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Membrane heme as a host factor in reducing effectiveness of dihydroartemisinin

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Abstract

Plasmodium falciparum infecting α-thalassemic erythrocytes are resistant to artemisinin and its derivatives. Binding of the drug to hemoglobin H resulting in drug inactivation was previously demonstrated. We now show that an additional host factor, membrane heme, significantly accounted for decreased antimalarial activity of artemisinin. The antimalarial activity of dihydroartemisinin in the presence of normal and thalassemic erythrocyte membranes showed a correlation with the heme content of the membrane ($r^2 = 0.466$, $P < 0.01$). The correlation was more clearly seen when the drug effectiveness was correlated with the heme content of α-thalassemic membrane ($r^2 = 0.636$, $P < 0.01$). However, the drug effectiveness showed no correlation to ferrozine-reactive (free or non-heme) iron content ($r^2 = 0.0001$, $P > 0.05$). α-Thalassemic erythrocytes contained higher amounts of membrane heme (11.04 ± 8.96 nmol/mg membrane protein) than those from normal and β-thalassemia/HbE erythrocytes (2.68 ± 1.28 and 3.98 ± 3.98 nmol/mg membrane protein, respectively, $P < 0.01$). Loss of drug effectiveness was also correlated with increment of heme content in membrane prepared from normal erythrocytes treated with phenylhydrazine. It is concluded that heme in both normal and thalassemic erythrocyte membranes is an important factor in drug inactivation.

Keywords: Artemisinin; Heme; Thalassemia; Malaria

1. Introduction

Artemisinin (Fig. 1) and its derivatives form a promising class of antimalarial drugs, which are now commonly used in the treatment of falciparum malaria [1,2]. Dihydroartemisinin (DHART, Fig. 1) is a derivative of artemisinin with the C-10 lactone group replaced by hemiacetal. It is more effective antimalaria than artemisinin, and is the active metabolite of a number of artemisinin derivatives. They are widely used in Thailand, Myanmar, Vietnam and China, where multidrug resistant parasites are common [3]. Up to the present, there has been no report of resistance to artemisinin and its derivatives in the treatment of falciparum malaria despite widespread clinical application [4,5]. Our previous studies have demonstrated the role of α-thalassemic erythrocytes, both of HbH and HbH/HbCS types, in reducing artemisinin effectiveness [6–8]. DHART, which apparently acts in a similar manner to artemisinin [4,5], also showed the same reduction in effectiveness with these erythrocytes [8]. It was found that the reduction in drug effectiveness is partly due to higher drug accumulation capacities of uninfected α-thalassemic erythrocytes than genetically normal cells and to drug inactivation by α-thalassemic erythrocytes. Greater drug binding affinity of hemoglobin H in the α-thalassemic erythrocytes than hemoglobin A partly accounts for the high drug accumulation capacities. Hemolysates, purified hemoglobin H and erythrocyte membranes have also been demonstrated to account for preferential drug inactivation [7]. Intact cells and the membrane compartment of β-thalassemia/HbE erythrocytes also had the capacity to inactivate the drug, albeit to a much lesser extent [7].

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Abbreviations: HbH, α-thalassemia 1/α-thalassemia 2; HbH/HbCS, α-thalassemia 1/hemoglobin constant spring; α-thal, both HbH and HbH/HbCS; β-thal/E, β-thalassemia/HbE; DHART, dihydroartemisinin; IC_{50}, 50% inhibitory concentration; r^2, correlation coefficient; *P*, *P*-value.

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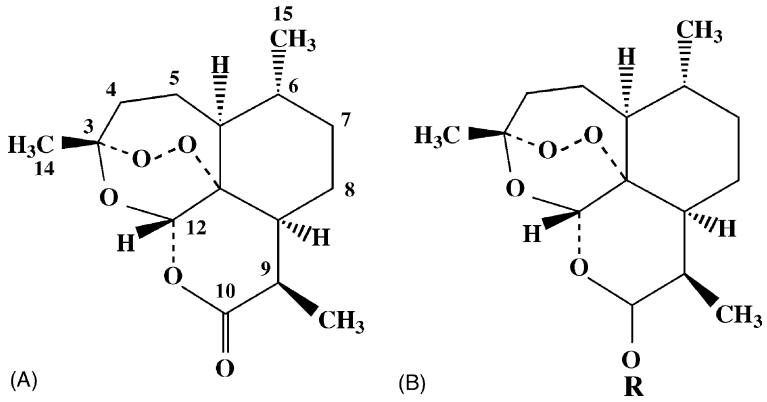

Fig. 1. Artemisinin (A) and its lactol derivatives (B) including dihydroartemisinin (R=H), artemether (R=CH_3), arteether (R=CH_2CH_3) and artesunate [R=$CO(CH_2)_2CO_2Na$].

Since the action of artemisinin is likely initiated by heme or molecular iron [9] and both heme and non-heme iron contents were reported to be present in higher amounts in thalassemic erythrocyte membranes than normal membranes [10,11], they can be significant factors interfering with artemisinin effectiveness. Premature activation of the drug outside the malaria parasite by erythrocyte components would lead to reduced effectiveness. Such interference, together with other contributing causes, may further lead to the development of true artemisinin resistance due to exposure of the parasite to sublethal drug concentrations. We present here the evidence that heme but not non-heme iron is the major factor in erythrocyte membrane causing reduction in effectiveness of DHART in both α-thalassemic and normal erythrocytes in which the heme content was artificially increased.

2. Materials and methods

2.1. Materials

Iron-free glassware was prepared by soaking in 50% hydrochloric acid overnight and then rinsing with deionized water [12]. Reagents used in sample preparations and iron study were prepared iron-free by using chelating resin (Chelex 100). Plasticware was purchased as iron-free grade (Axygen).

2.2. Samples

Heparinized blood samples were collected from 22 α-thalassemic patients (10 HbH and 12 HbH/HbCS patients, 8 males, 14 females) and 11 β-thalassemia/HbE patients (7 males, 4 females) from Division of Hematology, Department of Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, where hematologic parameters and hemoglobin typing were obtained. Normal blood samples were also collected voluntarily from nine healthy individuals (four males, five females) with normal hemoglobin typing. All subjects were non-splenectomized, ages during 20–50 years and had not been transfused for at least 3 months before the study. No subjects had previous history of malarial infection.

The samples were processed within 2 hr. Whole blood was centrifuged at 800 *g*, at 4° for 15 min, after which plasma and buffy coat were removed. To study drug inactivation by intact erythrocyte, the packed erythrocytes were washed for three times with serum-free culture medium (RPMI 1640 medium supplemented with 25 mM HEPES, pH 7.4, 0.2% $NaHCO_3$, 40 μg/mL of gentamicin) and resuspended to give 50% cell suspension in serum-free culture medium. For membrane studies, the erythrocytes were washed three times with ice-cold iron-free phosphate buffer saline (PBS, pH 7.4). Ghost membrane preparation was performed as described by Kuross and Hebbel [13]. Erythrocytes were lysed with ice-cold lysis buffer (5 mM phosphate buffer, 0.5 mM EDTA, pH 8.0, iron-free). Membrane was separated by centrifugation (10,000 *g*, at 4° for 15 min). The ghosts were extensively washed five times with ice-cold lysis buffer with multiple passages of the ghost pellet through 23 Gauge needle between washes and then twice with ice-cold washing buffer (5 mM phosphate buffer, pH 7.4, iron-free). The membrane was resuspended in 1 mL ice-cold washing buffer and kept in $<-20°$ until further experiments within 1 month.

2.3. Studies of dihydroartemisinin (DHART) inactivation by intact erythrocyte and membrane

To study the effect of intact erythrocyte on antimalarial activity of DHART, 70 μL of packed erythrocytes were incubated at 37° for 2 hr with 630 μL of radioactive [^{14}C]-DHART (a kind gift from Kenneth H. Davis, Jr., Chemistry and Life Sciences Division, Research Triangle Institute, NC, USA, specific activity 12.1 Ci/mol) in serum-free culture medium at final concentration of 1 μM [8]. For study of membrane effect, 200 μL of 1 mg/mL membrane protein (200 μg, equivalent to membranes prepared from 70 μL packed erythrocytes) were incubated with 800 μL of

the radioactive drug (1 μM final concentration) in the same condition as intact cells. At the end of the incubation, intact cells or membranes were pelleted by centrifugation at 10,000 *g* at 4° for 3 min. Amount of drug remaining in the supernatant was determined from its radioactivity. An aliquot of 500 μL supernatant was bleached with 500 μL of 15% H_2O_2 at 60° for 12 hr; 4 mL of Triton-based liquid scintillation fluid were added. The radioactivity was measured in a β-counter (LS1801, Beckman Instrument Inc.) and used for calculation of drug concentration in the supernatant. This drug concentration in the supernatant was then used as the starting drug concentration in the study of drug effectiveness which was determined from its antimalarial activity.

2.4. Antimalarial activity assay

Antimalarial activity of the drug in the supernatant was assayed by using the [^{3}H]-hypoxanthine incorporation method of Desjardins *et al*. [14]. An aliquot (25 μL) of appropriate dilution of the supernatant (ranging from 0.1 to 100 nM DHART) was pipetted into 96 flat-bottom well microtitration plate. Parasitized erythrocyte suspensions containing 1.5% hematocrit with 1–2% parasitemia at the early ring stage were added (200 μL each well). 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) were added into each well and the plate was incubated for another 18 hr under the same condition. Parasitic DNA was harvested onto glass filter paper (Whatman) by using cell harvester (Nunc). Each paper disk was washed with distilled water, dried and placed in toluene-based scintillation fluid for counting in β-counter. The 50% inhibitory concentration (IC_{50}) value was evaluated from the sigmoid graph of percent [^{3}H]-hypoxanthine incorporation vs. log of drug concentration. The drug effectiveness index was calculated from the ratio of IC_{50} of control (drug without cell or membrane)/IC_{50} of sample.

2.5. Measurement of membrane iron

Each membrane sample was diluted with 5 mM phosphate buffer (pH 7.4, iron-free) to an equivalent of 1 mg/mL of protein. An aliquot of 100 μL of erythrocyte membrane (100 μg) was used for each iron assay. Free or non-heme iron in membrane samples was determined by its reactivity with an iron chelator, ferrozine (Sigma) by the method of Kuross and Hebbel [13]. Free iron can rapidly react (within 2 min) with ferrozine in the presence of sodium dodecylsulfate (SDS) and reducing agents (ascorbic acid and sodium metabisulfite). Absorbance of the reaction was measured at 562 nm. Amounts of free iron were calculated and expressed as nmol-free iron/mg membrane protein. Total heme (i.e. free heme, hemoglobin, hemichrome) in membranes was measured by the absorbance at 398 nm after dissolving membrane samples in formic acid [13]. Amounts of heme iron were calculated using a hemoglobin standard (Biosystem) and expressed as nmol heme iron/mg membrane protein.

The protein contents of erythrocyte membrane samples were determined by the method of Lowry *et al*. [15] with bovine serum albumin as standard (Sigma).

2.6. DHART inactivation and membrane iron content in phenylhydrazine-treated normal erythrocyte

To study the possible mechanism of drug inactivation in α-thalassemic erythrocytes, 20% cell suspension of normal erythrocytes in serum-free culture medium was oxidized by adding 1, 10 or 100 μM phenylhydrazine hydrochloride (BDH) in PBS. The mixtures were incubated at 37° for 1 hr. Control cells were treated under the same condition without phenylhydrazine hydrochloride. After incubation, the erythrocytes were washed thrice with serum-free culture medium and resuspended for drug inactivation assay and iron assay as described above.

2.7. DHART accumulation and distribution in oxidized erythrocytes

To study the effect of oxidative stress on drug accumulation and distribution in the cell, normal and thalassemic erythrocytes were treated with 0.1 and 1.0 mM phenylhydrazine hydrochloride and washed by the same procedure as described above. Two sets of duplicate aliquots (70 μL each) of packed-treated erythrocytes were incubated with [^{14}C]-DHART (at final concentration of 1 μM) by the same procedure as the study of drug inactivation by intact erythrocyte. After incubation, the erythrocytes were pelleted and washed thrice with 1.0 mL serum-free culture medium. One set of erythrocytes was lysed with 2% SDS (500 μL each) and bleached with 500 μL of 15% H_2O_2 at 60° for 12 hr. The radioactivity of accumulated drug was measured by a β-counter after adding 4 mL of Triton-based liquid scintillation fluid. For drug distribution measurement, another set of erythrocytes was lysed with 10 vol. of lysis buffer. An aliquot of 500 μL lysate was incubated with an equal volume of 2% SDS prior to bleaching and radioactivity counting as described above. The membranes were washed five times with 1.0 mL of washing buffer and the above process was followed for radioactivity determination. Amounts of the drug accumulated and distributed in the erythrocytes were calculated as pmol/10^6 cells.

2.8. Statistical analysis

The statistical differences of the data were determined by using Mann–Whitney *U*-test for independent data and Sign-Rank test for dependent data. Correlation of parameters studied was determined from linear regression analysis.

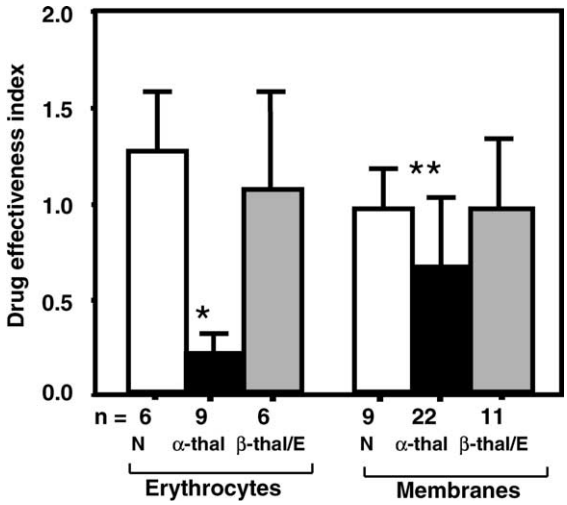

Fig. 2. Antimalarial effectiveness of DHART in the supernatant after incubating the drug (1 μM) with intact cells or membrane compartments of normal or thalassemic erythrocytes at 37° for 2 hr. The drug activity was measured by [^{3}H]-hypoxanthine incorporation assay. The drug effectiveness index was calculated from the ratio of IC_{50} of control (drug without cell or membrane)/IC_{50} of sample. Columns represent mean ± SD. $^*P < 0.01$, $^{**}P < 0.02$ when comparing α-thalassemia with the other cells or membranes; N represents normal samples in the study. For α-thalassemia, there were 4 HbH and 5 HbH/HbCS subtypes for the group of N = 9 and 10 HbH and 12 HbH/HbCS subtypes for the group of N = 22.

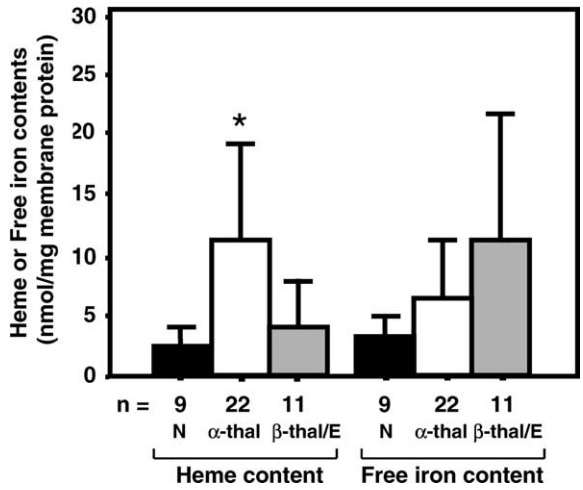

Fig. 3. Total heme and free iron contents in normal and thalassemic membranes. Free iron in membrane samples was determined from its reactivity with an iron chelator, ferrozine, and measuring the absorbance at 562 nm. Total heme was measured by the absorbance at 398 nm after dissolving membrane samples in formic acid. Columns represent mean ± SD. $^*P < 0.01$ when comparing α-thalassemic membrane heme content with the other cell types; N represents normal samples in the study. The α-thalassemia group comprised 10 HbH and 12 HbH/HbCS subtypes.

3. Results

3.1. Dihydroartemisinin inactivation by intact erythrocytes

It was earlier shown [7,8] that, similarly to the reduction of effectiveness of artemisinin by α-thalassemic erythrocytes, the effectiveness of DHART is also reduced. We show here further (Fig. 2) that a part of the reduction in effectiveness was due to preferential inactivation of the drug since pre-incubation with these erythrocytes resulted in reduced effectiveness of the free drug in the supernatant ($P < 0.01$). As shown in Fig. 2, the drug effectiveness index of DHART after incubation with both types of α-thalassemic erythrocytes was five times lower than that after incubation with normal or β-thalassemia/HbE erythrocytes.

3.2. Dihydroartemisinin inactivation by α-thalassemic erythrocyte membrane

Our previous report demonstrated that both α- and β-thalassemic erythrocyte hemolysates could inactivate artemisinin to greater extent than that of the normal hemolysate [7]. We now show that the membrane component of α-thalassemic erythrocytes was also involved in artemisinin inactivation. Significant reduction of DHART activity was demonstrated after incubating the drug with 200 μg membrane protein (Fig. 2, $P < 0.02$). Intact α-thalassemic erythrocytes could reduce the effectiveness of DHART by more than 80%, while the membrane fraction accounted for about 35% of the reduction.

3.3. Ferrozine-reactive iron and heme iron content in erythrocyte membranes

Membrane iron components, heme or non-heme, which are known to be present in higher amount in α-thalassemic erythrocytes than normal erythrocytes [10,11], may be significant factors in reducing drug effectiveness. In order to investigate this possibility, both free (non-heme) iron- and heme iron-bound membrane were determined. Fig. 3 shows the amounts of both forms of membrane iron in the various cell types. Although there was a wide variation in the free and heme-bound iron contents of the membranes (Fig. 3), it was found that α-thalassemic erythrocyte membrane contained significantly higher heme iron content (11.04 ± 8.96 nmol/mg membrane protein, $P < 0.01$) than membranes from normal (2.68 ± 1.28 nmol/mg membrane protein) or β-thalassemia/HbE erythrocytes (3.98 ± 3.98 nmol/mg membrane protein). Decrease in drug effectiveness by membrane was correlated to membrane heme content ($r^2 = 0.460$, $P < 0.01$, Fig. 4A) whereas there was no correlation with free iron content ($r^2 = 0.0001$, $P > 0.05$, Fig. 4B). The correlation between reduction in drug effectiveness index and membrane heme content was more clearly evident for α-thalassemic erythrocytes ($r^2 = 0.636$, $P < 0.01$, Fig. 4C). This indicated that membrane heme was an important factor accounting for drug inactivation by α-thalassemic erythrocytes.

3.4. Role of oxidative stress on membrane heme content, hemoglobin denaturation and DHART ineffectiveness

Under oxidative stress, hemoglobin H may be oxidized, and this oxidized form is associated with cellular

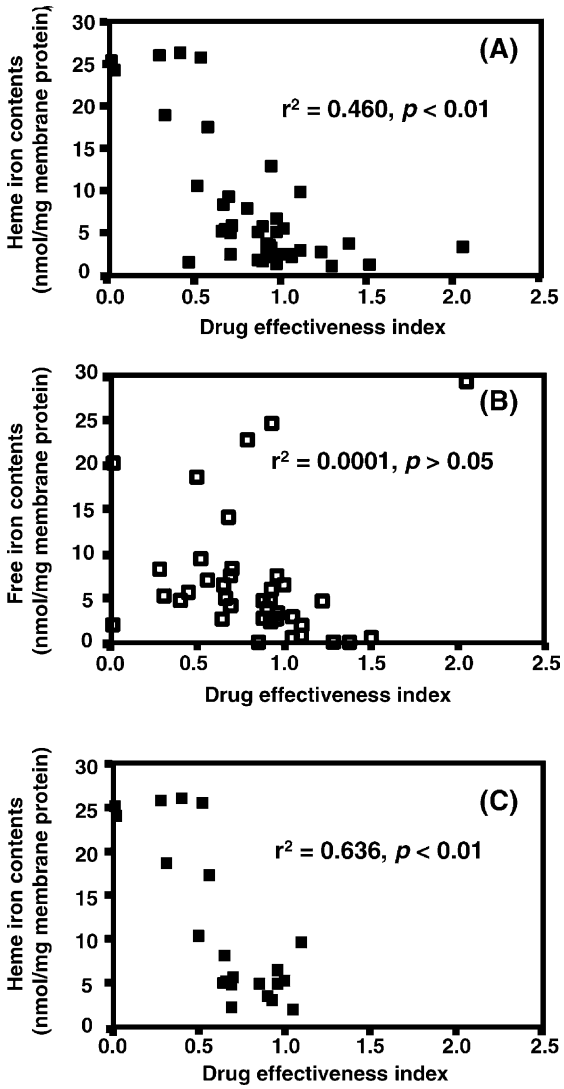

Fig. 4. Correlation of antimalarial effectiveness with membrane heme or iron contents. (A) Correlation of antimalarial effectiveness with membrane heme contents of all samples; (B) lack of correlation of antimalarial effectiveness with membrane-free iron contents of all samples; (C) increased correlation of antimalarial effectiveness with membrane heme contents of α-thalassemic membrane.

membrane [11,16]. In order to investigate the role of oxidative stress on membrane heme and drug ineffectiveness, normal erythrocytes were oxidized with various concentrations of phenylhydrazine. Upon oxidation, hemoglobin is oxidized and denatured to monomeric globin which can associate with cell membrane and causes membrane pathology [17]. After incubating DHART with oxidized genetically normal erythrocytes, the drug effectiveness index decreased in a dose-dependent manner and was significantly reduced when the cells were treated with ≥10 μM phenylhydrazine ($P < 0.015$, Fig. 5). Fold increment of the membrane heme and free iron contents (membrane heme or free iron content of oxidized cells/that of control cells) also directly depended on phenylhydrazine concentration. However,

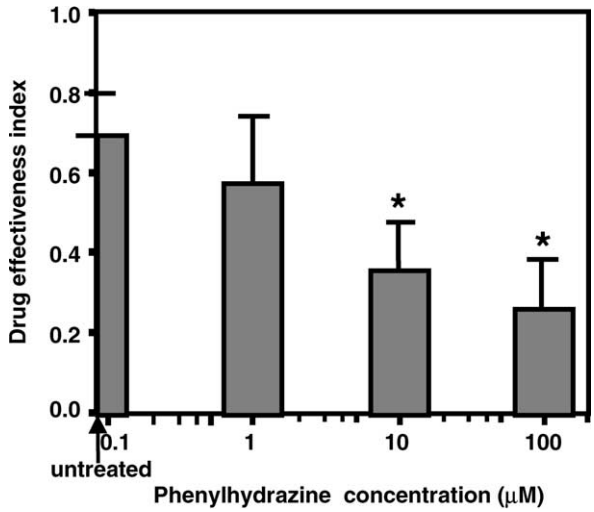

Fig. 5. Antimalarial effectiveness of DHART (1 μM) after incubating with phenylhydrazine-treated and untreated normal erythrocytes (N = 8). Columns represent mean ± SD. *$P < 0.015$ when comparing treated cells with untreated cells.

there was a higher increase of heme content compared to that of free iron (Fig. 6).

3.5. Role of oxidative stress on drug accumulation and distribution in erythrocytes

α-Thalassemic erythrocytes accumulate artemisinin or its derivatives to a greater extent than normal and β-thalassemia/HbE erythrocytes [7]. After treatment of normal and thalassemic erythrocytes with various concentrations of phenylhydrazine, all treated erythrocytes took up more labeled DHART in a dose-dependent manner than control untreated cells (Fig. 7A). DHART accumulation in intact cells showed significant differences between cells

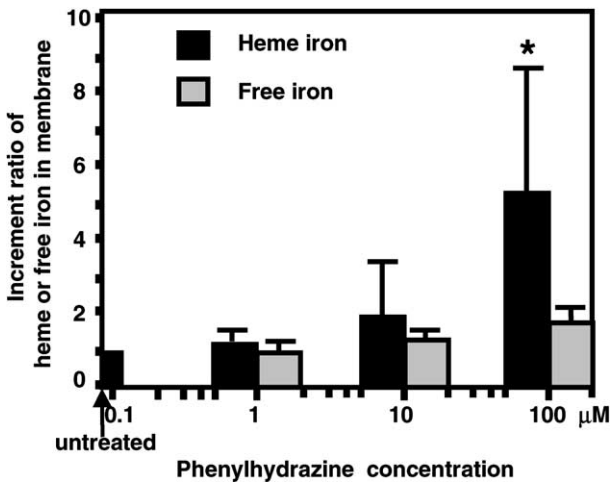

Fig. 6. Increment ratio of heme or free iron contents in membranes of phenylhydrazine-treated normal erythrocytes comparing to untreated cells (N = 8). Columns represent mean ± SD of increment ratio. *$P < 0.015$ when comparing treated cells with untreated cells.

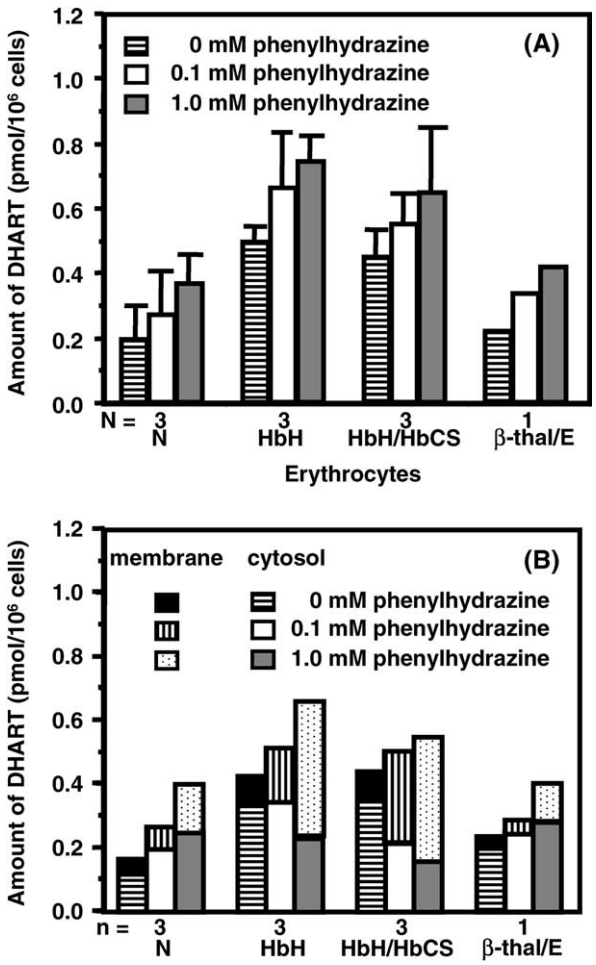

Fig. 7. DHART accumulation in phenylhydrazine-treated normal (N) and thalassemic erythrocytes. (A) Drug accumulation in intact cell; (B) drug distribution in cell compartments. Columns represent mean ± SD, N represents number of samples in the study.

treated with 1 mM phenylhydrazine and untreated cells of normal and HbH cells ($P < 0.05$). The distribution of the drug in the phenylhydrazine-treated erythrocytes is shown in Fig. 7B. Without treatment, there was a higher amount of drug in the cytosolic compartment than in membrane compartment (Fig. 7B). Binding of the drug to membrane compartment increased with increasing concentration of phenylhydrazine. At 1.0 mM phenylhydrazine, the amount of drug bound to membrane compartment was three-fold higher than in untreated samples (Table 1, Fig. 7, $P < 0.025$). The additional drug accumulation in intact erythrocytes after 1.0 mM phenylhydrazine-treated cells was preferentially accumulated in membrane compartment than in cytosol (Table 1).

4. Discussion

Resistance of *P. falciparum* to artemisinin and its derivatives, induced upon infection of α-thalassemic erythrocytes, has been previously reported [18]. The mechanism of host-induced drug resistance is partly due to competition of host cell components for binding with the drugs, and partly to inactivation of the drugs by the cell components [6–8]. In this report, we showed further that the membrane fraction of α-thalassemic erythrocytes was partly responsible for the reduction of effectiveness of DHART in the presence of these erythrocytes. Such reduction was likely due to the presence of heme on the membrane. In contrast, there was no correlation between the drug inactivation and non-heme iron content.

We also showed that reduction in effectiveness of DHART could also be caused by normal erythrocytes after treatment with phenylhydrazine. Phenylhydrazine induces hemoglobin oxidation and elevation of heme at the membrane [17], and therefore acts as agent which mimics the effect of α-thalassemia. Membranes prepared from severe types of thalassmic diseases are known to have an increased globin content (α-globin in β-thalassemia and β-globin in α-thalassemia) which is partially oxidized [10,19]. Heme release would consequently occur and lead to intercalation of free heme into the lipid bilayer [20]. Release of free iron from heme or hemichrome catalyzes membrane lipid peroxidation [16]. It was not clear whether the membrane-attached heme, which caused inactivation

Table 1
DHART accumulation and distribution in 1.0 mM phenylhydrazine (PHZ)-treated cells compared to untreated cells

Cell components	DHART accumulation (pmol/10^6 cells)[a]			
	Normal (N = 3)	HbH (N = 3)	HbH/HbCS (N = 3)	β-thal/HbE (N = 1)
Intact cells				
Untreated	0.20 ± 0.11	0.50 ± 0.05	0.46 ± 0.07	0.22
PHZ-treated	0.37 ± 0.09	0.75 ± 0.05	0.65 ± 0.04	0.42
Cytosol				
Untreated	0.12 ± 0.09	0.33 ± 0.05	0.35 ± 0.07	0.20
PHZ-treated	0.25 ± 0.08	0.23 ± 0.02	0.16 ± 0.01	0.28
Membrane				
Untreated	0.04 ± 0.02	0.10 ± 0.04	0.12 ± 0.04	0.04
PHZ-treated	0.15 ± 0.01	0.44 ± 0.03	0.39 ± 0.16	0.12

[a] Values represent mean ± SD.

of DHART, was free heme or associated with globin or both. In any case, the heme must be exposed enough to bind with DHART, resulting in its subsequent inactivation. This supports the role of hemoglobin oxidation and presence of erythrocyte membrane-bound heme in DHART inactivation. Asawamahasakda *et al*. [21] demonstrated intact cell did not bind [^{3}H]-DHART. However, our study found that about 10% of the drug could accumulate in normal intact erythrocyte, and about 30–40% of the drug accumulated in α-thalassemic erythrocytes. Alkylation of the drug to membrane proteins of Asawamahasakda *et al*. [21] supports our finding of higher drug accumulation in membrane after oxidation.

Inactivation of DHART by heme may occur through a mechanism similar to the mechanism of drug action leading to parasite death [22]. This probably involves cleavage of the peroxide bridge, mediated by heme iron, and production of reactive intermediates, which then undergo reaction with parasite protein targets [23]. However, reaction of DHART with erythrocyte membrane heme can lead to its premature inactivation, thereby rendering it ineffective in subsequent exposure to the parasite.

Hutagalung *et al*. [24] found the opposite phenomenon in malarial patients with HbE trait. They reported faster parasite clearance rate in HbE trait patients treated by artemisinin when compared to malarial patient controls with normal Hb type. HbE is by nature more similar to HbA and more stable than HbH, therefore, it should have little effect on artemisinin ineffectiveness. In our study, β-thalassemia/HbE erythrocytes which contain HbE showed effects of both intact cell and membrane compartment on drug effectiveness similar to normal erythrocytes (Fig. 2). Higher parasite clearance rate in HbE trait subjects may be due to impaired growth of parasites [25] and/or enhanced phagocytosis of infected HbE red blood cell by monocyte or macrophage [26].

Pharmacokinetic studies of artesunate, an artemisinin derivative, in α-thalassemic patients demonstrated significantly higher level of plasma drug concentration in α-thalassemia than normal individuals [27]. However, their study also showed a faster reduction rate of active drug in α-thalassemic plasma than normal plasma consistent with the phenomena reported here and elsewhere [7].

Although our observation was made only with α-thalassemic erythrocytes, it is possible that other abnormal erythrocytes, such as sickle erythrocytes, could undergo similar processes of drug inactivation because of high membrane heme and free iron content and its sensitivity to oxidation [28]. However, the β-thalassemia/HbE erythrocytes appear not to have such inactivating capacity. This may be ascribed to a greater uptake of artemisinin and its derivatives by α-thalassemic erythrocytes compared to β-thalassemia/HbE and normal cells [7,8]. Nevertheless, with the likelihood of wider deployment of artemisinin and derivatives in the near future, it is important to find out whether similar reduction in drug effectiveness exists with other types of genetic erythrocyte abnormalities.

Acknowledgments

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References

[1] Newton P, White N. Malaria: new developments in treatment and prevention. Ann Rev Med 1999;50:179–92.
[2] Price RN. Artemisinin drugs: novel antimalarial agents. Expert Opin Investig Drugs 2000;9:1815–27.
[3] Hien TT, White NJ. Qinghaosu. Lancet 1993;341:603–8.
[4] van Agtmael MA, Eggelte TA, van Boxtel CJ. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. Trends Pharmacol Sci 1999;20:199–205.
[5] White NJ. Artemisinin: current status. Trans R Soc Trop Med Hyg 1994;88(Suppl 1):S3–4.
[6] Kamchonwongpaisan S, Chandra-ngam G, Avery MA, Yuthavong Y. Resistance to artemisinin of malaria parasites (*Plasmodium falciparum*) infecting α-thalassemic erythrocytes *in vitro*. Competition in drug accumulation with uninfected erythrocytes. J Clin Invest 1994;93:467–73.
[7] Charoenteeraboon J, Kamchonwongpaisan S, Wilairat P, Vattanaviboon P, Yuthavong Y. Inactivation of artemisinin by thalassemic erythrocytes. Biochem Pharmacol 2000;59:1337–44.
[8] Vattanaviboon P, Wilairat P, Yuthavong Y. Binding of dihydroartemisinin to hemoglobin H: role in drug accumulation and host-induced antimalarial ineffectiveness of α-thalassemic erythrocytes. Mol Pharmacol 1998;53:492–6.
[9] Meshnick SR, Taylor TE, Kamchonwongpaisan S. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol Rev 1996;60:301–15.
[10] Schrier SL. Thalassemia: pathophysiology of red cell changes. Ann Rev Med 1994;45:211–8.
[11] Shinar E, Rachmilewitz EA. Oxidative denaturation of red blood cells in thalassemia. Semin Hematol 1990;27:70–82.
[12] Bothwell TH, Charlton RW. Current problems of iron overload. Recent Results Cancer Res 1979;69:87–95.
[13] Kuross SA, Hebbel RP. Non-heme iron in sickle erythrocyte membranes: association with phospholipids and potential role in lipid peroxidation. Blood 1988;72:1278–85.
[14] Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. Antimicrob Agents Chemother 1979;16:710–8.
[15] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[16] Hebbel RP, Eaton JW. Pathobiology of heme interaction with the erythrocyte membrane. Semin Hematol 1989;26:136–49.
[17] Chiu D, Lubin B. Oxidative hemoglobin denaturation and RBC destruction: the effect of heme on red cell membranes. Semin Hematol 1989;26:128–35.

[18] Yuthavong Y, Butthep P, Bunyaratvej A, Fucharoen S. Decreased sensitivity of artesunate and chloroquine of *Plasmodium falciparum* infecting hemoglobin H and/or hemoglobin constant spring erythrocytes. J Clin Invest 1989;83:502–5.

[19] Advani R, Sorenson S, Shinar E, Lande W, Rachmilewitz E, Schrier SL. Characterization and comparison of the red blood cell membrane damage in severe human α- and β-thalassemia. Blood 1992;79:1058–63.

[20] Cannon JB, Kuo FS, Pasternack RF, Wong NM, Muller-Eberhard U. Kinetics of the interaction of hemin liposomes with heme binding proteins. Biochemistry 1984;23:3715–21.

[21] Asawamahasakda W, Benakis A, Meshnick SR. The interaction of artemisinin with red cell membranes. J Lab Clin Med 1994;123:757–62.

[22] Gilliars PLHR, Meunier B, Yuthavong Y. Possible modes of action of the artemisinin-type compounds. Trends Parasitol 2001;17:122–6.

[23] Jefford CW, Vicente MGH, Jacquier Y, Favarger F, Mareda J, Millasson-Schmidt P, Brunner G, Burger U. The deoxygenation and isomerization of artemisinin and artemether and their relevance of antimalarial action. Helv Chim Acta 1996;79:1475–87.

[24] Hutagalung R, Wilairatana P, Looareesuwan S, Brittenham GM, Gordeuk VR. Influence of hemoglobin E trait on the antimalarial effect of artemisinin derivatives. J Infect Dis 2000;181:1513–6.

[25] Vernes A, Haynes J, Tang D, Dutoit E, Diggs C. Decreased growth of *Plasmodium falciparum* in red cells containing hemoglobin E, a role for oxidative stress, and a sero-epidemiological correlation. Trans R Soc Trop Med Hyg 1986;80:642–8.

[26] Bunyaratvej A, Butthep P, Yuthavong Y, Fucharoen S, Khusmith S, Yoksan S, Wasi P. Increased phagocytosis of *Plasmodium falciparum*-infected erythrocytes with hemoglobin E by peripheral blood monocytes. Acta Hematol 1986;76:155–8.

[27] Ittarat W, Looareesuwan S, Pootrakul P, Sumpunsirikul P, Vattanavibool P, Meshnick SR. Effects of α-thalassemia on pharmacokinetics of the antimalarial agent artesunate. Antimicrob Agents Chemother 1998; 42:2332–5.

[28] Repka T, Shalev O, Reddy R, Yuan J, Abrahamov A, Rachmilewitz EA, Low PS, Hebbel RP. Non-random association of free iron with membranes of sickle and β-thalassemic erythrocytes. Blood 1993; 82:3204–10.