHb pocket (competitively with DPG) and reduces the affinity of oxygen for hb.[6,7] In contrast, PL has been reported to increase the affinity of oxygen binding to Hb,[8,9] presumably by binding to the N-terminus of the α chains of oxyHb, stabilizing the oxy- form of Hb.[10,11]

These studies suggest that the concentration of PL and PLP in the erythrocyte may have significant effects on the oxygen-carrying capacity of the cells. The effects of these endogenous effectors on oxygen-Hb binding parameters may have importance in SCD. It has been hypothesized that antisickling agents act by reducing the P_{50} (the partial pressure at which half of the sites are occupied) of oxygen binding to Hb, whereas agents that exacerbate sickling increase the P_{50} of oxygen binding to Hb. Previous studies suggest that DPG and IHP, which decrease the oxygen P_{50}, act as sickling-enhancing agents,[12,13] whereas PLP and PL have been reported to have antisickling characteristics.[8,9,14]

In previous optical binding studies there was one potentially confounding technical difficulty. Because of its intense absorption in the visible region of the spectrum, low concentrations of Hb (50 μmol/L or less) were used. The concentration of Hb in erythrocytes is nearly 100 times greater, 5 mmol/L. The concentration of Hb used in binding studies is an important variable because at concentrations below 600 μmol/L, dissociation of Hb tetramers to dimers occurs to a significant extent,[15,16] and the shape of the oxygen binding curves of Hb dimers differ markedly from the tetramers.[17] Thus, published values obtained wtih low Hb concentrations in which it is likely that a significant portion of the total Hb was in the form of dimers, are likely to differ from those obtained with concentrations of Hb above 1 mmol/L in which the proportion of dimers is negligible.[15,16] For this study we have determined the parameters of oxygen binding to HbA_0 in the presence of DPG, IHP, PLP, and PL with high concentrations of Hb. Because of the general binding characteristics of HbS and HbA are very similar,[10,11] the P_{50} of oxygen binding to HbA_0 in the presence of an effector has been used as an initial screen of potential antisickling agents.[10] Our results suggest that enthalpy change or reduction in Hill coefficient may be a more reliable index of a potential antisickling agent than the change of P_{50} for oxygen binding alone.

Methods and materials

Preparation of HbA_o

All reagents except Hb were obtained from Sigma Chemical Corporation (St. Louis, MO USA). Hemoglobin A_0 was prepared from freshly drawn, EDTA anticoagulated blood.[18] The Hb was stripped of organic phosphates, dialyzed against 0.2 mol/L potassium phosphate, 1 mmol/L Na_2EDTA buffer, pH 7.6 (phosphate/EDTA). The dialyzed hemoglobin was adjusted to a concentration of approximately 2.5 mmol/L and stored as pellets in liquid nitrogen until used. Potassium phosphate buffer was used throughout because its relatively low enthalpy of protination (–0.8 kcal/mol)[19] is advantageous for calorimetric studies. The heterotopic effectors DPG, IHP, PLP, and PL were prepared as 10 mmol/L stock solutions in phosphate/EDTA. The final solution conditions of HbA_0 (1.0–1.25 mmol/L), 5 mmol/L effector, and phosphate/EDTA were obtained by volumetric dilution of the stock HbA_0 solution with an equal volume of stock effector solution. An enzymatic reducing system[20] was employed to decrease the amount of oxidized hemoglobin to less than 1% before and during the course of each experiment. Hemoglobin concentrations were determined using a precisely calibrated (0.0101 cm) thin layer optical cell in a Cary 210 spectrophotometer. The Hb spectra and published extinction coefficients[6,21] were used to calculate the concentration of oxyHb, deoxyHb hemoglobin, and methemoglobin.

Optical binding curves of HbA_o in the presence of effectors

Optical binding data were obtained using the Gill thin-layer optical cell and valve[17] that was fitted to the sample compartment of a Cary 219 spectrophotometer. A concentrated Hb solution was suspended between a glass window and an optically transparent, gas-permeable membrane by a stainless steel shim defining the optical path length (typically 0.005 cm). The gas-permeable membrane was in direct contact with the gas reservoir of a stainless steel cell, connected to a precision gas dilution valve. The apparatus was thermostated at 15 ± 0.01°C by a Lauda circulating water bath and monitored by a quartz thermometer fitted to the copper cooling plates of the apparatus.

Initially, Hb-effector solutions were placed in the cell and equilibrated by flushing with buffer-equilibrated oxygen for 15 min. Oxygen binding data were measured as a decrease in optical density of the Hb solution at 576 nm due to the displacement of oxygen by stepwise addition of nitrogen through the dilution valve. The partial pressure of oxygen after each dilution step, n, is given by the relationship:

$$P_n = P_O D^n \qquad \text{(Equation 1)},$$

where P_n is the partial pressure of oxygen at dilution step n, P_O is the partial pressure of oxygen at the beginning of the experiment, and D is the dilution factor of the valve. The dilution factor of the valve was determined for each experiment by least squares analysis of oxygen partial pressure (Clark oxygen electrode YSI 5331, Yellow Springs Inc., Yellow Springs, OH, USA) versus dilution step.

The changes in optical density (ΔOD_i) versus oxygen partial pressure (P_i) at each step were used to determine the equilibrium constants in the Adair formalism:

$$Hb + iO_2 \rightleftharpoons Hb(O_2)_i = \beta_i \qquad \text{(Equation 2)},$$

where β_i is the overall equilibrium constant for each step i for the four ligated forms of Hb (i = 1 through 4). The oxygen-binding system was expressed in terms of the various ligated populations by the binding polynomial, P, which, in terms of β_i is given by:[17]

$$P = 1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4 \qquad \text{(Equation 3)}.$$

The fractional saturation, θ, is defined as the sum of ligated molecules per macromolecule relative to the number of binding sites, and was expressed in terms of the equilibrium constants and ligand activity:

$$\theta = \frac{(\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3 + 4\beta_4 x^4)}{(4(1 + \beta x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4))} \qquad \text{(Equation 4)}.$$

These experiments give optical binding curves as a function of oxygen partial pressure. The theoretical reaction absorbencies (A) are given by:

$$(\Delta A)^{theor} = (\theta_i - \theta_{i-1})\Delta A_T \qquad \text{(Equation 5)},$$

where ΔA_T is the change of absorbance from the completely unligated state to the fully ligated states. ΔA^{theor} is the calculated absorbance change corresponding to the geometric mean of the partial pressures x_i and $x_{(i-1)}$. The binding parameters were estimated by least squares optimization using the Marquardt-Levenburg algorithm[23] in which the experimentally measured absorbance changes were compared with the theoretically calculated absorbance changes (Equation 5). In this model, it is assumed that there were only two molar extinction coefficients, ligated and unligated, for each subunit; and thus, it was represented as a linear system.

For this study we measured P_m, but the data have also been converted to P_{50} to facilitate comparison with earlier studies. The P_m is a thermodynamic parameter which is defined as the partial pressure at which half of the work of saturation is completed; whereas P_{50} is a kinetic parameter that is defined as the partial pressure at which half of the sites are ligated. The P_m is equal to the P_{50} when the saturation curve is symmetrical.

Enthalpy of Oxygen Binding to HbA_o

Enthalpy values were obtained using the thin-layer gas-solution microcalorimeter described previously.[24,25] A dilution valve at the top of the calorimeter, similar to the one described in the thin layer apparatus, allows for successive additions of gaseous ligands. The calorimeter was immersed in a water bath regulated (Tronac, Orem, UT USA) to a temperature deviation of less than ±0.0003°C. The instrument was calibrated electrically by determining the heats obtained by inserting a nominal 100-Ω resistor wire wrapped around a thin-walled copper dowel into the cell.

A 50 μL aliquot of Hb solution (approximately 1.2 mmol/L, containing the reducing system and effectors) was placed on a 1-cm square of Whatman No. 1 filter paper in a modified 2.5 cm × 7 mm NMR tube.[25] The modified NMR tube in the reference cell contained filter paper with a 50 μL aliquot of buffer, effector and enzymatic reducing system. The system was flushed with buffered equilibrated nitrogen until thermal and vapor equilibrium were established. Oxygen was then introduced into the reaction cell. The heat effect of the dissolution of gas was nullified by the reference cell.

Results

Oxygen-binding curves

Figure 1 shows the oxygen-binding curves for HbA_o and HbA_o in the presence of 5 mmol/L PL, 5 mmol/L PLP, 5 mmol/L DPG and 5 mmol/L IHP, as well as that for a reference standard, human myoglobin. All oxygen binding studies were measured with 1 mmol/L HbA_o in phosphate/EDTA, pH 7.6, at 15°C. The data for myoglobin were calculated from published measurements.[26] The oxygen P_m values determined from the curves in *Figure 1* are shown in *Table 1*. The P_m for HbA_o was found to be 533 Pa. In agreement with a previous study,[27] the P_m for oxygen binding were increased by 5 mmol/L concentrations of DPG and IHP to 600 Pa and 1583 Pa, respectively. The two B-6 vitamers were found to have opposite effects on the P_m. The oxygen-binding curve for HbA_o was shifted slightly to the right by PLP (P_m = 733 Pa), whereas that for PL was markedly left shifted (P_m = 187 Pa).

The cooperativity of oxygen binding to HbA_o is reflected by the shape of curve and is quantified by the maximum Hill coefficient, n_{max}. The calculated n_{max} for HbA_o for each of the conditions shown in *Figure 1* is reported in *Table 1*. The n_{max} for both HbA_o and HbA_o with DPG was 2.9. All of the other effectors, IHP, PLP, and PL, caused a decrease in cooperativity as indicated by the decreased n_{max} values.

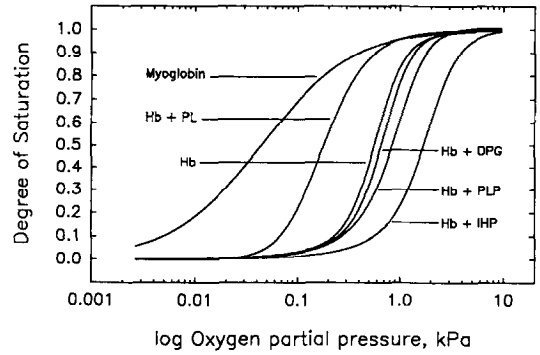

Figure 1 Oxygen-binding curves for HbA_o in the absence and presence of effectors. Measurements were made with 1.2 mmol/L HbA in 0.2 mol/L potassium phosphate, pH 7.6 at 15°. The binding curves plotted for HbA and for HbA plus effectors (5 mmol/L) are the average of three separate determinations. Values for human myoglobin were calculated from published measurements.[26]

Enthalpy

The overall enthalpies (ΔH) for oxygen binding to HbA_o in the presence of effectors are reported in *Table 2*. The ΔH for the binding of oxygen to HbA_o was −48.1 kcal/mol Hb. The ΔH for HbA_o + DPG was unchanged (−48.0 kcal/mol Hb) and that for binding HbA_o + IHP was increased to −42.2 kcal/mol Hb. Both PLP and PL reduced the ΔH for the binding of oxygen to HbA_o to −57.0 kcal/mol Hb and −51.1 Kcal/mol Hb, respectively. Although this study is limited to four effectors, these results suggest that antisickling agents tend to decrease the ΔH of oxygen binding to HbA_o regardless of their opposite effects on P_m, whereas those that enhance sickling have similar (DPG) or increased (IHP) ΔHs. This suggests that the effect of an agent on the ΔH of oxygen binding may be a better index for identifying effectors with potential antisickling properties than P_{50} or P_m alone.

Calculated entropies of oxygen binding

Table 2 lists the free energy (ΔG) and entropy (ΔS) changes accompanying the binding of oxygen to deoxyHbA_o to pro-

Table 1 Calculated parameters from oxygen-binding curves

Hb and effector	P_m (Pa)	P_{50} (Pa)	n_{max}
HbA_o + IHP	1583 ± 39	1746	2.6
HbA_o + DPG	600 ± 11	653	2.9
HbA_o	533 ± 7	560	2.9
HbA_o + PLP	733 ± 27	840	2.3
HbA_o + PL	187 ± 5	187	2.1

The binding parameters, ± standard errors, were calculated from the oxygen-hemoglobin binding curves shown in *Figure 1* as described under "Methods and materials." Concentrations of effectors were 5 mmol/L.

Table 2 Thermodynamic parameters of HbA_o and HbA_o with effectors

Hb and effector	ΔG Kcal/mol Hb	ΔH Kcal/mol Hb	ΔS cal/(mol Hb*K)	Description
HbA_o + IHP	−24.2	−42.2	−62.5	enhances sickling
HbA_o + DPG	−26.6	−48.0	−74.3	enhances sickling
HbA_o	−26.6	−48.1	−73.6	
HbA_o + PLP	−26.1	−57.0	−107.2	antisickling
HbA_o + PL	−29.2	−51.1	−76.0	antisickling

The thermodynamic parameters were calculated from the binding curves shown in *Figure 1* as described under "Methods and materials." Concentrations of effectors was 5 mmol/L.

duce oxyHbA_o. The free energies were calculated from the relationship $\Delta G = 4RT\ln P_m$; the entropies were calculated from the relationship $\Delta G = \Delta H - T\Delta S$. The ΔS for oxygen binding to HbA_o, HbA_o + DPG and HbA_o + PL were all similar: −73.6 cal/(mol Hb*K), −74.3 cal/(mol Hb*K) and −76 cal/(mol Hb*K), respectively. The ΔS for oxygen binding to HbA + PLP was −107.2 cal/(mol Hb*K), whereas that for HbA + IHP was −62.5 cal/(mol Hb*K).

Discussion

Over 80,000 Americans and millions of people worldwide have SCD. These people undergo intermittent, often severe crises characterized by localized pain in the joints, back, chest, and abdomen. These painful crises are caused by capillary occlusion and localized ischemia produced when erythrocytes change from normal flexible cells to rigid sickled-shaped cells. The deformation is caused by the polymerization of HbS at low oxygen tensions. In the deoxy state, polymerization is favored due to the relatively low solubility of deoxyHbS_1. One approach to prevent sickling is to increase the oxygen binding affinity of HbS, reduce the proportion of insoluble deoxyHbS, and decrease the likelihood of polymerization.[3] Thus, the ability of an agent to increase the oxygen affinity of HbA_o, as indicated by a decrease in P_{50}, has been used as an initial screening process to identify potential antisickling drugs.[2,10] The best candidates would be those that increase oxygen binding most. However, oxygen transfer from erythrocytes to tissues is accomplished because myoglobin has a higher affinity for oxygen than does hemoglobin, and one potential disadvantage of increasing the oxygen binding affinity of Hb may be to compromise the efficiency of oxygen transport to tissue.[3]

The hypothesis that sickling-enhancing agents increase the affinity for oxygen was supported by our findings that DPG and IHP increased the oxygen P_{50} from 560 to 653 and 1746 Pa, respectively (*Table 1*). The second part of the hypothesis, that antisickling agents should decrease the P_{50}, was not supported by our findings. PL and PLP had opposite effects on the oxygen P_{50}: PL caused a marked decrease in the P_{50} for HbA_o from 560 to 187 Pa, whereas PLP increased the P_{50} to 840 Pa. These results are inconsistent with the hypothesis that antisickling agents should decrease the P_{50}. Our results for PL are in disagreement with studies by Benesch and coworkers[11,28] who reported that the HbA oxygen-binding curve was shifted to the left only slightly by PL (from 473 to 462 Pa). This inconsistency is most likely due to differences in experimental conditions, especially that their measurements were made with low HbA_o concentrations (50 μmol/L); whereas, ours were performed at higher, presumably more physiological, concentrations of Hb (1.2 mmol/L). Our findings of opposite effects on the oxygen P_{50} induced by PL and PLP are, however, supported by studies by Kark and coworkers,[8,9] who reported similar results for oxygen binding in hemolysate samples. Based on these findings, we suggest that the shift in P_{50} (or P_m) alone is not sufficient to predict the ability of a compound to act as an antisickling agent.

PLP induces a shift-to-the-right of the oxygen/Hb binding curve, as does DPG. These effectors share common, mutually exclusive binding sites, along with other sites unique to each.[29,30] Both DPG and PLP bind to the positively charged cavity in deoxyHb, and both bind to the N-terminus of one of the β chains of deoxyHbA. DPG is held by three salt bridges to Val 1β and His 2β of one β chain and to His 143β of the opposite β chain. The binding of PLP is through a Schiff base to Val 1β and three salt bridges to Lys 82β of one β chain, and His 143β and His 146β of the opposite chain.[29,30] In the oxyHbA conformation, both compounds bind to the N-terminus of one of the α chains. The equilibrium-binding constant of PLP, which interacts through a Schiff base, is more than an order of magnitude stronger than that for DPG, which binds to the N-terminus through electrostatic interactions only. It is likely that the shift-to-the-right of the oxygen/Hb curve common to both compounds results from interactions with binding sites that they share; whereas, their opposite effects on polymerization of deoxyHbS results from their unique sites of interaction.

The difference is the effect of PL and PLP on the oxygen binding curve is likely to be due to the differences in interaction of the two vitamers with Hb. The shift-to-the-left of the oxygen/Hb binding curve in the presence of PL is believed to be caused by the binding of PL to the N terminus of the α chain of oxyHbA.[8] The resulting, enhanced stability of the oxy- conformation has been reported for other agents that bind at this site.[5,10] The shift-to-the-right of the oxygen/Hb binding curve induced by PLP is believed to result from interaction with the N terminus of the β chain of the deoxy- conformation.[29] Whether PL-Hb and PLP-Hb complexes prevent sickling by a common mechanism remains unknown, but it does not appear to be due to an increase in oxygen affinity alone.

The structural transition of deoxyHb to oxyHb is respon-

sible for Hb cooperativity, and a reduction of the n_{max} suggests an attenuation of this structural change. All of the effectors except DPG reduced the cooperativity of oxygen binding to HbA_o. The B-5 vitamers reduced the n_{max} more than DPG or IHP. The n_{max} was reduced from 2.9 to 2.1 and 2.3 by PL and PLP, respectively; whereas, the n_{max} was found to be 2.9 and 2.5 for DPG and IHP, respectively. The n_{max} occurred at oxygen saturation greater than 50% for all effectors except PL, for which the n_{max} occurred below 50% oxygen saturation. These data suggest that the cooperativity of oxygen binding to Hb in the presence of an effector may be a more useful guide for identifying potential antisickling agents than is the P_{50}. However, further functional and structural studies are needed to determine the significance of the decrease of the n_{max} for these effectors.

The enthalpies of reaction of oxygen binding to HbA_o in the presence of each of the effectors were determined by thin-layer gas-solution microcalorimetry. There are several advantages to measuring the enthalpies of ligand binding to Hb directly, rather than by calculation from the van't Hoff relationship. The free energies for binding in the van't Hoff relationship are often calculated from an optical response to binding of a ligand to a macromolecule. This requires the assumptions that the heat capacity of the reaction associated with ligand binding is zero, and that the optical response is independent of temperature, both of which may be violated. Aside from the simplicity of measurement, direct calorimetry does not require any additional assumptions.

Both PL and PLP decreased the ΔH, IHP increased it, and DPG did not affect the ΔH. This suggests that a decrease in ΔH may be an additional useful index for identifying effectors with potential antisickling properties. Further studies including the screening of additional effectors and stepwise enthalpy measurements are planned to determine if the decrease in enthalpy reported here is a general property associated with the mechanism of action of B-6 compounds.

These results may have clinical relevance. Investigations of patients with sickle cell disease have identified a variety of nutritional abnormalities, including vitamin B-6.[31,32] Physiological improvements have been achieved through specific nutritional supplements which may improve the quality of life for many patients.[31] For example, when supplemented with 100 mg vitamin B-6 per day in the form of pyridoxine-HCl, a 10 fold increase in erythrocyte PLP concentrations resulted. In one of five patients, there was a dramatic reduction in severity, frequency, and duration of painful crises.[32] Although beneficial effects from vitamin B-6 supplementation appear to be significant, the mechanism by which it acts remains undetermined.

In summary, the efforts for developing therapeutic antisickling agents have involved methods to increase the affinity of oxygen binding to Hb. However, a potential disadvantage of modifying Hb for higher oxygen binding may be an adverse effect on oxygen transport to tissues. Our studies indicate that increasing oxygen binding affinity of Hb may not be a necessary requirement for an antisickling agent, and that a decrease in ΔH for oxygen binding to Hb, accompanied by a large reduction in cooperativity, may be better indices. We suggest that instead of a single index of oxygen-binding affinity, a thorough description of the physical properties of oxygen binding to HbA_o and HbS in the presence of potential effectors may aid the development of effective antisickling drugs.

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