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ENHANCEMENT OF HEMIN-INDUCED MEMBRANE DAMAGE BY ARTEMISININ

NAIMIN WEI and S. M. HOSSEIN SADRZADEH*

Department of Pathology, New England Deaconess Hospital and Harvard Medical School, Boston, MA, U.S.A.

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Abstract—Artemisinin is an effective antimalarial agent, and its action on the malarial parasite is suggested to be mediated by oxidative processes. Since malarial parasites contain a high concentration of hemin, and hemin may induce the formation of reactive oxygen species, we investigated the interaction of artemisinin, iron and hemin. We used erythrocyte membrane-bound Ca^{2+} pump ATPase (basal) and calmodulin (CaM)-activated Ca^{2+} pump ATPase as our model. Membranes were incubated with artemisinin in the presence or absence of iron–ascorbate or hemin at 37° for 1 hr. Following incubation, ATPase activity was measured. Our results showed that artemisinin (500 μM) had no effect on ATPase activities. However, artemisinin enhanced the inhibitory effect of iron (50 μM)–ascorbate (500 μM) on ATPase activity (46.3 ± 3.9 vs 63 ± 2.1% for basal; 57.2 ± 2.5 vs 74.8 ± 2.1% for CaM-activated). Desferrioxamine (DFO, 200 μM) blocked significantly the effect of iron–ascorbate–artemisinin on ATPases ($P < 0.01$). Hemin inhibited ATPase activity in a concentration-dependent fashion. Artemisinin enhanced hemin (10 μM)-induced inhibition of basal (36.0 ± 6.0 vs 73.7 ± 3.0%) and CaM-activated Ca^{2+} pump ATPase (31.6 ± 2.8 vs 70.0 ± 1.5%). Iron chelators (DFO, ferene, 8-hydroxyquinoline, 1,10-phenanthroline, and 1,2-dimethyl-3-hydroxypyrid-4-one) had no effect on artemisinin plus hemin-induced enzyme inhibition. Catalase (2000 U/mL) had a minor effect on the artemisinin–hemin or hemin-mediated effect. Thiourea (1 mM) had no effect. However, superoxide dismutase (500 U/mL) and dithiothreitol blocked artemisinin–hemin or hemin-mediated ATPase inhibition significantly ($P < 0.001$). In conclusion, these results suggest that, in our model, artemisinin enhances the damage of hemin-induced ATPases via oxidation of thiol groups on the enzymes. Free iron or hydroxyl radical does not seem to be involved. This interaction between artemisinin and hemin may contribute to the antimalarial action of artemisinin against malarial parasites.

Key words: artemisinin; qinghaosu; hemin; desferrioxamine; membrane; ATPase

Artemisinin (qinghaosu) is an effective antimalarial agent that was isolated from an ancient Chinese herbal remedy for malaria, *Artemisia annua* [1]. It is a unique molecule because it contains bridged endoperoxide, which is required for its antimalarial activity [2]. Peroxides can lead to the formation of reactive oxygen species; therefore, it has been suggested that the endoperoxide bridge is involved in its antimalarial effect, probably by producing free radicals that damage the parasite membrane. Indeed, several lines of evidence suggest an oxidant mode of action for artemisinin. Artemisinin was shown to produce activated oxygen species and lipid peroxidation *in vitro* [3]. In addition, oxidant drugs have been shown to potentiate the effect of artemisinin, while antioxidants such as catalase, DTT† and α-tocopherol have been shown to antagonize artemisinin-mediated antimalarial action [4]. Iron may be involved in the antimalarial activity of artemisinin. Meshnick *et al.* [5] showed that artemisinin-induced free radical formation is iron dependent. In addition, iron chelators, such as DFO, have been shown to inhibit artemisinin-mediated action [6].

Hemin has also been suggested to play a role in the antimalarial action of artemisinin [7, 8]. Hemin has been shown to catalyze the decomposition of artemisinin [7], and to interact with artemisinin and cause oxidation of protein thiols [8]. *Plasmodium falciparum*, the causative agent of malaria, contains large amount of hemin [9]. Hemin has been shown to inhibit plasma membrane CaM-activated Ca^{2+} pump ATPase activity, presumably by interacting with the thiol groups of the enzyme [10]. In the present study, we investigated the interactions of artemisinin and hemin, using erythrocyte membrane-bound basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase as our model.

MATERIALS AND METHODS

Calmodulin and saponin were purchased from the Calbiochem Corp. (La Jolla, CA). Desferrioxamine and L1 were from CIBA-Geigy Limited (Basel, Switzerland). PMSF and artemisinin were from the Aldrich Chemical Co. (Milwaukee, WI). Copper-zinc SOD, catalase and all other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

* Corresponding author: S. M. Hossein Sadrzadeh, Ph.D., Department of Pathology, M323, New England Deaconess Hospital, 185 Pilgrim Road, Boston, MA 02215. Tel (617) 632-9030; FAX: (617) 632-0167.

† Abbreviations: DTT, dithiothreitol; CaM, calmodulin; DFO, desferrioxamine; L1, 1,2-dimethyl-3-hydroxypyrid-4-one; PMSF, phenylmethylsulfonyl fluoride; SOD, superoxide dismutase; and RBC, red blood cells.

Membrane preparation. Blood was obtained from a local blood bank. Erythrocyte membranes were prepared according to the method of Farrance and Vincenzi [11] with minor modification. Briefly, blood cells were washed with saline three times, and lysed in a hypotonic imidazole buffer (pH 7.1, 20 mM) with EGTA (40 μM) and PMSF (4 μM). Membranes were washed twice with imidazole buffer containing EGTA and PMSF and three times with 20 mM imidazole alone. The final wash was in 40 mM histidine–imidazole buffer (pH 7.1), and the membranes were stored in a refrigerator on ice under nitrogen.

Experimental model. To study the interaction of artemisinin and iron, membranes (0.75 mg/mL) were incubated (37°, 60 min) with artemisinin (500 μM) and/or iron ($FeSO_4$, 50 μM)–ascorbate (500 μM) in the presence or absence of an iron chelator, DFO (200 μM), in 50 mM Tris buffer (pH 7.1). To study the interaction between artemisinin and hemin, membranes were first washed with 50 mM Tris buffer (pH 7.1) to eliminate histidine and imidazole, and then incubated with hemin (5–50 μM) and/or artemisinin (500 μM) at 37° for 60 min. At the end of the incubation period, membrane ATPase activities were measured.

To study the effects of antioxidants (SOD 500 U/mL, catalase 2000 U/mL), iron chelators (DFO 40 μM, ferene 40 μM, 8-hydroxyquinoline 40 μM, L1 40 μM, and 1,10-phenanthroline 40 μM), hydroxyl radical scavenger (thiourea 1 mM), or DTT (10 mM), these agents were added to membranes prior to the addition of hemin or hemin–artemisinin. Artemisinin was made up as a 50 mM stock solution in absolute ethanol and stored at −20°. The final concentration of artemisinin was 500 μM in 1% ethanol (1% ethanol had no effect on ATPase activities). Hemin (4 mM) was prepared daily in 0.1 N NaOH and diluted in 50 mM Tris buffer (pH 7.1). Catalase, DFO and ferene were prepared daily in 50 mM Tris buffer (pH 7.1). The concentrations of membrane protein were estimated by the BCA method (Pierce Co.) [12].

ATPase assays. Membrane ATPase activities were measured as described by Sadrzadeh *et al.* [13]. Basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase were measured simultaneously in multi-well plates. Briefly, membranes were washed with assay buffer, following incubation with different agents. The typical assay mixture contained RBC membrane (75 μg/mL), 18 mM histidine-imidazole (pH 7.1), 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM ouabain and 30 nM CaM (only for CaM-activated Ca^{2+} pump ATPase). After a 15-min preincubation at 37°, 1% SDS was added to the control groups. The enzymatic reaction was started with 3 mM ATP. After 60 min at 37°, the reaction was stopped with 1% SDS, and the inorganic phosphate released was measured with ammonium molybdate at A_{810}.

Statistical analysis. Results are presented as means ± SEM. Comparison between groups was done using Student's *t*-test.

RESULTS

The interaction of artemisinin with iron–ascorbate is shown in Fig. 1. Incubation of membranes with artemisinin at 500 μM did not affect ATPase activities markedly. However, as expected, iron (50 μM)–ascorbate (500 μM) resulted in a significant inhibition of both basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase activities ($P < 0.001$). Addition of artemisinin further enhanced the inhibitory effect of iron–ascorbate on ATPases (Fig. 1). DFO, a potent ferric iron chelator, when added prior to the addition of artemisinin plus iron, reversed the inhibitory effect of iron (Fig. 2). Incubation of ascorbate and artemisinin with membranes at 37° for 60 min had no effect on ATPases (data not shown).

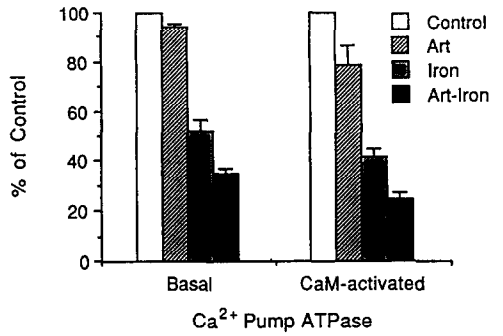

Fig. 1. Effect of artemisinin on iron-mediated ATPase inhibition. RBC membranes were incubated with artemisinin (Art, 500 μM) and/or iron (50 μM $FeSO_4$, 500 μM ascorbate) at 37° for 60 min. ATPases were measured as described in Materials and Methods. Specific ATPase activities (nmol P_i/min/mg protein) for controls were: 21.40 ± 1.12 and 39.96 ± 2.78 for basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase, respectively. Each bar represents the mean ± SEM (N = 4).

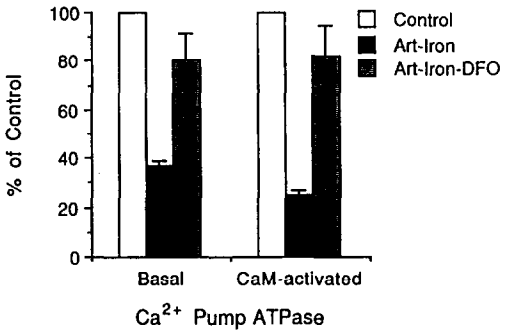

Fig. 2. Effect of DFO on artemisinin–iron-induced ATPase inhibition. RBC membranes were incubated with artemisinin (500 μM) and iron (50 μM) plus ascorbate (500 μM) with or without DFO (200 μM) at 37° for 60 min. ATPases were measured as described in Materials and Methods. Specific activities (nmol P_i/min/mg protein) for controls were: 18.95 ± 1.36 and 36.84 ± 1.47 for basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase, respectively. Each bar represents the mean ± SEM (N = 3).

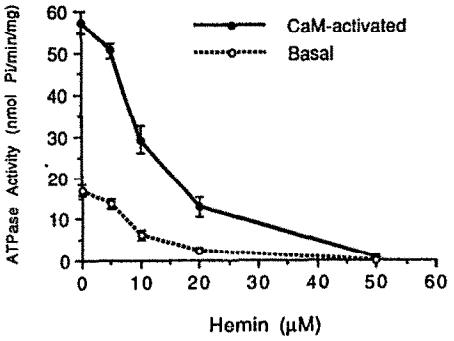

Fig. 3. Effect of hemin on ATPases. RBC membranes were incubated with different concentrations of hemin at 37° for 60 min. ATPases were measured as described in Materials and Methods. Each point represents the mean ± SEM (N = 3).

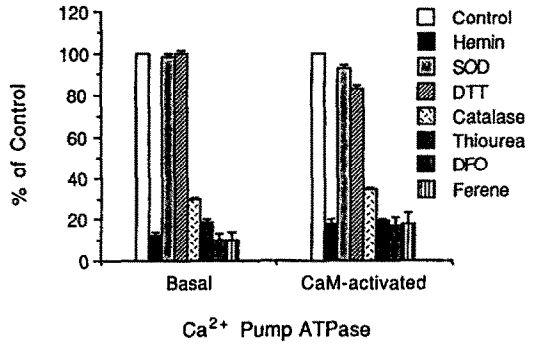

Fig. 5. Effects of antioxidants and iron chelators on hemin-induced ATPase inhibition. RBC membranes were incubated with hemin (20 μM) with or without SOD (500 U/mL), DTT (10 mM), catalase (2000 U/mL), thiourea (1 mM), DFO (40 μM) or ferene (40 μM) at 37° for 60 min. ATPases were measured as described in Materials and Methods. Specific activities (nmol P_i/min/mg protein) for controls were: 14.13 ± 1.91 and 48.52 ± 5.87 for basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase, respectively. Each bar represents the mean ± SEM (N = 3).

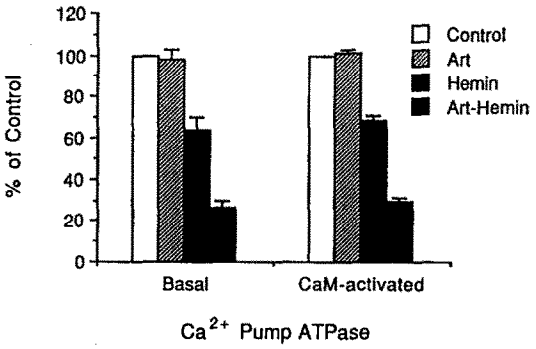

Fig. 4. Enhancement by artemisinin of the hemin-induced effect on ATPases. RBC membranes were incubated with artemisinin (500 μM) and/or hemin (10 μM) at 37° for 60 min. ATPases were measured as described in Materials and Methods. Specific activities (nmol P_i/min/mg protein) for controls were: 15.82 ± 2.45 and 48.41 ± 4.15 for basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase, respectively. Each bar represents the mean ± SEM (N = 3).

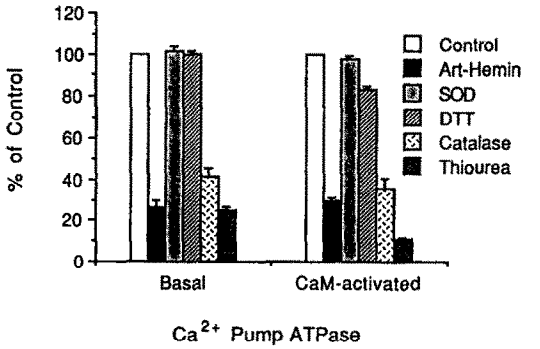

Fig. 6. Effects of antioxidants on hemin–artemisinin-mediated ATPase inhibition. RBC membranes were incubated with hemin (10 μM)–artemisinin (500 μM) with or without SOD (500 U/mL), DTT (10 mM), catalase (2000 U/mL), or thiourea (1 mM) at 37° for 60 min. ATPases were measured as described in Materials and Methods. Specific activities (nmol P_i/min/mg protein) for controls were: 15.85 ± 0.74 and 41.47 ± 4.08 for basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase, respectively. Each bar represents the mean ± SEM (N = 3).

The inhibition of ATPase activities by hemin is shown in Fig. 3. Hemin inhibited basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase in a concentration-dependent fashion with complete inhibition at a concentration of 50 μM.

The effect of artemisinin and hemin on membrane ATPases is shown in Fig. 4. Again, artemisinin (500 μM) alone had no effect on ATPase activities. Hemin (10 μM) inhibited basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase activities by 36.0 ± 6.0 and 31.6 ± 2.8%, respectively. Addition of artemisinin (500 μM) to hemin (10 μM) resulted in more inhibition of the enzymes (basal = 73.7 ± 3%, and CaM-activated = 70.0 ± 1.5%). Therefore, although artemisinin alone had no effect, artemisinin potentiated the inhibitory effect of hemin on ATPase activities.

To investigate the specific reactive oxygen species involved in the inhibition of ATPases, we used various antioxidants in our model (Figs. 5 and 6). As shown in Figs. 5 and 6, SOD significantly blocked the effect of hemin or hemin–artemisinin ($P < 0.001$). Catalase, however, had only a minor effect on hemin- or hemin–artemisinin-induced membrane damage. DTT also blocked the hemin- or hemin–artemisinin-mediated effect significantly ($P < 0.001$). However, thiourea, a hydroxyl radical scavenger, had no effect. In addition, we studied the effect of iron chelators in this system. We used both

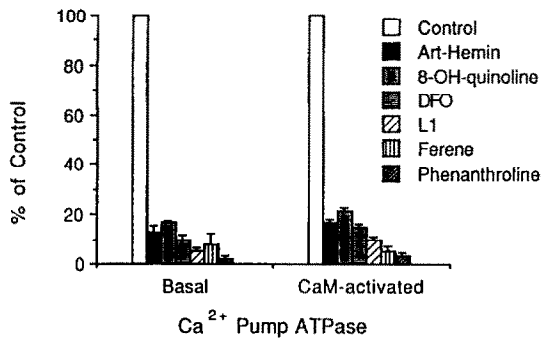

Fig. 7. Effects of iron chelators on hemin–artemisinin-mediated ATPase inhibition. RBC membranes were incubated with hemin (10 μM)–artemisinin (500 μM) with or without 8-hydroxyquinoline (40 μM), DFO (40 μM), L1 (40 μM), ferene (40 μM) or 1,10-phenanthroline (40 μM) at 37° for 60 min. ATPases were measured as described in Materials and Methods. Specific activities (nmol P_i/min/mg protein) for controls were: 15.79 ± 1.63 and 39.09 ± 0.49 for basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase, respectively. Each bar represents the mean ± SEM (N = 3).

hydrophilic iron chelators (DFO and ferene) and hydrophobic chelators (8-hydroxyquinoline, 1,10-phenanthroline, and L1), and as shown in Fig. 7, none of these chelators had any effect on hemin–artemisinin-induced enzyme inhibition.

DISCUSSION

The exact mechanism for the artemisinin-mediated antimalarial effect is not clear, although some evidence suggests an oxidant mode of action. Since membrane ATPases have been shown to be sensitive to oxidant stress [13], we used erythrocyte membrane basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase as a model to study the interaction between artemisinin and hemin.

Based on a recent observation by Meshnick *et al.* [5], iron is apparently involved in the antimalarial activity of artemisinin. Also, it was reported that DFO, an iron chelator, antagonizes the artemisinin-mediated antimalarial effect [6]. To study the interaction between iron and artemisinin, we incubated RBC membranes with iron–ascorbate, in the presence or absence of artemisinin at 37° for 60 min. We found that artemisinin alone had no effect on membrane ATPase activities; however, as expected, iron resulted in 46 and 57% inhibition of basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase, respectively (Fig. 1). The inhibitory effect of the iron is probably via the generation of reactive oxygen species. Interestingly, artemisinin enhanced the effect of iron–ascorbate significantly ($P < 0.01$, Fig. 1). It is possible that artemisinin may behave similarly *in vivo*. That is, *Plasmodium falciparum* usually engulfs a large quantity of the host hemoglobin and degrades it to hemin [9], which possibly results in the release of free iron. Although catalytic free iron can cause tissue damage under certain circumstances, it may not affect the parasite similarly. That is, the free iron released during hemoglobin degradation, by itself, may not affect the parasites; however, in the presence of artemisinin, the combined effect of iron–artemisinin may damage the parasite. In our model, DFO blocked the artemisinin–iron effect significantly ($P < 0.01$, Fig. 2). It is possible that activated oxygen species formed by iron–artemisinin either directly oxidize the essential -SH groups on the enzymes or initiate the peroxidation of membrane lipids and indirectly damage the membrane-bound enzymes.

Another mechanism of the artemisinin-mediated antimalarial effect may involve hemin. Hemin has been shown to inhibit CaM-activated Ca^{2+} pump ATPase activity [10]. Our results demonstrated that hemin inhibits basal Ca^{2+} and CaM-activated Ca^{2+} pump ATPase in a concentration-dependent fashion (Fig. 3). Since *P. falciparum* accumulates a high concentration of hemin, the antimalarial effect of artemisinin may be via its interaction with hemin. Our results suggest that such interaction might occur *in vivo*. As shown in Fig. 4, artemisinin enhanced the effect of hemin on membrane ATPases significantly ($P < 0.01$).

Hemin has been shown to interact with thiol groups of Ca^{2+} pump ATPase, resulting in the inhibition of the enzyme [10]. The latter study suggests an oxidative-mediated process, since DTT partially restored the enzyme activity [10]. To further investigate the mechanism(s) of hemin–artemisinin-induced enzyme inhibition, we used SOD (to remove $O_2^{\cdot-}$), catalase (to remove H_2O_2), DTT, and thiourea (hydroxyl radical scavenger). Figure 5 shows that SOD and DTT blocked hemin-induced inhibition of ATPase activities significantly ($P < 0.001$). Since SOD completely inhibited the hemin and artemisinin–hemin-induced reaction (Figs. 5 and 6), we suggest that superoxide anion was probably involved in the damaging effect of hemin to the membrane ATPases. Such an effect was apparently via the oxidation of essential -SH groups on the enzymes, because DTT also blocked the effect of hemin. Hydrogen peroxide is probably not involved since catalase did not have a marked effect on hemin or artemisinin–hemin-induced damage (Figs. 5 and 6). In addition, hydroxyl radical is probably not involved in these reactions, since thiourea had no effect on hemin–artemisinin-induced damage.

Furthermore, we used hydrophilic ferrous and ferric iron chelators (ferene and DFO, respectively), as well as hydrophobic chelators such as 8-hydroxyquinoline, 1,10-phenanthroline, and L1, in this system. The lipid-soluble chelators were included to chelate free irons that are possibly released from hemin and buried in the lipid bilayer. To our surprise, none of these chelators had any effect on the hemin–artemisinin-induced reactions (Fig. 7). Thus, the latter results suggest that, in this model, free iron is not involved in the above reactions. It is possible that iron, still attached to the protoporphyrin ring, catalyzes the formation of oxygen free radicals that can subsequently damage the membrane-bound enzymes. Indeed, some years ago, we showed that hemoglobin (heme iron) can

act as a Fenton reagent, generate reactive oxygen species and initiate lipid peroxidation [14].

In conclusion, these results suggest that hemin induces damage to membrane-bound enzymes via oxidation of the essential thiol groups on the enzymes. Apparently, free iron or hydroxyl radicals are not involved in these reactions. Also, artemisinin enhanced hemin-mediated inhibition of membrane-bound enzymes. Such interaction between artemisinin and hemin may contribute to the antimalarial activity of the drug.

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REFERENCES

1. Klayman DL, Qinghaosu (artemisinin): An antimalarial drug from China. *Science* **228**: 1049–1055, 1985.
2. Brossi A, Venugopalan B, Gerpe LD, Yeh HJC, Flippen-Anderson JL, Buchs P, Luo XD, Milhous W and Peters W, Arteether, a new antimalarial drug: Synthesis and antimalarial properties. *J Med Chem* **31**: 645–650, 1988.
3. Meshnick SR, Tsang TW, Lin FB, Pan HZ, Chang CN, Kuypers F, Chiu D and Lubin B, Activated oxygen mediates the antimalarial activity of qinghaosu. *Prog Clin Biol Res* **313**: 95–104, 1989.
4. Krungkrai SR and Yuthavong Y, The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents that modulate oxidant stress. *Trans R Soc Trop Med Hyg* **81**: 710–714, 1987.
5. Meshnick SR, Yang Y-Z, Lima V, Kuypers F, Kamchonwongpaisan S and Yuthavong Y, Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). *Antimicrobial Agents Chemother* **37**: 1108–1114, 1993.
6. Kamchonwongpaisan S, Vanitchareon N and Yuthavong Y, The mechanism of antimalarial action of artemisinin (qinghaosu). In: *Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications* (Eds. Ong ASH and Packer L), pp. 363–372 Birkhauser, Basel, 1992.
7. Zhang F, Gosser DK Jr and Meshnick SR, Hemin-catalyzed decomposition of artemisinin (qinghaosu). *Biochem Pharmacol* **43**: 1805–1809, 1992.
8. Meshnick SR, Thomas A, Ranz A, Xu CM and Pan HZ, Artemisinin (qinghaosu): The role of intracellular hemin in its mechanism of antimalarial action. *Mol Biochem Parasitol* **49**: 181–190, 1991.
9. Goldberg DE, Slater AFG, Cerami A and Henderson GB, Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: An ordered process in a unique organelle. *Proc Natl Acad Sci USA* **87**: 2931–2935, 1990.
10. Leclerc L, Vasseur C, Bursaux E, Marden M and Poyart C, Inhibition of membrane erythrocyte ($Ca^2 + Mg^{2+}$)-ATPase by hemin. *Biochim Biophys Acta* **946**: 49–56, 1988.
11. Farrance ML and Vincenzi FF, Enhancement of ($Ca^{2+} + Mg^{2+}$)-ATPase activity of human erythrocyte membranes by hemolysis in isosmotic imidazole buffer. II. Dependence on calcium and a cytoplasmic activator. *Biochim Biophys Acta* **471**: 59–66, 1977.
12. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85, 1985.
13. Sadrzadeh SMH, Vincenzi FF and Hinds TR, Simultaneous measurement of multiple membrane ATPases in microtiter plates. *J Pharmacol Toxicol Methods* **30**: 103–110, 1993.
14. Sadrzadeh SMH, Graf E, Panter SS, Hallaway PE and Eaton JW, Hemoglobin: A biologic Fenton reagent. *J Biol Chem* **23**: 14354–14356, 1984.