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Binding of Dihydroartemisinin to Hemoglobin H: Role in Drug Accumulation and Host-Induced Antimalarial Ineffectiveness of α-Thalassemic Erythrocytes

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ABSTRACT

Dihydroartemisinin and other artemisinin derivatives are relatively ineffective against *Plasmodium falciparum* infecting α-thalassemic erythrocytes, namely hemoglobin (Hb) H or HbH/Hb Constant Spring erythrocytes, as compared with those infecting genetically normal erythrocytes. The variant erythrocytes accumulate radiolabeled dihydroartemisinin to a much higher extent than the normal ones, and the accumulated drug was retained after extensive washing, in contrast to the drug in normal erythrocytes which was mostly removed. At initial drug concentration of 1 mM, most (82–88%) of the drug was found in the cytosol fraction of both variant and normal erythrocytes. Binding of the drug to hemoglobins accounted for 40–70% of the total uptake. Hb H accounted for 10.9 ± 2.7% and 12.4 ± 6.2% of total protein in HbH and HbH/Hb Constant Spring erythrocytes. HbH bound with 28.7 ± 6.7% of the drug, whereas HbH/Hb Constant Spring erythrocytes bound with 21.8 ± 8.3% of the drug. Binding experiments showed that Hb H had 5–7 times the drug-binding capacity of Hb A. For Hb H, the maximum binding capacity (B_{max}) = 1.67 ± 0.17 mol/mol Hb, and the dissociation constant (K_d) = 66 ± 17 μM, and for Hb A, B_{max} = 0.74 ± 0.18 mol/mol Hb and K_d = 224 ± 15 μM. It is concluded that preferential binding of dihydroartemisinin to Hb H over Hb A accounts partly for the higher accumulation capacity of the α-thalassemic erythrocytes, which leads to its antimalarial ineffectiveness.

The artemisinins form a group of antimalarials derived from *Artemisia annua*, an herbal plant long used in China for the treatment of fevers (United Nations Development Program *et al.*, 1997; Klayman, 1993). They are sesquiterpenoids with an endoperoxide essential for antimalarial activity. DHART is more active than artemisinin against *Plasmodium falciparum*, and is probably the metabolically active form of the derivatives already in use or in advanced stages of development. With the threat of multidrug-resistant malaria on the rise, the artemisinins, which have proven to be effective against parasites resistant to chloroquine and other drugs, will be playing an increasing role in antimalarial chemotherapy. Although no resistance to these drugs has been reported so far from the field, it is important to understand factors that may contribute to the development of resistance and that may reduce the efficacy of the drugs in the future.

We have shown previously that the genetic type of the host erythrocytes can influence the efficacy of artemisinin derivatives against *P. falciparum* (Yuthavong *et al.*, 1989; Kamchonwongpaisan *et al.*, 1994). Parasites in culture infecting α-thalassemic erythrocytes, both of the genetic type α-thalassemia 1/α-thalassemia 2 (--/-α) and α-thalassemia1/Hb Constant Spring (--/$\alpha^{CS}\alpha$), or of the phenotypes HbH and HbH/HbCS respectively, are more resistant to the artemisinins than the same parasites infecting genetically normal erythrocytes. Resistance is therefore generated from the host, not the parasite, and is caused by the competition from the erythrocytes, which take up the drugs in large quantities, resulting in low medium concentration and low drug uptake of the parasite. Drug-binding sites may therefore be present in the variant erythrocytes and be responsible for the uptake. The search for such possible binding sites is important in the understanding of the apparent drug resistance of the parasite infecting α-thalassemic erythrocytes, and may yield information on the nature of the drug receptor. This article reports the results of the study on distribution and localization of dihydroartemisinin in α-thalassemic and normal erythrocytes. It was found that Hb H binds with the

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ABBREVIATIONS: DHART, dihydroartemisinin; Hb, hemoglobin; HbH, α-thalassemia 1/α-thalassemia 2; HbH/HbCS, α-thalassemia 1/hemoglobin Constant Spring; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

drug with much higher avidity than Hb A and that the former accounts for a significant portion of the drug taken up.

Experimental Procedures

Sample preparations. About 15 ml of venous blood from α-thalassemic patients (both HbH and HbH/HbCS phenotypes) and normal individuals was collected with citrate-phosphate-dextrose as anticoagulant. Whole blood was centrifuged at 800 × *g*, at 4° for 15 min, after which plasma and the buffy coat were removed. The packed erythrocytes were washed twice with culture medium composed of RPMI 1640 supplemented with 25 mM HEPES, pH 7.4, 0.2% $NaHCO_3$, and 40 μg/ml gentamicin. The erythrocytes were then resuspended in an equal volume of culture medium, and cell numbers were counted by an automated cell counter (Technicon, Bayer Diagnostics, Tarrytown, NY). For DHART inhibition assay, the packed erythrocytes were resuspended in a 10% human-serum-supplemented culture medium.

DHART inhibition assay. The antimalarial activity of DHART was measured against *P. falciparum* infecting normal and α-thalassemic erythrocytes using the [^{3}H] hypoxanthine incorporation method of Desjardins *et al.* (1979). Aliquots (25 μl) of serially diluted DHART in dimethylsulfoxide were pipetted into a microtitration plate containing 96 flat-bottomed wells. Parasitized erythrocyte suspension (200 μl) containing 1.5% hematocrit with 0.5% parasitemia were added. After 24-hr incubation in a candle jar at 37°, 25 μl of [^{3}H]hypoxanthine (0.5 μCi, specific activity 20–30 Ci/mmol; Amersham, Paisley, UK) were added into each well and the plate was reincubated under the same condition for 18 hr. Using a cell harvester (Nunc, Roskilde, Denmark), the cell suspension was aspirated through glass filter paper (no. 934-AH; Whatman, Maidstone, UK), and washed with distilled water. The disks were dried and placed in toluene-based scintillation fluid for counting in a b-counter (LS1801; Beckman Instruments, Palo Alto, CA). IC_{50} values were evaluated from the sigmoidal graph of percent [^{3}H]hypoxanthine incorporation versus log of drug concentration.

[^{14}C]dihydroartemisinin accumulation. Aliquots (140 μl) of 50% red blood cell suspension were incubated with 560 μl of 1.25 mM [^{14}C]DHART [specific activity 12.1 mCi/mmol; final concentration, 1.0 mM in 0.1% dimethylsulfoxide (a kind gift from Dr. Kenneth H. Davis, Jr., Chemistry and Life Sciences Division, Research Triangle Institute, NC)] in 1.5-ml microcentrifuge tube at 37° for 2 hr (Kamchonwongpaisan *et al.*, 1994). Cells were pelleted by centrifugation at 10,000 × *g* for 5 min. The packed erythrocytes were washed with 1 ml of culture medium three times and were then incubated with 700 μl of 2% sodium dodecyl sulfate solution at 60° for 1 hr. Solutions were bleached with 400 μl of 15% hydrogen peroxide at 60° for 12 hr. Four milliliters of Triton-based liquid scintillation fluid was added and the radioactivity was determined.

To study drug retention, 70 μl of [^{14}C]DHART-labeled packed erythrocytes were incubated in 1 ml of culture medium in triplicates for a further 3 hr, and the amounts of radioactivity remaining within the cells were measured hourly as described above for comparison with the initial unwashed cells.

[^{14}C]dihydroartemisinin distribution within red blood cells. One volume (70 μl) of packed [^{14}C]DHART-labeled erythrocytes was mixed with half a volume of lysis buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.8), and the cell suspension was then freeze-thawed to lyse the intact erythrocytes. The membrane fraction was separated by centrifugation at 10,000 × *g* for 15 min. Radioactivity in 105 μl of hemolysate was measured, and the amount of drug was calculated. The membrane fraction was washed five times with a buffer containing 1 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride in 5 mM Tris·HCl, pH 7.6, and then incubated with 500 μl of 2% sodium dodecyl sulfate solution at 60° for 1 hr. Four milliliters of Triton-based liquid scintillation fluid was added and radioactivity was determined for calculation of the amount of the drug in the membrane fraction.

Hemoglobin typing by cellulose acetate gel-electrophoresis. Two microliters of hemolysate from [^{14}C]DHART-labeled erythrocytes was electrophoresed on a cellulose acetate plate (cellogel; Chemetron, Milano, Italy) in Tris-glycine buffer, pH 8.6, at 280 V for 30 min. The cellulose acetate plate was stained with Ponceau S solution and destained with 5% acetic acid. Cellogel was dehydrated and dried, and the percentages of hemoglobin types were quantified using a densitometer (eDC; Helena, Beaumont, TX). Total hemoglobin concentrations were assayed by the cyanmethemoglobin method (Brown, 1988).

Hemoglobin binding capacity. Hemolysates (10–20 μl) from normal and thalassemic red blood cells was separated on a cellulose acetate plate as described above. Each lane was cut into areas containing band at origin, Hb A, Hb A_2, Hb H, Hb CS and area(s) with no Hb band. Corresponding areas from the same sample were pooled and eluted with 5 ml of distilled water by shaking overnight at room temperature. The radioactivity was determined after bleaching with 15% hydrogen peroxide in Triton-based liquid scintillation fluid. Drug-binding capacity of each Hb was calculated as moles of [^{14}C]drug per mole of Hb.

Hemoglobin isolation by carboxy methyl cellulose chromatography. Hemolysates, prepared from drug-free erythrocytes using the freeze-thaw technique as described above, were dialyzed in bis-Tris buffer (0.03 M bis-Tris, pH 6.1, with 0.01% potassium cyanide) at 4° for 12 hr. The dialyzed hemolysates were loaded onto a carboxy methyl cellulose column (1 × 20 cm, CM-52 cellulose; Whatman), and washed with 1–2 column volumes of bis-Tris buffer at a flow rate of 50 ml/hr, followed by 800 ml of salt gradient (between 0.030 and 0.065 M sodium chloride in bis-Tris buffer) (Schroeder and Huisman, 1980). Ten-milliliter fractions of the effluent were collected. Conductance and absorption at 280 and 415 nm were measured. Fractions from the same peak of Hb were pooled, dialyzed in 10 mM phosphate buffer, pH 7.4, and concentrated. Hb concentrations were assayed by the cyanmethemoglobin method.

Binding constant measurements. Binding constants of DHART with Hb A and Hb H were measured by dialysis technique (Kabat and Mayer, 1961). The isolated hemoglobin was diluted to 10 μM with 10 mM phosphate buffer, pH 7.4, and 1 ml aliquots were placed in dialysis tubes (16 mm in diameter, retaining protein of molecular mass ≥ 12,000 Da; Sigma, St. Louis, MO). Each tube was incubated in 1 ml of [^{14}C]DHART (varying from 1×10^{-7} M to 5×10^{-4} M) in the same buffer at 37° for 20 hr. Then 500 μl of the solutions within and outside the tube was collected, and bleached with 500 μl of 15% hydrogen peroxide. Four milliliters of Triton-based liquid scintillation fluid was added, and radioactivity was measured. The concentrations of bound and free drugs were calculated and the binding curves were evaluated using the program ENZFITTER (Cambridge Biosoft, Northwich, UK).

Results

P. falciparum was found to be more resistant to dihydroartemisinin when infecting α-thalassemic erythrocytes, both of the HbH and the HbH/HbCS types, than when infecting genetically normal erythrocytes. The IC_{50} values were 9.6 ± 1.2 nM for HbH and 13.7 ± 7.2 nM for HbH/HbCS, which were 8.0 and 11.4 times higher than that of infected normal erythrocyte (1.2 ± 0.5 nM).This result was similar to those for artesunate (Yuthavong *et al.*, 1989) and artemisinin (Kamchonwongpaisan *et al.*, 1994) reported earlier. The variant erythrocytes took up higher amounts of dihydroartemisinin than normal erythrocytes, another finding similar to the previous result for artemisinin (Kamchonwongpaisan *et al.*, 1994), although the magnitude of the difference was lower for

dihydroartemisinin. Under the experimental conditions used, drug uptake of HbH erythrocytes (0.25 ± 0.13 pmol/106 cells) was 2.8 times, and of HbH/HbCS erythrocytes (0.44 ± 0.11 pmol/106cells) was 4.9 times, that for genetically normal erythrocytes (0.09 ± 0.05 pmol/106cells).

Most of the drug accumulated by the HbH and HbH/HbCS erythrocytes remained in the cells even after extensive washing (88% and 90%, respectively; (Fig. 1). In contrast, only 43% of dihydroartemisinin in genetically normal erythrocytes remained in the cells after similar washing. This result indicated that the drug in the thalassemic erythrocytes was much more tightly bound than that in genetically normal erythrocytes.

To investigate the factors responsible for high drug uptake by thalassemic erythrocytes, the cells were lysed after exposure to the radiolabeled drug and fractionated into membrane (pellet) and cytosol (supernatant) fractions. Fig. 2 shows that most (82–88%) of the drug was associated with the cytosol fraction for both genetically normal and thalassemic erythrocytes. The remaining drug was located in the membrane fraction. Drug-binding capacity of various cytosolic components was investigated further by electrophoresis of the lysates. Table 1 shows the amount and percentages of Hb A, Hb H, and bound dihydroartemisinin calculated from the associated radioactivity. For both HbH and HbH/HbCS erythrocytes, Hb H in the cells accounts for about 22–29% (mean 25.3 ± 7.7%) of the total drug found in the lysate, although it accounts for only 11–12% (mean 11.7 ± 4.4%) of the total Hb. In contrast, Hb A, accounting for 74–81 (mean 77.4 ± 5.7%) of total Hb, has only about 27% (mean 27.2 ± 7.3%) of the total drug associated with it. The drug-binding capacities of the two types of hemoglobin in these cells, calculated as mmole of drug per mole of hemoglobin, are shown in Fig. 3. Hb H has about five to seven times as much dihydroartemisinin bound as Hb A. The drug binding capacity of Hb H isolated from HbH and HbH/HbCS erythrocytes was 1.79 ± 0.24 and 1.35 ± 0.52 mmol/mol Hb, respectively, which were 7.5 and 4.7 times higher than drug-binding capacity of Hb A (0.24 ± 0.14 and 0.29 ± 0.16 mmol/mol Hb, respectively). The drug-binding capacity of Hb A in the thalassemic cells was not different from that in genetically normal cells.

The maximum binding capacities (B_{max}) and dissociation constants (K_d) for the binding between dihydroartemisinin and Hb H, as well as Hb A, were studied by equilibrium dialysis using isolated Hb of both types. Typical binding curves are shown in Fig. 4, and the values for B_{max} and K_d are shown in Table 2. B_{max} for Hb H binding was 1.67 ± 0.17 mol/mol Hb, whereas B_{max} for Hb A binding was 0.74 ± 0.18 mol/mol Hb. The K_d value for Hb H binding was 66 ± 17 μM, about 3-fold lower than the value of 224 ± 15 μM for Hb A binding.

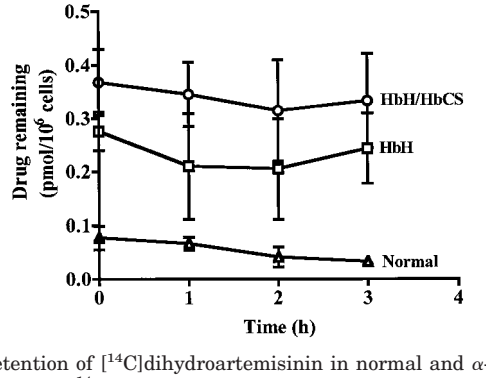

Fig. 1. Retention of [^{14}C]dihydroartemisinin in normal and α-thalassemic erythrocytes. [^{14}C]DHART-labeled normal and α-thalassemic erythrocytes were incubated with culture medium at 37°. At zero time and hourly intervals, cells were sedimented and radioactivity remaining in the cells was determined compared with initially unwashed cells. *Vertical bars*, standard deviation of three experiments.

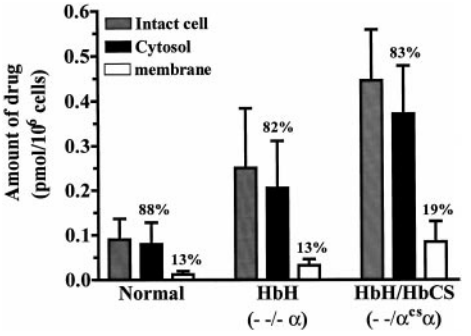

Fig. 2. Distribution of [^{14}C]dihydroartemisinin in normal and α-thalassemic erythrocytes. [^{14}C]DHART-labeled normal and α-thalassemic erythrocytes were lysed by hypotonic solution. Hemolysate and membrane fractions were separated by centrifugation. Drug content in cytosolic (hemolysate) and membrane compartments was measured. ▨, mean ± standard deviation of drug in intact erythrocytes; ■, □, mean ± standard deviation of drug in cytosolic and membrane compartments, respectively. Amount of drug in both compartments is also demonstrated as percentages of intact cell.

Discussion

As in findings for artesunate (Yuthavong *et al.*, 1989) and artemisinin (Kamchonwongpaisan *et al.*, 1994), dihydroartemisinin showed less activity against *P. falciparum* in culture when the parasite infected α-thalassemic (Hb H or Hb H/Hb Constant Spring) erythrocytes than when it infected genetically normal red blood cells. We also show here that, as in the case of artemisinin (Kamchonwongpaisan *et al.*, 1994), dihydroartemisinin was preferentially accumulated by the α-thalassemic erythrocytes.

DHART and other derivatives are hydrophobic molecules and it is possible that the high uptake in the thalassemic erythrocytes was caused by binding with erythrocyte membrane. Indeed, it has been shown (Asawamahasakda *et al.*, 1994) that dihydroartemisinin binds with isolated erythrocyte membrane, although not with intact erythrocytes. The membranes of α-thalassemic erythrocytes have many unique features (Schrier, 1994), which may account for preferential binding with dihydroartemisinin. However, Fig. 2 shows that although a significant proportion was associated with the membrane fraction, most of the drug was located in the cytosol fraction. Although Asawamahasakda *et al.* (1994) showed that the drug binds with membrane proteins to a greater extent than cytosolic proteins on a drug per protein basis, the membrane fraction may account for only a small portion of the drug taken up, in view of the relatively small amount of membrane proteins compared with cytosolic proteins and of the possibility that not all the drug in the cytosol is protein-bound. Nevertheless, the possibility remains open that the erythrocyte membrane may play a crucial role in drug transport. It has been shown earlier (Kamchonwongpaisan *et al.*, 1994) that drug accumulation in both variant and

TABLE 1

Hemoglobin content and its associated [^{14}C] dihydroartemisinin in normal and α-thalassemic hemolysates

Hemolysates obtained from [^{14}C] DHART-labelled normal and α-thalassemic erythrocytes were separated on cellulose acetate plates in Tris-glycine buffer, pH 8.6. Percentages of each hemoglobin band were estimated by a densitometer (shown in parentheses). The amount of Hb (pmol/10^6 cells) in each band was calculated from percent Hb and total Hb assayed by cyanmethemoglobin method. [^{14}C] DHART in each Hb band was eluted and measured for calculation as pmol/10^6 cells and percent of total drug (shown in parentheses).

Hemolysates	Hb A		Hb H		Total Hb	Total drug in hemolysate
	Amount of Hb	Amount of bound drug	Amount of Hb	Amount of bound drug		
	pmol / 10^6 cells (% of total)					
HbH/HbCS						
Patient 1	179 (78.1)	0.032 (26.4)	24 (10.5)	0.026 (21.5)	229	0.121
Patient 2	171 (67.4)	0.037 (26.6)	19 (7.4)	0.019 (13.7)	254	0.139
Patient 3	158 (75.6)	0.075 (28.7)	40 (19.4)	0.079 (30.3)	208	0.261
	169 ± 11[a] (73.7 ± 5.6)[a]	0.048 ± 0.024[a] (27.2 ± 1.3)[a]	28 ± 11[a] (12.4 ± 6.2)[a]	0.041 ± 0.033[a] (21.8 ± 8.3)[a]	230 ± 23[a]	0.174 ± 0.076[a]
HbH[c]						
Patient 4	142 (84.3)	0.014 (15.2)	13 (7.8)	0.020 (21.7)	169	0.092
Patient 5	143 (78.0)	0.052 (38.2)	23 (12.7)	0.046 (35.1)	183	0.136
Patient 6	165 (80.9)	0.044 (28.0)	25 (12.2)	0.046 (29.3)	204	0.157
	150 ± 13[a] (81.1 ± 3.2)[a]	0.037 ± 0.020[a] (27.2 ± 8.2)[a]	20 ± 6[a] (10.9 ± 2.7)[a]	0.037 ± 0.015[a] (28.7 ± 6.7)[a]	185 ± 18[a]	0.128 ± 0.033[a]
Normal	300 (93.2)	0.067 (56.8)	—	—	322	0.118

[a] mean ± standard deviation

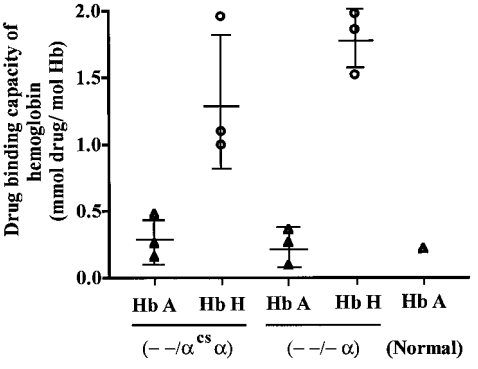

Fig. 3. [^{14}C]dihydroartemisinin binding capacity of hemoglobin. Hemolysates obtained from [^{14}C]DHART-labeled normal and α-thalassemic erythrocytes were separated on cellulose acetate plates in Tris-glycine buffer, pH 8.6. Hb contents and the amounts of associated drug were measured (shown in Table 1). This figure shows mean ± standard deviation of drug binding capacity of Hb A and H [millimoles of drug per mole of Hb (three experiments for α-thalassemic hemolysates and one experiment for normal hemolysate).

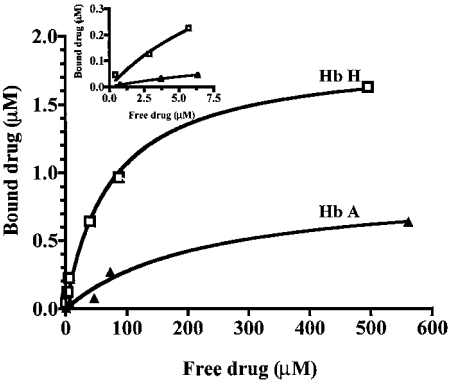

Fig. 4. Binding curve of DHART to hemoglobin. Hb A and Hb H were isolated from α-thalassemic erythrocytes by ion exchange chromatography. Binding of [^{14}C]DHART was conducted using the dialysis technique as described in Experimental Procedures. Maximum binding capacity (B_{max}) and dissociation constant were calculated from this curve (the values shown in Table 2).

normal erythrocytes depends on metabolic energy, possibly required for membrane transport of the drug.

In studies with isolated hemoglobin using the reversible binding model, it was found that Hb H-bound dihydroartemisinin with a higher affinity (lower K_d) and a higher maximum binding capacity (B_{max}) than did Hb A (Table 2). The binding affinity for Hb H was more than 3-fold that for Hb A, and the maximum binding capacity of Hb H with the drug was about 2-fold that of Hb A. The former seemed to bind two molecules of dihydroartemisinin, whereas the latter only bound one per molecule. Because Hb H has four β-globin subunits, and Hb A only two, it is possible that each molecule of the drug binds with a β-globin dimer. The mode of binding of Hb H with the drug remains to be further investigated.

Yang *et al.* (1994) reported that artemisinin binds covalently with hemoproteins including hemoglobin. However, from their data, it can be calculated that only approximately 0.003 molecule of the drug was covalently bound per hemoglobin molecule. Our reversible binding model is not invalidated by a small extent of covalent binding. Nevertheless, there may be a higher extent of covalent binding of dihydroartemisinin with hemoglobin in the cellular environment, because the drug can be activated by intracellular heme and iron (Meshnick *et al.*, 1996; Paitayatat *et al.*, 1997).

In α-thalassemic red blood cells, Hb H bound dihydroartemisinin five to seven times as much as Hb A on a molar basis, although it accounts for only about 12% of the total Hb content. Hb H and Hb A in these erythrocytes account for a major portion (40–70%) of drug accumulation, but they are

TABLE 2

Maximum binding capacities (B_{max}) and dissociation constants (K_d) of dihydroartemisinin-hemoglobin complexes

The maximum drug bound per Hb molecule (B_{max}) and K_d of Hb binding to DHART were assayed by equilibrium dialysis. The values were calculated from binding curve (shown in Fig. 4) using the ENZFITTER program.

Hb	B_{max} (moles of drug/moles of hemoglobin)	K_d
		μM
Hb A		
Sample 1	0.55	236
Sample 2	0.77	208
Sample 3	0.90	229
	0.74 ± 0.18[a]	224 ± 15[a]
Hb H		
Sample 1	1.86	74
Sample 2	1.59	46
Sample 3	1.56	77
	1.67 ± 0.17[a]	66 ± 17[a]

[a] Mean ± standard deviation.

not the only factors responsible for preferential accumulation of the drug. Other factors, not yet identified, must also be responsible for drug accumulation of the variant erythrocytes.

A fraction of the drug may also be covalently bound to Hb and other protein components of the thalassemic erythrocytes. There is evidence that iron and/or heme are important in the action of artemisinin and its derivatives, which proceed through formation of free radicals (Meshnick *et al.*, 1996; Paitayatat *et al.*, 1997). Because thalassemic erythrocytes have been shown to be under oxidative stress (Shinar and Rachmilewitz, 1990), a portion of the drug may be induced to bind covalently with cellular protein components, through the enhanced stress, possibly through increased Fe(II)-Fe(III) cycling, after which the drug will become inactive. Failure to remove the bound drug by repeated washing may reflect both the tight noncovalent binding of the drug with HbH and its covalent binding with red cell protein components including hemoglobins. Inactivation of the drug may additionally help to explain the apparent resistance of malaria parasites infecting α-thalassemic erythrocytes.

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