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Artemisinin effectiveness in erythrocytes is reduced by heme and heme-containing proteins

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ABSTRACT

Artemisinin loses its antimalarial activity on prolonged exposure to erythrocytes, especially α -thalassemic erythrocytes. In this report, we show that the major artemisinin-inactivating factor in cytosol of normal erythrocytes was heat-labile but a heat-stable factor from α -thalassemic cells also played a significant role in reducing artemisinin effectiveness, which was shown to be heme released from hemoglobin (Hb). Studies of fractionated lysate from genetically normal erythrocytes revealed that the protein fraction with molecular weight greater than 100 kDa was capable of reducing artemisinin effectiveness more readily than lower molecular weight fraction. Catalase and Hb A, but not selenoprotein glutathione peroxidase, were capable of reducing artemisinin effectiveness. Hemin (ferriprotoporphyrin IX) also reduced artemisinin effectiveness in a concentration- and time-dependent manner. It is concluded that heme and heme-containing proteins in erythrocyte are largely responsible for reducing artemisinin effectiveness and may contribute to resistance of *Plasmodium falciparum* infecting α -thalassemic erythrocytes observed in vitro.

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1. Introduction

Plasmodium falciparum malaria remains one of the most lethal and widespread diseases in tropical regions due to the resistance of the parasite to most available antimalarial drugs [1]. Artemisinin or qinghaosu, a sesquiterpene lactone containing an endoperoxide bridge, and its derivatives are effective antimalarials that act rapidly to clear parasites in multidrug resistant malaria with no serious side effects. They form the prominent class of drugs for treatment of falciparum

malaria [2,3]. Several successful regimens have been developed with artemisinin derivatives in combination with other antimalarials that have more prolonged drug action [4].

Host-dependent artemisinin resistance has been shown in *P. falciparum* infecting α -thalassemic erythrocytes, including those containing hemoglobin (Hb) H alone (α -thal1/ α -thal2) or Hb H with Hb Constant Spring (CS) (Hb H/Hb CS or α -thal1/Hb CS) [5–8]. The resistance is associated with the fact that these genetically variant erythrocytes are capable of taking up a large portion of artemisinin drugs [6] due to a higher binding

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Abbreviations: Hb H, α -thalassemia1/ α -thalassemia2; Hb H/Hb CS, α -thalassemia1/hemoglobin Constant Spring; Hb A, hemoglobin A; GSH-Px, glutathione peroxidase; IC₅₀, 50% inhibitory concentration; MW, molecular weight; kDa, kilodalton; p, p-value

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affinity of the drugs to Hb H in α -thalassemic erythrocytes than to Hb A [7]. In addition, both α -thalassemic and normal erythrocytes are capable of interacting with artemisinin, thereby reducing artemisinin effectiveness. Factors responsible for reducing artemisinin effectiveness are found in both cytosolic and, to a lesser extent, membrane fractions. Purified Hb H and membrane heme have been reported to markedly reduce artemisinin effectiveness [8,9]. Since the effect in modifying artemisinin effectiveness is more pronounced in the cytosolic fraction, both in normal and Hb H erythrocytes, and that Hb H is present in limited amounts, we hypothesize that in addition to Hb H and membrane heme, other cytosolic components present in both normal and thalassemic cytosol may also play a major role in reducing artemisinin effectiveness.

In this report, we investigated cytosolic fractions of normal and α -thalassemic erythrocytes that are responsible for reducing artemisinin effectiveness, and determined that in addition to Hb H and membrane heme, free heme and hemoproteins in the cytosol, such as catalase and Hb A, are also responsible for reducing artemisinin effectiveness.

2. Methods and materials

2.1. Materials

Artemisinin was purchased from the National Center of Natural Science and Technology, Hanoi, Vietnam, and recrystallized as white needles from a dichloromethane/hexane mixture (mp 154–156 °C). Radioactive 15- ^{14}C -artemisinin (26.1 Ci/mol) was a gift from the Research Triangle Institute (NC, USA). 8- ^3H -Hypoxanthine (27.1 Ci/mmol) was purchased from Moravex Biochemicals (CA, USA). Bovine liver catalase, human glutathione peroxidase (GSH-Px), human Hb A and hemin were purchased from the Sigma Chemical Co. (MO, USA). Deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one) was a generous gift from Dr. Kovit Pattanapanyasat, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Microcon[®] filters and Centrprep[®] tubes were obtained from Amicon (MA, USA).

2.2. Subjects

Blood samples, in citrate-phosphate-dextrose solution, were obtained from the Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University, Thailand. All thalassemic subjects (α -thal1/ α -thal2 and α -thal1/Hb CS) were non-splenectomized and had received no blood transfusion for at least 3 months before the collection of blood. Samples from normal individuals were obtained voluntarily and were examined for their hematological parameters. No subjects had a previous history of malaria infection.

2.3. In vitro culture of *P. falciparum*

P. falciparum K1 strain (chloroquine- and pyrimethamine-resistant), originally isolated from a malaria patient in Kanchanaburi province, Thailand [10], was maintained using

the ‘candle jar’ culture technique as described by Trager and Jensen [11]. Parasites were grown in human erythrocytes (blood group O) using RPMI 1640 medium supplemented with 25 mM HEPES, pH 7.4, 0.2% NaHCO_3 , 40 $\mu\text{g/mL}$ of gentamycin and 10% human serum. For assay of antimalarial activity, parasite growth was synchronized at the ring stage by 5% sorbitol treatment [12] and growth monitored by measuring incorporation of ^3H -hypoxanthine into parasites (see below).

2.4. Preparation of membrane-free erythrocyte cytosol

One milliliter of 50% (v/v) erythrocyte suspension was lysed by addition of 4.5 mL of hypotonic solution containing 10 mM sodium phosphate buffer, pH 7.4, by a cycle of freeze-thawing in liquid nitrogen to obtain complete lysis. The lysed erythrocyte suspension was centrifuged at $10,000 \times g$ (Mikro 24–48R centrifuge, Hettich, Tuttlingen, Germany) for 10 min at 4 °C to sediment membrane. The membrane pellet was discarded and the supernatant used as membrane-free lysate (cytosolic fraction) in further experiments [8].

In some experiments, membrane-free lysates from normal and α -thalassemic individuals (equivalent to 10%, v/v, erythrocytes) were heated in boiling water for 5 min. Following centrifugation at $10,000 \times g$ for 5 min to remove precipitated proteins, the supernatant (heated lysate) was further filtered through Microcon-10[®] filter to remove protein remnants. These fractions were tested for their effects on artemisinin effectiveness.

2.5. Fractionation of cytosol

To investigate components responsible for artemisinin inactivation, 15 mL aliquot of the membrane-free lysate was filtered by centrifugation through a Centrprep-10[®] tube at $1366 \times g$ until 9 mL of the filtrate (designated as F0, MW less than 10 kDa) were collected. A 3 mL aliquot of retentate (L, MW higher than 10 kDa) was further filtered through a Centricon-100[®] tube at $1300 \times g$ until the retentate was reduced to 20% of its initial volume. To the retentate (R100, MW higher than 100 kDa) were added four volumes of F0 (F0 did not inactivate artemisinin) and the resulting solution was filtered as described above to remove any remaining small molecules with MW less than 100 kDa. This procedure was repeated once more and the retentate (R100) was adjusted to its original volume (3 mL) with F0. The first filtrate and the other filtrates (MW less than 100 kDa) were combined and fractionated through Centricon-50[®] tube to give R50 (50 kDa < MW < 100 kDa) and then through a Centricon-10[®] tube, to give R10 (10 kDa < MW < 50 kDa). Aliquots of 270 μL from each fraction (L, R10, R50 and R100) were then used to determine their effects on artemisinin activity.

2.6. Determination of effect of cytosolic fractions on artemisinin activity

Aliquots (270 μL) of the membrane-free cytosol, cytosolic fractions, or proteins equivalent to 10% (v/v) erythrocytes, were incubated at 37 °C with 30 μL of 10 μM ^{14}C -artemisinin, for 0, 2, 6, 12 and 24 h. The final concentration of the radiolabeled drug was 1 μM . As control, phosphate-buffered

saline was mixed with [^{14}C]-artemisinin and treated under the same conditions. In some experiments, the lysate was pre-treated with 5 μM deferiprone for 30 min at 37 °C prior to incubating with artemisinin. After incubation, the mixture was filtered through 10 kDa cut-off membrane (Microcon-10[®]) by centrifuging at $22,620 \times g$ for 15 min at 4 °C to separate free drug from the mixture. The concentrations of the free drug in the filtrates were determined from radioactivity counting in a liquid scintillation counter (Packard, IL, USA). These filtrates were then tested for their effects on artemisinin effectiveness.

Artemisinin effectiveness of each sample was determined by the [^3H]-hypoxanthine incorporation method [13]. In brief, the filtrate was four-fold serially diluted with culture medium. Aliquots of 25 μL of diluted samples were placed in a 96-well plate together with 200 μL of a 1.5% parasite-infected erythrocyte suspension, 1% parasitemia at the early ring stage. The cell suspensions were incubated in a candle jar at 37 °C for 24 h, and then an aliquot of 25 μL (0.25 μCi) of [^3H]-hypoxanthine was added to each well. The suspensions were incubated under the candle-jar condition for a further 18–24 h. Parasites were harvested onto glass filters (Unifilter[®], Packard), which were then dried and radioactivity measured in a liquid scintillation counter (Packard). Less than 1% of the radioactivity was from the [^{14}C]-artemisinin in the diluted filtrate. The IC_{50} value was determined from the sigmoid curve of percent [^3H]-hypoxanthine incorporation versus log of artemisinin concentration. Drug effectiveness index was defined as IC_{50} of control/ IC_{50} of test sample [8].

2.7. Statistical analysis

Statistical analysis was performed using Mann–Whitney U-test for data from independent random samples and Sign-Rank test for data from dependent samples.

3. Results

3.1. Effect of erythrocyte lysate and membrane-free lysate on artemisinin effectiveness

Reduction of antimalarial activity of artemisinin by cytosolic and membrane fractions of normal and both types of α -thalassemic (Hb H and Hb H/Hb CS) erythrocytes has been reported previously [8]. The effect of membrane fraction on limiting artemisinin effectiveness correlates with membrane heme content of these cells [9]. We investigated factors other than Hb H in the membrane-free lysates of normal and Hb H-containing erythrocytes that could reduce artemisinin effectiveness. As shown in Fig. 1, upon 2 h exposure of membrane-free lysates of normal, Hb H and Hb H/Hb CS erythrocytes to 1 μM [^{14}C]-artemisinin, the effectiveness index of artemisinin of membrane free lysates was significantly decreased from 1.0 ± 0.12 in control buffer to 0.67 ± 0.06 for normal ($n = 5$, p -value ≤ 0.01), 0.57 ± 0.01 for Hb H ($n = 5$, p -value ≤ 0.01) and 0.37 ± 0.01 for Hb H/Hb CS cells ($n = 5$, p -value ≤ 0.02). After 12 h of incubation, drug effectiveness index was further decreased to 0.35 ± 0.1 , 0.27 ± 0.07 and 0.25 ± 0.06 for normal, Hb H and Hb H/Hb CS lysate, respectively, and remained unchanged for up to 24 h of

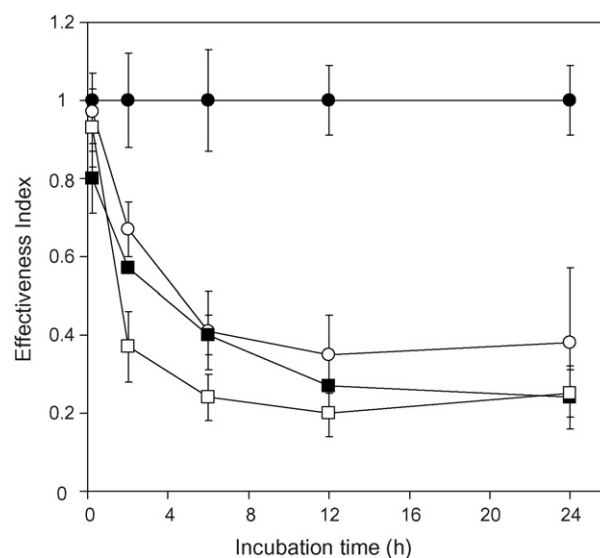


Fig. 1 – Antimalarial effectiveness of free [^{14}C]-artemisinin exposed to membrane-free lysate of normal and α -thalassemic lysate. A 270 μL aliquot of lysate sample was incubated at 37 °C with 1 μM [^{14}C]-artemisinin for 0, 2, 6, 12 and 24 h. After incubation, free drug was separated by ultracentrifugation through 10 kDa cut-off membrane and its effectiveness index was determined. Phosphate buffer control (●), normal (○), Hb H lysate (■), Hb H/Hb CS lysate (□). Mean and S.E.M. of five subjects are shown.

incubation of [^{14}C]-artemisinin with these membrane-free lysates. These data confirmed previous observation [8] that membrane-free lysates from normal, Hb H and Hb H/Hb CS erythrocytes have the ability to reduce artemisinin effectiveness and that the inactivation is more pronounced in lysates from Hb H-containing red cells.

3.2. Effect of heated erythrocyte lysate on artemisinin effectiveness

We hypothesized that proteins in the cytosol may be responsible for reducing artemisinin effectiveness. In order to demonstrate this, lysates were heated in boiling water for 5 min and centrifuged to sediment any precipitated protein. The supernatants were then subjected to artemisinin effectiveness assay. As summarized in Table 1, heated lysate from normal erythrocytes lost its ability to reduce artemisinin effectiveness during the first 6 h of incubation compared to unheated lysate (0.90 ± 0.10 versus 0.41 ± 0.10 , p -value ≤ 0.02). However, after 24 h exposure, the effect of heated lysate on reducing artemisinin effectiveness was similar to that of unheated lysate (0.29 ± 0.13 versus 0.38 ± 0.19). This suggests that factors responsible for reducing artemisinin effectiveness in normal erythrocyte lysate are heat-labile components, presumably proteins, but heat stable components were also present either at low concentrations and/or with low capability to reduce artemisinin effectiveness.

In contrast to the results from heated normal erythrocyte lysate, heated lysates of Hb H-containing erythrocytes

Table 1 – Comparison between antimalarial effectiveness of artemisinin following exposure to membrane-free unheated and heated hemolysates

| Type of lysate | $[^{14}\text{C}]$ -Artemisinin effectiveness index | | |
|----------------------------------|--|-----------------|-----------------|
| | Normal | Hb H | Hb H/Hb CS |
| Lysate (6 h) | 0.41 ± 0.10 | 0.4 ± 0.05 | 0.24 ± 0.06 |
| Lysate (24 h) | 0.38 ± 0.19 | 0.24 ± 0.08 | 0.25 ± 0.06 |
| Heated lysate (6 h) | 0.90 ± 0.10 | 0.35 ± 0.06 | 0.26 ± 0.05 |
| Heated lysate (24 h) | 0.29 ± 0.13 | 0.22 ± 0.04 | 0.26 ± 0.08 |
| Filtrate of lysate (24 h) | 0.98 ± 0.04 | 1.08 ± 0.07 | 0.97 ± 0.04 |
| Filtrate of heated lysate (24 h) | 0.91 ± 0.13 | 1.08 ± 0.11 | 1.06 ± 0.06 |

A 270 μL aliquot of lysate sample was incubated 1 μM $[^{14}\text{C}]$ -artemisinin at 37 °C for 0, 6, 12 and 24 h. Free drug was separated and effectiveness index determined after incubation. Numbers of samples in the tests were five for control and unheated lysate and four for heated lysate. The results are presented as mean and S.E.M.

retained their ability to reduce artemisinin effectiveness to a similar extent as their corresponding unheated lysates following 6 and 24 h incubation (Table 1). As SDS-PAGE of these heated lysates indicated the presence of only small amounts of proteins (data not shown), these samples (heated and non-heated) were filtered through a 10 kDa cut-off membrane to eliminate the proteins. Surprisingly, the filtrate from both the heated lysate of normal and Hb H-containing erythrocytes exhibited no significant effect on artemisinin effectiveness for up to 24 h of incubation.

3.3. Effect of heme in heated thalassemic erythrocyte lysate on artemisinin effectiveness

From the above observations we hypothesized that the supernatant of heated lysate from Hb H-containing erythrocytes contained heme, thus causing the reduction in artemisinin effectiveness. Spectrophotometric properties of these lysates and their filtrate fractions, both before and after heating, were examined at wavelengths between 250 and 650 nm. Heme and hemin solutions in 10 mM phosphate-buffered saline, pH 7.4, containing 1% SDS (to maintain heme and hemin in monomeric form) were used as reference spectra. Heme and hemin possess absorption peaks around 350–450 nm with their maximum absorption at approximately 415 and 407 nm, respectively (Fig. 2). The absorption peak of hemin was removed by filtration, suggesting that hemin was unable to pass through the membrane. Likewise, lysate and heated lysate also showed absorption peaks in the same region with their maximum absorption at about 415 and 407 nm, respectively. These absorption peaks of lysate and heated lysate were not observed in their filtrate fractions, indicating the presence of heme/hemin in the heated lysates, presumably released from Hb H.

3.4. Effect of heme on artemisinin effectiveness

Artemisinin activity was found to decrease following incubation with hemin in a concentration- and time-dependent manner (Fig. 3). It is worth noting that the concentration of hemin needed to exert an artemisinin inactivation effect equivalent to 10% thalassemic erythrocyte lysate was greater than 0.2 μM . It has been reported that both heme- and non-heme iron are found at higher levels in α -thalassemic than normal erythrocytes [14,15]. It is possible

that such iron-containing compounds may lead to a faster rate of drug inactivation by Hb H-containing lysate than that by normal lysate. To study the possible role of non-heme iron in the hemolysate in inactivating artemisinin, deferiprone (L1), an effective oral iron chelator used in treatment of thalassemia [16], was added to chelate non-heme iron. The effectiveness index (0.57 ± 0.09) of artemisinin following 2 h exposure to deferiprone-treated Hb H lysate was not significantly different from untreated Hb H lysate (0.62 ± 0.07) (Fig. 4B). Similar effect was obtained with deferiprone-treated normal erythrocyte lysate (effectiveness index of 0.84 ± 0.1 versus 0.81 ± 0.05 for untreated hemolysate) (Fig. 4A). The effectiveness index of artemisinin continued to decrease up to 24 h of incubation with no significant differences between deferiprone-treated and non-treated lysates from normal (0.47 ± 0.05 versus 0.43 ± 0.06) and Hb H (0.3 ± 0.12 versus 0.37 ± 0.03) erythrocytes. Altogether, these results indicate that chelatable non-heme iron in the hemolysates is not responsible for reducing artemisinin effectiveness.

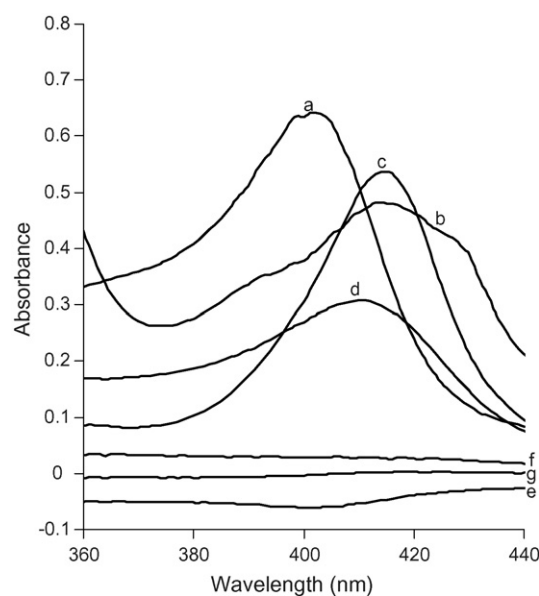


Fig. 2 – Spectra of heme (a), hemin (b), lysate (c) and heated lysate (d) and filtrates of hemin (e), lysate (f) and heated lysate (g).

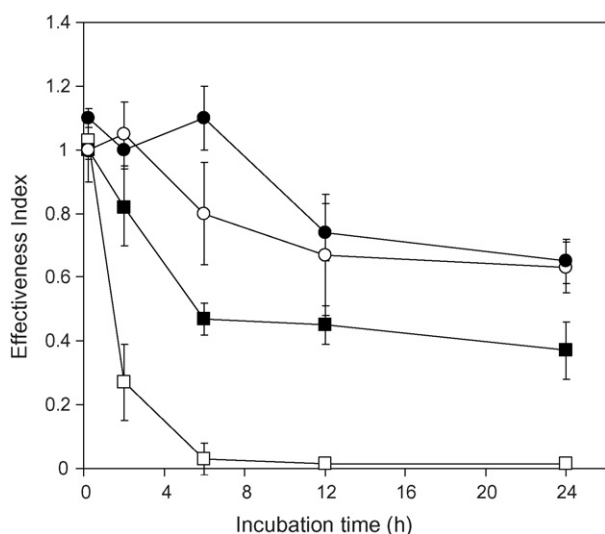


Fig. 3 – Antimalarial effectiveness index of [^{14}C]-artemisinin (1 μM) following exposure to various hemin concentrations at 37 °C. 0.002 μM (●), 0.02 μM (○), 0.2 μM (■) and 1 μM (□).

3.5. Identification of proteins in normal hemolysate that interact with artemisinin

In order to identify proteins responsible for reducing artemisinin effectiveness, lysate (L) from normal erythrocytes was fractionated using filter membranes with different MW cut-off levels to obtain fractions R10, R50 and R100 containing proteins with molecular weight less than 50 kDa, between 50 and 100 kDa, and higher than 100 kDa, respectively. An aliquot of each fraction was subjected to artemisinin effectiveness assay by incubating with [^{14}C]-artemisinin for 15 min and 6 h. As shown in Fig. 5, the effectiveness index of artemisinin after 15 min incubation with fraction L, R10, R50 and R100 was 0.7 ± 0.05 , 0.84 ± 0.11 , 0.9 ± 0.09 and 0.58 ± 0.11 , respectively, suggesting that fraction R100 with proteins of greater than

100 kDa contained the major artemisinin-inactivating factors, while fractions R50 and R10 had only minimal effects on artemisinin effectiveness at this short period of treatment. After 6 h of incubation, the effectiveness index was further reduced to 0.57 ± 0.06 , 0.50 ± 0.07 , 0.34 ± 0.12 and 0.37 ± 0.11 for L, R10, R50 and R100, respectively, indicating that, given sufficient time, all the fractions were capable of reducing artemisinin effectiveness, albeit to different extents.

3.6. Effect of antioxidant enzymes and hemoproteins on artemisinin effectiveness

The above observation indicates that fraction R100 has a more pronounced role in affecting artemisinin effectiveness than fraction R50. This led us to investigate possible cytosolic proteins with molecular weight higher than 100 kDa and between 50 and 100 kDa on artemisinin effectiveness. On SDS-PAGE, the majority of proteins stained with Coomassie blue in lysate, R100 and R50 was hemoglobin. In fraction R50 and R10, protein band of molecular weight of 32 kDa was also observed. Immunoblot of the gel on nitrocellulose membrane with anti-Hb antibody revealed approximately equal amounts of hemoglobin. Although the band of catalase was not observed on Coomassie blue-stained gel in these fractions, it was detected by anti-catalase antibody in fraction R100 and R50, with higher amount in R100 fraction (data not shown).

Based on the detection of catalase and hemoglobin in fractions R100 and R50, which are capable of reducing artemisinin effectiveness, catalase, glutathione peroxidase (GSH-Px) and Hb were therefore selected for artemisinin effectiveness assay. Interestingly, artemisinin effectiveness decreased following exposure to 1080–10,800 units/mL (0.4–4 μM) of catalase (Fig. 6A) and 0.1–200 μM Hb A (Fig. 6B) in a time- and dose-dependent manner whereas drug effectiveness was not affected following exposure to 0.01–1.0 U/mL of GSH-Px (in the presence of 2 mM glutathione), remaining at 0.98–1.0 for up to 12 h of incubation. These results suggest that Hb and catalase may be the major proteins in the erythrocytes responsible for reducing artemisinin effectiveness.

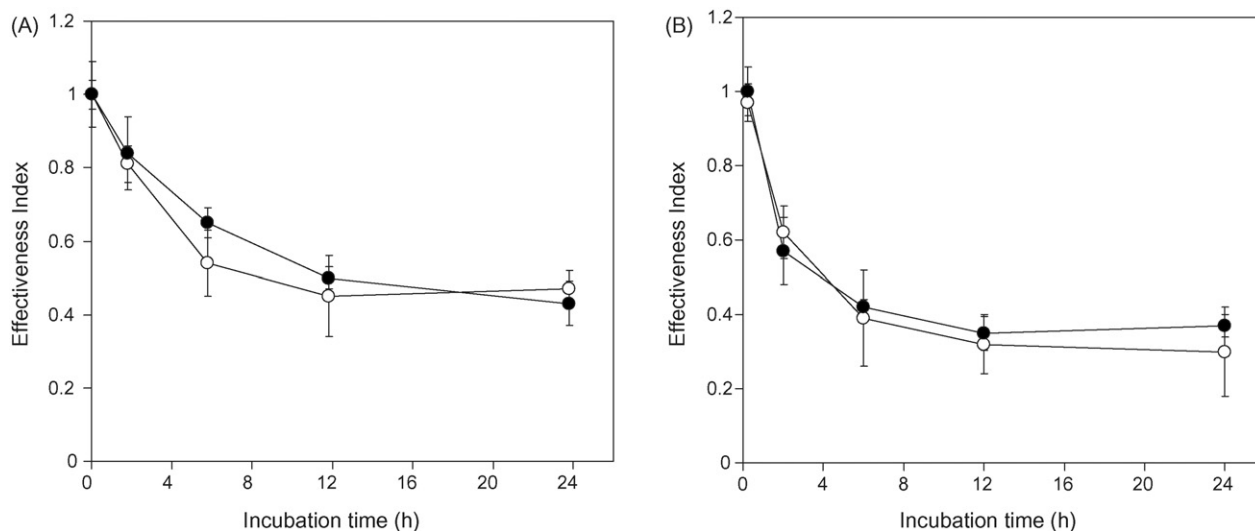


Fig. 4 – Ineffectiveness of deferipone (L1) (5 μM) in reversing inactivation of artemisinin by normal (A) and Hb H (B) lysate. (●) without L1 and (○) with L1.

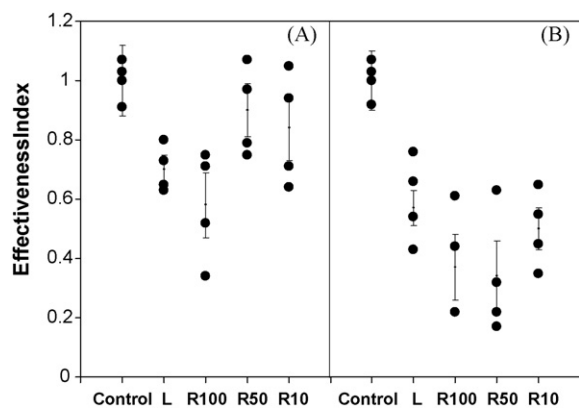


Fig. 5 – Artemisinin effectiveness indices of cytosolic (L) and its fractions with molecular weight higher than 100 kDa (R100), 100–50 kDa (R50) and 50–10 kDa (R10). An aliquot of 270 μ L from each fraction was incubated with 1 μ M [14 C]-artemisinin at 37 $^{\circ}$ C for 15 min (A) and for 6 h (B). Phosphate buffer served as a control.

4. Discussion

Artemisinin-resistant parasites are found in vitro when α -thalassemic erythrocytes, both Hb H and Hb H/Hb CS, are used for cultivation [5,6]. The reduced artemisinin effectiveness against *P. falciparum*-infecting thalassemic erythrocytes is due partly to competition of the host cell components for binding with the drug [6,7], and partly due to an inactivation of the drug by cell components in both cytosolic and membrane fractions [8,9]. The cytosolic fraction is mostly responsible for the reduction in drug effectiveness, but the membrane fraction also has a significant effect. Our previous studies showed that Hb H in the cytosol and heme in the membrane

could reduce artemisinin effectiveness [8,9]. In this study, such effects on reducing artemisinin effectiveness were confirmed in membrane-free lysate of normal, Hb H and Hb H/Hb CS erythrocytes. We also demonstrated that hemoprotein (catalase), free heme and non-chelatable iron in the lysate were the major factors responsible for reducing artemisinin effectiveness.

In membrane-free lysate prepared from normal and α -thalassemic erythrocytes, protein components played a major role in reducing artemisinin effectiveness. In normal hemolysate, the effect on reducing artemisinin effectiveness was abolished upon heating, as well as by filtration to remove the protein content. On the other hand, both heated and unheated lysate of Hb H-containing samples readily inhibited artemisinin, probably due to their high content of accessible heme, especially in the heated lysate. However, the inactivating effects of heated and unheated Hb H lysates on artemisinin effectiveness were abolished upon filtration when both protein and heme (released from Hb H) were filtered out. In the presence of cyanide, a strong pi-binding ligand which forms a six-coordinated low spin stable complex with heme as characterized by the shift of absorption peak from 403 to 419 and 550 nm [17], the filtrate of the cyanide-treated heated-HbH lysate could resume its capability of reducing artemisinin effectiveness, with an effectiveness index of 0.61 ± 0.06 after 6 h of incubation, thus confirming the presence of heme in the heated lysate. It was estimated from measurements of the absorption spectra that heated Hb H-containing lysate contained 1.5 times higher level of released heme than normal heated lysate.

In screening fractions of normal lysate for proteins that may be capable of reducing artemisinin effectiveness, the potency of each fraction was found in the rank order of R100 > lysate > R50 > R10. The presence of catalase in higher amounts in fraction R100 as detected by immunoblot (data not

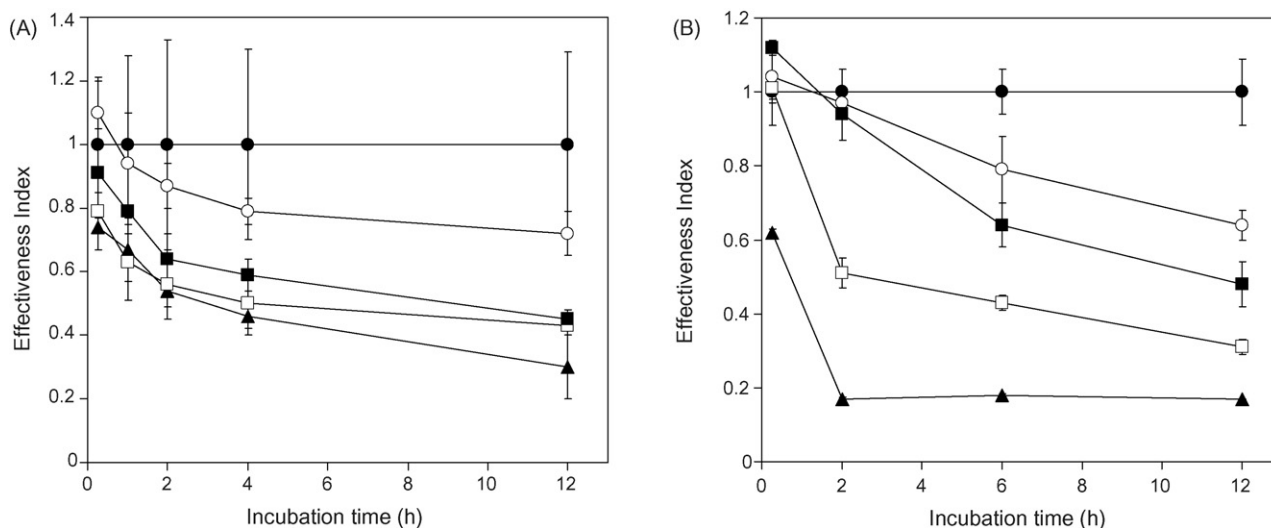


Fig. 6 – The effect of catalase (A) and Hb A (B) on reducing antimalarial effectiveness of artemisinin. One micromolar [14 C]-artemisinin was incubated with various concentrations of the hemoproteins in 10 mM phosphate buffer pH 7.4 at 37 $^{\circ}$ C for 0, 1, 2, 4 and 12 h. Control (●), 1080 unit/mL (○), 2700 unit/mL (■), 5400 unit/mL (□) and 10,800 unit/mL (▲) of catalase (A) and control (●), 0.1 μ M (○), 1 μ M (■), 10 μ M (□) and 200 μ M (▲) of Hb A (B). Free drug was separated from drug-enzyme mixture by ultracentrifugation through a 10 kDa cut-off membrane. The filtrate fractions were then tested for the drug effectiveness against *P. falciparum*.

shown) makes it a more likely candidate than Hb A in reducing artemisinin effectiveness. However, Hb A at 200 μ M, a concentration lower than that present in 10% lysate (approximately 500 μ M), was much more potent in reducing artemisinin effectiveness than the amount of catalase present in 10% lysate (approximately 7000 units/mL). This discrepancy may be due to the presence of other factors that can protect artemisinin from interacting with Hb A. The effect of catalase in reducing artemisinin effectiveness is in fact in line with previous observation where addition of catalase to culture media could diminish artemisinin antimalarial activity in vitro [18].

Although the role of catalase in reducing the effectiveness of artemisinin was investigated due to its presence in erythrocyte, other heme-containing proteins, such as cytochrome C, readily inactivated artemisinin following a brief exposure (data not shown). Detoxification of artemisinin by hemoproteins in various tissues and organs may provide an explanation for the rapid clearance of the drug from the human body [2].

Iron chelators have been reported to antagonize the antimalarial activity of artemisinin, and thus chelatable iron was postulated to play a role in the mode of action of artemisinin [19,20]. However, addition of deferiprone, an iron chelator, to the hemolysate did not prevent artemisinin from inactivation, in line with a previous report [9]. This may possibly be due to low amount of chelatable iron in normal erythrocyte cytosol [21], thus ruling out the role of chelatable iron in this inactivation phenomenon in erythrocyte lysate. The ability of catalase and cytochrome C, but not glutathione peroxidase (a selenoprotein), to detoxify artemisinin emphasizes the role of the heme prosthetic group in this process.

It has been reported that interaction of catalase with *t*-butylhydroperoxide leads not only to detoxification of peroxides but also to destruction of the heme moiety of catalase in the absence of hydrogen donor such as NADPH [22]. Similarly, it is possible that interaction of artemisinin with catalase in the malaria parasite where reducing power is diminished [23] may lead to inactivation of catalase, thus promoting oxidative killing of the parasite both by the drug and from cellular metabolism. If this is the case, catalase may serve as one of the targets of artemisinin, and being of host origin, resistance of *P. falciparum* to this drug should not be easily developed.

The reduced artemisinin effectiveness described here is due in part to the interaction of artemisinin with erythrocyte lysate. It was observed that upon interaction with erythrocyte lysate, approximately 30–40% of the radiolabelled artemisinin were retained in the retentate fractions of normal and thalassemic lysates, presumable covalently attached to proteins [24] and as non-filterable heme [25], while 60–70% of the radioactive drug were recovered in the filtrate fraction as free drug with reduced antimalarial effectiveness. Thus, after activation, in addition to the known alkylation of artemisinin to nearby biological molecules, the drug may become inactivated and released in forms whose structures have yet to be elucidated. The phenomenon is not a random chemical activity as it has been shown that in the presence of human serum proteins, artemisinin is not inactivated but is

protected from inactivation by intact normal erythrocytes [8]. Other non-heme protein such as glutathione peroxidase, a selenium-containing protein, could not reduce artemisinin effectiveness.

It is still unclear whether artemisinin derivatives exert their antimalarial activities through reaction with heme [2,26,27]. Among other mechanisms, it was proposed that reductive scission of the peroxide by ferroprotoporphyrin IX gives rise to an active intermediate which can alkylate both heme and essential parasite proteins leading to parasite death [27–29]. However, a number of 10-deoxo artemisinin derivatives, which are active antimalarials, have been reported not to react with ferroprotoporphyrin IX [30]. While this result is still a matter of controversy [31,32], the ability of artemisinin to interact with heme, as reported earlier [24], and to interact with a number of heme-containing proteins, resulting in reduction in effectiveness of the drug, emphasizes the role of heme in the mechanism of inactivation as well as action of this drug. A number of other host components outside the erythrocytes may also interact with artemisinin and its derivatives, thereby reducing its effectiveness.

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