

Resistance to Artemisinin of Malaria Parasites (*Plasmodium falciparum*) Infecting α -Thalassemic Erythrocytes In Vitro

Competition in Drug Accumulation with Uninfected Erythrocytes

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Abstract

Plasmodium falciparum infecting hemoglobin (Hb)H and/or Hb Constant Spring erythrocytes has higher resistance to artemisinin in vitro than when infecting normal erythrocytes. This is due to low drug accumulation of infected erythrocytes resulting from competition with uninfected variant erythrocytes, which have a higher accumulation capacity than genetically normal cells. Drug accumulation of the parasite was shown to be saturable and dependent on metabolic energy. The 50% inhibitory concentrations (IC₅₀'s) for the parasite in HbH/Hb Constant Spring erythrocytes were decreased when normal erythrocytes were added to the infected cells, and correspondingly, the IC₅₀'s in normal erythrocytes were increased when HbH/Hb Constant Spring erythrocytes were added to the infected cells. The changes of IC₅₀ corresponded to the variation in drug accumulation of mixtures of normal and variant erythrocytes of different compositions. The IC₅₀'s for the parasite in variant erythrocytes were also greatly decreased when the hematocrit of the culture was lowered, while the IC₅₀'s in normal erythrocytes were independent of the hematocrit. The increase in IC₅₀ values for the parasites infecting variant erythrocytes was also related to the decrease in parasite accumulation, indicating that drug accumulation capacity of the parasite also has a role in determining drug sensitivity. Artemisinin sensitivity therefore is determined by its accessibility to the parasite, which is decreased in infected variant erythrocytes. (*J. Clin. Invest.* 1994; 93:467–473.) Key words: malaria • *Plasmodium falciparum* • α -thalassemia • variant erythrocytes • artemisinin

Introduction

Resistance of the malaria parasite *Plasmodium falciparum* to chloroquine and other drugs in various parts of the world necessitates the development of new effective antimalarials (1). Derivatives of artemisinin (qinghaosu) (2, 3) comprise a new family of such drugs presently undergoing development and clinical trials. Their fast action and effectiveness against chloroquine-resistant strains of the parasites make them potentially valuable drugs, especially in such areas of high resistance as

Southeast Asia and the Pacific. These areas also have a high incidence of thalassemia and abnormal hemoglobins (4), some genotypes for which are associated with innate resistance to falciparum malaria (5, 6). Hence, hemoglobin (Hb)¹H-containing erythrocytes from α -thalassemic (genotype α -thalassemia 1/ α -thalassemia 2, --/ α) or α -thalassemic/Hb Constant Spring individuals (genotype α -thalassemia 1/Hb Constant Spring, --/ $\alpha^{CS}\alpha$) have been shown to be resistant to infection by *P. falciparum* in vitro (7, 8). Enhanced immune recognition and clearance of parasitized erythrocytes have also been implicated as contributing to the apparent protective effect of thalassemia against falciparum malaria (9). These experimental results support an earlier observation that prevalence of the genes for α -thalassemia parallels the endemicity of falciparum malaria in the Pacific Islands (5). The high frequencies of genes for various types of thalassemia and abnormal hemoglobins in Southeast Asia and various other parts of the world make it relevant to ask whether antimalarial drugs would have the same efficacy in malaria patients carrying these genes as in genetically normal patients. This question also carries implications for development of drug resistance, which originated in Southeast Asia for a number of antimalarials, including chloroquine (1). If the drug concentrations that are sufficient to kill the parasites in normal patients were suboptimal for genetically variant patients, these would impose selective pressure on the parasites to become drug resistant.

We have reported earlier that *P. falciparum* infecting HbH and/or Hb Constant Spring erythrocytes displays more resistance to artesunate, a derivative of artemisinin, and chloroquine than the same parasite strain infecting genetically normal erythrocytes (10). This novel host effect on sensitivity to artesunate is especially striking in view of the facts that these genetically variant erythrocytes are already subject to high oxidative stress (11), that *P. falciparum* is sensitive to oxidative stress (12–14), and that artemisinin probably exerts its action through an oxidative mode (15–17). Two possible explanations of the observation were raised: the higher levels of antioxidant enzymes in the variant erythrocytes could give rise to a compensation effect lessening the oxidative damage potential of artemisinin, and/or the infected variant erythrocytes could not accumulate as much drug as infected normal erythrocytes. In this paper, we show that the latter explanation is correct. Furthermore, we show that the reduction in artemisinin accumulation of infected variant erythrocytes is due partly to competition with uninfected erythrocytes for the drug, and partly to lower drug accumulation capacities of the parasite.

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1. Abbreviations used in this paper: Hb, hemoglobin; IC₅₀, 50% inhibitory concentration.

Methods

Culture of *P. falciparum*. Chloroquine-resistant K₁ strain was obtained from Kanchanaburi Province, Thailand, and maintained in vitro by the candle jar technique of Trager and Jensen (18). The cultures were synchronized at ring stage by 5% sorbitol treatment (19) and concentrated at schizont stage by Percoll layering technique (20) to obtain ~95% parasitemia. The concentrated schizonts were subcultured with normal and variant erythrocytes for at least 72 h before antimalarial testing and for 90 h for drug accumulation study, at which time the synchrony was still largely maintained. The culture medium used was RPMI 1640 supplemented with 25 mM Hepes, 0.2% NaHCO₃, 40 µg/ml gentamicin, and 10% human serum (blood group AB), pH 7.4.

50% inhibitory concentration (IC₅₀) testing. The antimalarial activity of artemisinin against *P. falciparum*-infected normal and thalassemic erythrocytes was measured by using the [³H]hypoxanthine incorporation method of Desjardins (21). Briefly, artemisinin was dissolved in DMSO and diluted with culture medium to the appropriate concentration. The final concentration of DMSO was 0.001%, which had no effect on parasite growth. 25 µl of medium containing drug and 200 µl of 1.5% cell suspension (or as indicated) with 1–2% parasitemia at immature schizont or ring stage (or as indicated) were cultured in triplicated manner for 24 h. 25 µl of 0.5 µCi [³H]hypoxanthine was added. After an additional 18 h of culture, the cells were harvested onto glass fiber filters. The radioactivity was measured by a liquid scintillation counter (LS-1801; Beckman Instrs., Inc., Fullerton, CA). The IC₅₀'s (the drug concentrations required for 50% reduction of the radioactivity as compared with control without the drug) of artemisinin against these infected cells were obtained from dose–response curves.

In some experiments, a constant amount of *P. falciparum*-infected erythrocytes (ring stage) was diluted with a known composition of uninfected normal and variant erythrocytes, with the final hematocrit of 1.5%. In other experiments, the infected erythrocytes (immature schizont stage) were diluted with various amounts of normal and variant cells to obtain a set of cells with various hematocrit values. These cells were similarly tested for the IC₅₀.

[¹⁴C]Artemisinin accumulation. After a 90-h culture, *P. falciparum*-infected (mostly at late trophozoite and immature schizont stage) and control uninfected erythrocytes (both normal and variant) were washed with the medium without serum supplemented. Duplicated samples of 70-µl packed cells were incubated with 630 µl of 130 nM [¹⁴C]artemisinin (dissolved in 0.001% toluene and 0.1% DMSO in incomplete medium without serum), or at various concentrations for study of concentration effect, at 37°C for 2 h in a shaking water bath. The cells were pelleted. 600 µl of supernatant was bleached with 400 µl of 15% hydrogen peroxide at 60°C. 4 ml of liquid scintillation fluid was added and the radioactivity was measured. The drug accumulated by the cells, defined as drug accumulation capacity, was calculated and expressed as the amount of drug in 70-µl pellet per unit concentration of the drug remaining in the supernatant (nmol/µM).

In some experiments, drug accumulation by concentrated infected erythrocytes was measured directly. Concentrated infected cells with parasites in late trophozoite–early schizont stage were prepared. A synchronized culture with 30–40% parasitemia was put in the medium supplemented with 2.75 µg/ml hypoxanthine. The culture was then left under standard incubation condition for 40 h, and the parasitized cells, mostly at late trophozoite–early schizont stage were harvested and washed with incomplete medium. 15% cell suspension in incomplete medium was overlaid on an equal volume of 60% Percoll and centrifuged at 600 g for 20 min. The dark band at the interface of the two media, containing infected cells at ~95% parasitemia, was collected and washed thrice with incomplete medium. Duplicated samples of 10-µl packed infected cell pellets were used for determining the drug accumulation as above. In addition to the measurement in supernatant, the drug accumulation in the cell pellets was also determined directly. The pellets were washed thrice with incomplete medium, incubated with 700 µl of 2% SDS solution at 60°C for 1 h, then bleached

with 400 µl of 15% hydrogen peroxide at 60°C overnight. 4 ml of liquid scintillation fluid was added and the radioactivity was determined.

In the study of effect of iodoacetamide on drug uptake, the cells were preincubated at 37°C with 500 µl of 100 µM iodoacetamide in incomplete medium. After 1 h of incubation, 130 µl of [¹⁴C]-artemisinin was added to the cell suspension to obtain a final concentration of 100 nM and a 10% cell suspension. The cells were mixed and incubated further for 2 h, and the drug accumulation was determined as above.

Statistical analysis. The Mann-Whitney U test was used for comparing the data from normal and variant erythrocytes based on independent random samples.

Results

As has been observed before, the IC₅₀'s for artemisinin against *P. falciparum* infecting HbH and HbH/Hb Constant Spring erythrocytes are significantly higher than against the parasite in genetically normal erythrocytes (Fig. 1). The basis for the differences in IC₅₀'s was earlier (10) suggested as being due to differences in oxidative stresses in the infected variant and normal erythrocytes, or due to differences in drug accumulation. The latter possibility was explored through the use of radiolabeled artemisinin. Fig. 2 A shows the accumulation of [¹⁴C]-artemisinin at the initial concentration of 130 nM by infected genetically normal erythrocytes (6.6% parasitemia), infected HbH erythrocytes (6.6% parasitemia), and HbH/Hb Constant Spring erythrocytes (5.3% parasitemia), while Fig. 2 B shows the drug accumulation by control erythrocytes that were not exposed to the parasite. Since the final medium concentration was decreased to different extents by the different amounts of the drug accumulated into the cells, the amounts of accumulation were divided by the final medium concentration in order to obtain normalized accumulation, which can be defined as accumulation capacities. Fig. 2, D and E, show the accumulation per unit medium concentration of whole infected and control erythrocytes, and Fig. 2, C and F, show the difference between the two for genetically normal, HbH, and HbH/Hb Constant Spring erythrocytes. The values in Fig. 2, C and F, can be regarded, respectively, as the accumulation and accumulation capacities of the parasite infecting the three different

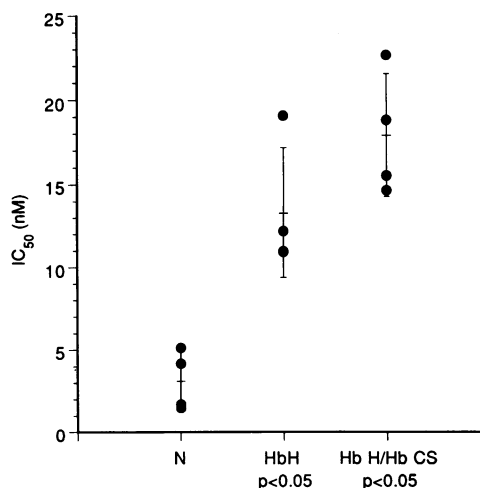


Figure 1. The IC₅₀ values with means±SD for artemisinin against *P. falciparum* infecting normal (N), HbH, and HbH/Hb Constant Spring (H/CS) erythrocytes.

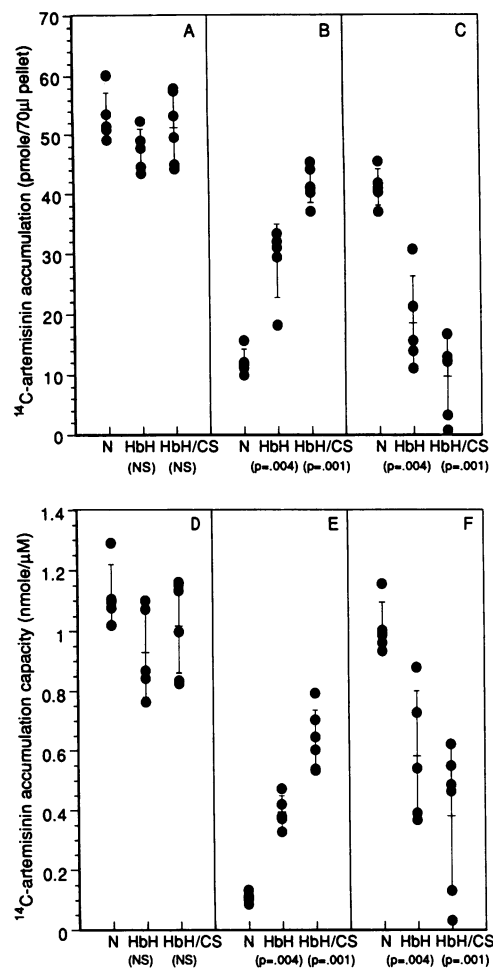


Figure 2. The [¹⁴C]artemisinin accumulation (A–C) and accumulation capacity (D–F), with means±SD, by infected erythrocytes with ~ 6% parasitemia (A and D), uninfected control erythrocytes (B and E), and calculated data for the parasite (C and F). 70 µl of packed cells was incubated with 630 µl of medium with initial artemisinin concentration of 130 nM for 2 h before accumulation measurements. Statistical analysis using Mann-Whitney U test was shown. N, normal; HbH/CS, HbH/Hb Constant Spring; NS, not significantly different.

genetic types of erythrocytes. While the accumulation and accumulation capacities of whole infected (nonparasitized plus parasitized) cells were comparable for the three genetic types (Fig. 2, A and D), the accumulation and accumulation capacities for uninfected HbH and, to a greater extent, HbH/Hb Constant Spring erythrocytes were much higher than those of uninfected normal erythrocytes with very little accumulation (Fig. 2, B and E). By contrast, the accumulation and accumulation capacity for the parasite infecting HbH/Hb Constant Spring erythrocytes were smaller than that infecting HbH erythrocytes, in turn smaller than that infecting genetically normal erythrocytes (Fig. 2, C and F).

The accumulation capacities of infected erythrocytes decreased rapidly with increasing initial concentration of the drug up to ~ 2 µM for all three genetic types, indicating saturability of the binding sites (Fig. 3). In contrast, the accumulation capacity for uninfected, genetically normal erythrocytes remained constant over the concentration range, while the accumulation capacities for uninfected HbH and HbH/Hb Con-

stant Spring gradually declined with increasing initial drug concentration. The accumulation capacity of the parasites infecting genetically normal erythrocytes, as obtained by subtracting those of uninfected from infected erythrocytes (Fig. 3 A), was about two and three times that of the parasites infecting HbH (Fig. 3 B) and HbH/Hb Constant Spring erythrocytes (Fig. 3 C), respectively, at therapeutic concentrations (< 100 nM), indicating differences in availability of specific sites. The accumulation capacities of the parasites infecting three different genetic types of erythrocytes declined to approximately the same level at concentrations > 2 µM, indicating the predominance of nonspecific binding at these high concentrations.

The measurement of the drug accumulation capacity of the parasites from the difference in accumulation of infected erythrocytes (6–10% parasitemia) and uninfected erythrocytes was validated by direct measurement of drug accumulation by concentrated infected cells (95% parasitemia). Fig. 3 D shows the results of such measurement on a suspension of concentrated infected genetically normal and HbH/Hb Constant Spring erythrocytes, which are comparable to the results obtained by the subtraction method. These results indicate further that the parasites in parasitized cells account for the majority of drug accumulation.

Metabolic energy requirement for the drug accumulation of these cells was also studied by examining the effect of temperature or of addition of iodoacetamide. Fig. 4 A shows that the decrease in incubation temperature markedly decreased the accumulation capacity of both infected normal and infected HbH erythrocytes. Iodoacetamide has a partial effect on drug accumulation of the parasites, but not of uninfected erythrocytes, for both genetically normal and HbH erythrocytes (Fig. 4 B).

When the data from drug sensitivity and accumulation experiments, which were obtained from the same set of samples, were combined to explore further the basis of differences in drug sensitivities, an inverse relationship was found between the final medium concentration of the drug incubated with uninfected erythrocytes of different genetic types and the IC₅₀ values for parasites infecting these erythrocytes (Fig. 5 A). This indicates that the increase in observed IC₅₀ values of parasites infecting variant erythrocytes was at least partly due to lowered medium concentration. Fig. 5 B shows that the IC₅₀ values were correlated with the accumulation capacities of uninfected erythrocytes, which can account for the lowered medium concentration and increased IC₅₀ values for infected variant erythrocytes. In agreement with the results in Fig. 2, the accumulation capacity of the parasite, corrected for lowered medium concentration, was also influenced by the genetic type of the erythrocyte that it has infected. Fig. 5 C shows an inverse correlation between the IC₅₀ values and normalized parasite drug accumulation capacities, indicating that the former is also influenced by the capacities for drug accumulation of the parasite inside the variant cells.

To prove further that the differences in IC₅₀ values for parasites infecting various genetic types of erythrocytes were due to different drug accessibilities, and to assess the relative importance of competition by uninfected cells and parasite drug accumulation capacities, ring-stage-infected erythrocytes were cultured in the presence of the drug together with mixtures of uninfected genetically normal and variant cells of known compositions for 40 h. The IC₅₀ values, obtained before the parasite

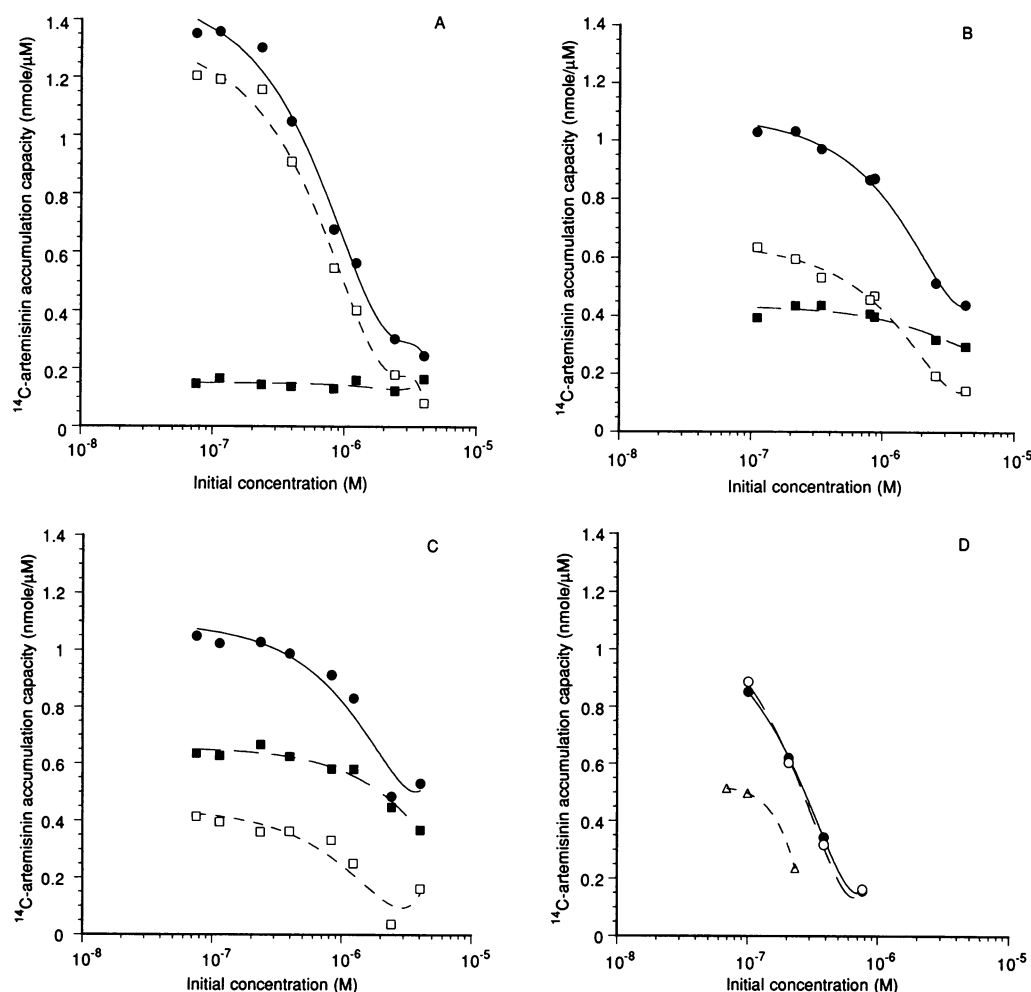


Figure 3. Effect of concentration on the [^{14}C]artemisinin accumulation capacity of infected normal (A), HbH (B), HbH/Hb Constant Spring (C) erythrocytes, and concentrated infected normal and HbH/Hb Constant Spring (D) erythrocytes, with 9.8, 6.0, 8.9, and 95% parasitemia, respectively (●), uninfected control erythrocytes (■), and calculated data for the parasites (□). 70 μl (A–C) or 10 μl (D) of packed cells were incubated with 630 μl (A–C) or 600 μl (D) of medium with varying artemisinin concentrations at 37°C for 2 h before measurements. (●, ■, and □) Points obtained from concentration differences in the supernatants (● in D, those for parasitized normal erythrocytes); (○ and △) obtained from direct measurement in pellet of 95% parasitized normal and HbH/Hb Constant Spring erythrocytes, respectively.

could begin a new cycle, should be independent of the composition of uninfected cells with regard to their genetic types if these were determined only by the accumulation capacities of the infected cells, but would be dependent on the uninfected cell composition if the accumulation capacities are wholly or partly determined by competition between infected and uninfected cells. Data in Fig. 6 show that the IC_{50} values for both infected genetically normal and HbH/Hb Constant Spring erythrocytes indeed depend on the composition of uninfected cells with regard to their genetic types, so that the values of IC_{50} for both genetic types converge as the compositions of the uninfected cells approach one another. The variation in IC_{50} values of the parasite infecting both types of cells was reflected by the variation in accumulation capacities of uninfected erythrocytes with different compositions of normal and HbH/Hb Constant Spring genotypes (Fig. 7). It can therefore be concluded that the IC_{50} values are to a large extent influenced by drug accumulation of uninfected erythrocytes, thereby lowering the medium concentration. This conclusion is also supported by another set of experiments determining the IC_{50} values for infected genetically normal and variant erythrocytes at different hematocrit values in the culture. As Fig. 8 shows, the IC_{50} values for infected HbH and HbH/Hb Constant Spring

erythrocytes were decreased with decreasing hematocrit values, whereas the IC_{50} values for infected genetically normal erythrocytes were independent of the hematocrit. These experiments show that the major factor leading to apparent drug resistance of the parasite infecting the variant erythrocytes is competition in drug accumulation by uninfected variant erythrocytes.

Discussion

The resistance to artemisinin of *P. falciparum* infecting HbH and HbH/Hb Constant Spring erythrocytes (Fig. 1) supports our previous finding on similarly increased resistance to artesunate (10). In this report, we have shown further that such increased resistance is mainly due to the fact that uninfected HbH and HbH/Hb Constant Spring erythrocytes have high accumulation capacity for artemisinin (Figs. 2, B and D, 3, and 5, A and B), hence, competing for the drug with infected erythrocytes. Therefore, a higher initial concentration of the drug is required so that the infected erythrocytes can accumulate enough drug to achieve the lethal concentration. Uninfected genetically normal erythrocytes, on the other hand, have low drug accumulation capacity, and did not significantly compete for the drug with infected normal erythrocytes. In support of this explanation, it was found (Fig. 6) that a decrease in the

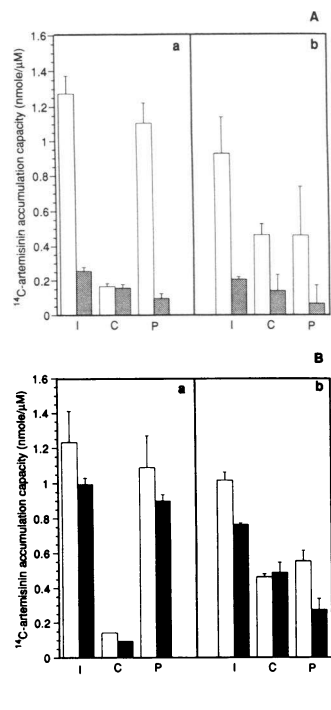


Figure 4. (A) Effect of temperature on the [^{14}C]artemisinin accumulation capacity of normal (a) and HbH (b) erythrocytes. 70 μL of packed cells was incubated with 630 μL of medium with initial artemisinin concentration of 130 ± 7 nM at 37°C (□) and 4°C (■) for 2 h before accumulation measurements. I, infected erythrocytes; C, uninfected control erythrocytes; P, calculated data for parasites. Error bars indicate ranges of the results from three experiments. (B) Effect of iodoacetamide on accumulation capacity of normal (a) and HbH (b) erythrocytes. 70 μL of packed cells was pretreated with 500 μL of 100 μM iodoacetamide at 37°C for 1 h, and 130 μL of [^{14}C]artemisinin was added to a concentration of 100 nM before further incubation for 2 h (■). (□) Untreated control. I, infected erythrocytes; C, uninfected control erythrocytes; P, calculated data for parasites. Error bars indicate ranges of the results from duplicate samples.

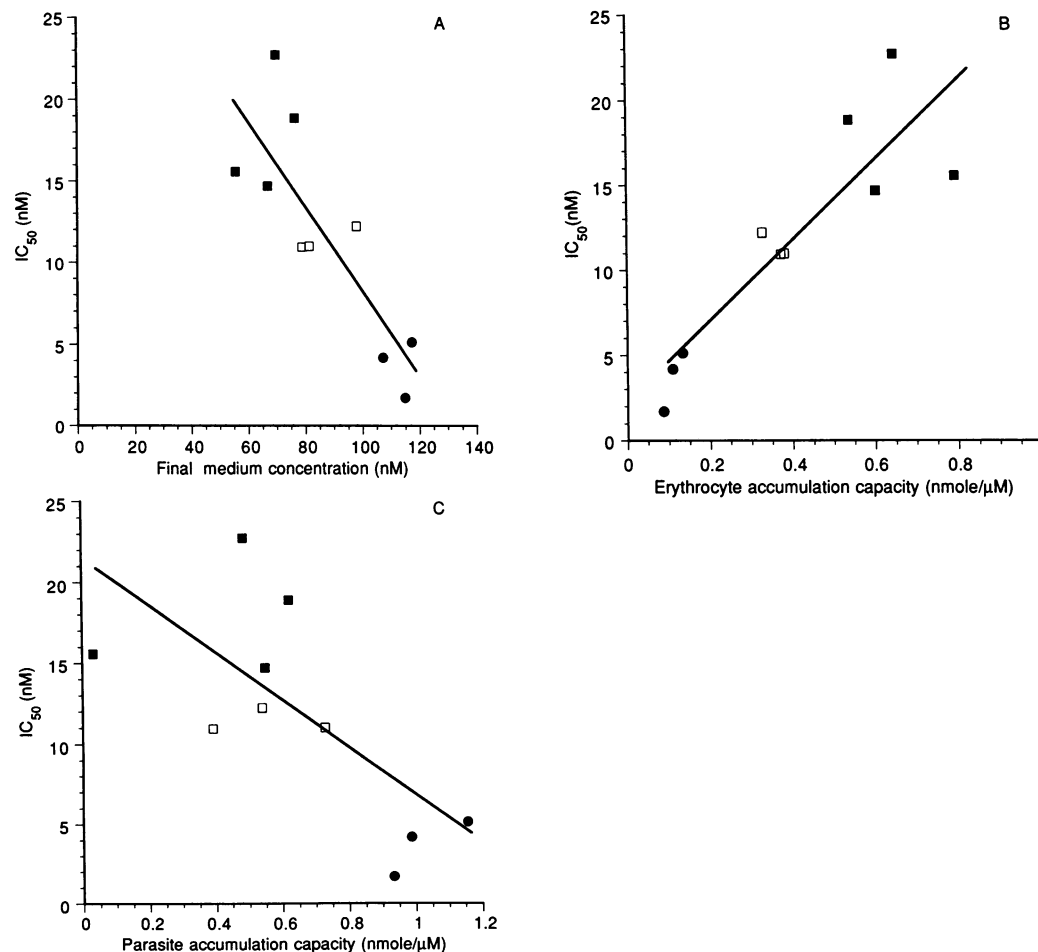


Figure 5. Correlation between the IC_{50} values and (A) final [^{14}C]artemisinin concentration in the medium of uninfected erythrocytes, $r = 0.83$; (B) accumulation capacities of uninfected erythrocytes, $r = 0.88$; and (C) calculated data for the accumulation capacities of intracellular *P. falciparum*, $r = 0.68$. (●) Normal erythrocytes, (□) HbH erythrocytes, and (■) HbH/Hb Constant Spring erythrocytes.

proportion of uninfected HbH/Hb Constant Spring erythrocytes in the culture caused a decrease in the IC_{50} for *P. falciparum* infecting HbH/Hb Constant Spring erythrocytes, and an increase in the proportion of uninfected erythrocytes caused a corresponding increase in the IC_{50} for the parasite infecting genetically normal erythrocytes. The variation in IC_{50} values was paralleled by that in drug accumulation capacities of erythrocyte mixtures (Fig. 7). Decrease of the hematocrit value in the culture with variant erythrocytes, thereby leading to less drug accumulation by uninfected erythrocytes, also led to a corresponding decrease in IC_{50} , while the hematocrit value had little influence on IC_{50} of parasites infecting genetically normal erythrocytes (Fig. 8).

Although drug accumulation of uninfected HbH and HbH/Hb Constant Spring erythrocytes was far higher than uninfected genetically normal erythrocytes, the accumulation of all genetic types was linear with external drug concentration to > 2 μM . By contrast, the accumulation by the parasites was saturable, consistent with a previous report in which the accumulation of dihydroartemisinin showed saturation at drug concentrations of > 3.86 μM (22). Furthermore, temperature dependence and partial inhibition by iodoacetamide of parasite drug accumulation (Fig. 4) indicated at least partial dependence on metabolic energy. Except for dependence on temperature of HbH erythrocytes, there was little effect of temperature or iodoacetamide on drug accumulation of uninfected erythrocytes.

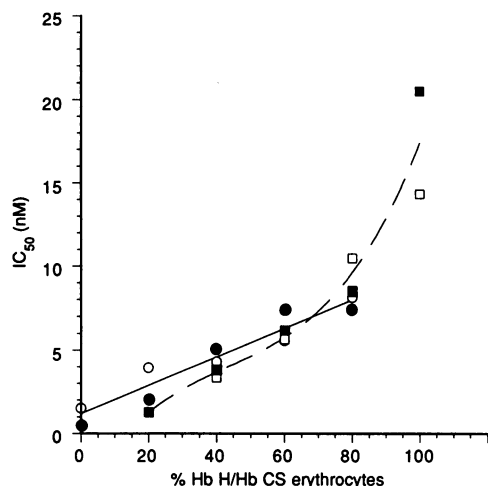


Figure 6. The IC_{50} values for artemisinin against *P. falciparum* infecting normal (\circ and \bullet) and HbH/Hb Constant Spring (\square and \blacksquare) erythrocytes in cultures with various composition of uninfected normal and HbH/Hb Constant Spring erythrocytes. Data for two experiments are shown.

Although the data in Figs. 6 and 8 indicate that the IC_{50} values are mainly determined by the genetic types and concentrations of uninfected cells that compete for the drug in the medium, other data (Fig. 2, C and F, and 5 C) suggest that drug accumulation capacity of the parasite infecting the variant erythrocytes, i.e., the accumulation corrected for altered medium concentration, may also be another determining factor, albeit a less important one. The low drug accumulation of the parasite infecting genetically variant erythrocytes may be due to limitation of transit of the drug from erythrocyte cytoplasm to the parasite or a true decrease in drug accumulation capacity of the parasite infecting the variant cells.

Our results reported here carry implications for the treatment of *P. falciparum*-infected patients with artemisinin or their derivatives, especially in the areas with high prevalence of thalassemic/hemoglobinopathic genes, which are largely the same as those with malaria endemicity (5). Hence, if the differences in sensitivity also exist at the clinical level, there will be a

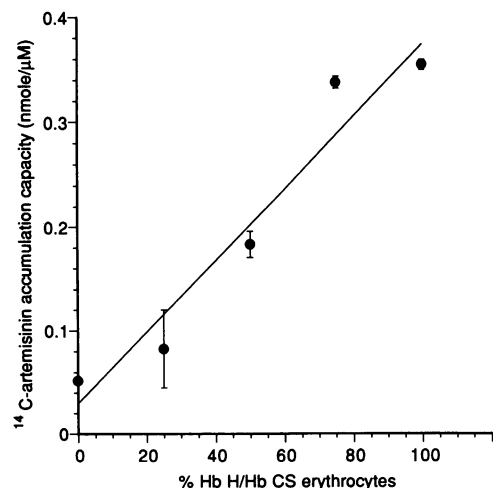


Figure 7. The $[^{14}C]$ artemisinin accumulation capacities of mixtures of uninfected normal and HbH/Hb Constant Spring erythrocytes as a function of the percentage of the latter.

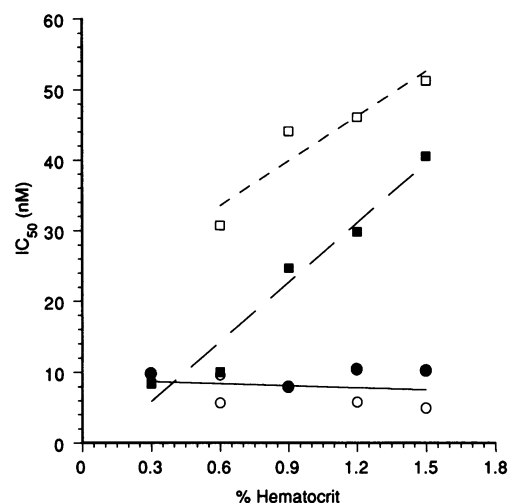


Figure 8. The IC_{50} values for artemisinin against *P. falciparum* infecting normal (\circ and \bullet), HbH (\square), and HbH/Hb Constant Spring (\blacksquare) erythrocytes at various hematocrit values. Data for two experiments are shown.

potential problem of possible induction of resistance to artemisinin due to exposure of the parasite infecting variant erythrocytes to subcurative doses of the drug. In addition, there may also be problems related with drug toxicity to patients who carrying the genetically variant genes. It has been shown that hemin forms an adduct with artemisinin, which leads to oxidative damage on membranes (23). In addition, it was also shown that ferrous, ferric, hemoglobin, and hemin enhanced the generation of superoxide and hydrogen peroxide by artesunate (24). Variant erythrocytes have increased hemichrome (25), nonheme iron (26). These components and other hemoglobin degradative products containing iron may potentiate artemisinin-induced oxidative damages to the erythrocytes, leading to such consequences as decreased erythrocyte deformability and increased methemoglobin level. Even in the absence of these drugs, such damages have already been found to be more pronounced in erythrocytes containing unstable Hb (HbSS, HbEE, and HbAE) than in normal erythrocytes (16). Therefore, the variation in effective dosage and therapeutic index of artemisinin for treatment of *P. falciparum* infection in these groups of patients should be investigated.

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