

Heme Polymerase Activity and the Stage Specificity of Antimalarial Action of Chloroquine

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ABSTRACT

Plasmodium falciparum lysate, prepared from 2.7×10^7 ring-infected erythrocytes and incubated with hemoglobin in sodium acetate at pH 5, incorporated a mean of 1.6 nmol of ferriprotoporphyrin IX (FP) into hemozoin in 18 to 22 hr. A similar preparation of trophozoite lysate incorporated a mean of 3.6 nmol of FP into hemozoin in 4 to 6 hr. These findings indicate differences between heme polymerase activity (hemozoin pro-

duction) at the ring and trophozoite stages of malaria parasites. Intracellular hemozoin production was 90% inhibited at the ring and trophozoite stages by 0.5 and 7 nmol of chloroquine/ 10^6 infected erythrocytes, respectively. The inhibition killed the rings but not the trophozoites, suggesting that mature parasites may have a mechanism for protecting themselves against chloroquine-FP toxicity.

Shortly after the invasion of erythrocytes, the protozoan parasite *Plasmodium* enters its ring stage of development and begins to ingest and digest hemoglobin as it grows to trophozoite and schizont stages (Olliaro and Goldberg, 1995). In the 48-hr asexual life cycle of *P. falciparum*, these developmental stages cover the first 20 to 24 hr, the next 12 to 18 hr and the remaining period, respectively (Yayon *et al.*, 1983). The rate of hemoglobin catabolism is highest at the trophozoite stage (Orjih *et al.*, 1994). Heme polymerase is located in the acidic food vacuole of the parasite and uses FP that has been released from hemoglobin as substrate for biosynthesis of hemozoin (Chou and Fitch, 1992; Slater and Cerami, 1992). In this process, FP is polymerized to β -hematin (the principal component of hemozoin) and stored in the food vacuole (Fitch and Kanjanangulpan, 1987; Slater *et al.*, 1991). Incorporation of FP into hemozoin is believed to be a protective measure by the parasite against self-destruction. Nonpolymerized FP has been shown to be highly toxic, damaging proteases and cell membranes (Chou and Fitch, 1980; Gluzman *et al.*, 1994; Orjih *et al.*, 1981; Vander Jagt *et al.*, 1987). It also binds chloroquine with high affinity, which may account for the selective accumulation of this drug in parasitized erythrocytes (Chou *et al.*, 1980). Chloroquine promotes but can also delay FP toxicity (Orjih *et al.*, 1981).

Consistent with a proposed mechanism of the antimalarial action of chloroquine (Orjih *et al.*, 1981), various studies have shown that the drug inhibits FP polymerization (Chou and Fitch, 1992; Egan *et al.*, 1994; Slater and Cerami, 1992). However, chloroquine kills *P. falciparum*, specifically at the

ring stage of development (Orjih *et al.*, 1994; Ter Kuile *et al.*, 1993; Zang *et al.*, 1986), although it has been shown in cell-free preparations that it inhibits the heme polymerase activity of trophozoites (Slater and Cerami, 1992).

The present study presents a comparison of the heme polymerase activity in *P. falciparum* rings and trophozoites, measurement of chloroquine inhibition of this activity in parasitized erythrocytes and determination of the effect of the inhibition on the viability of the parasites. The findings of the study may provide plausible explanations for the stage specificity of the antimalarial action of chloroquine.

Materials and Methods

Parasite cultures. RPMI 1640 medium was used for growing malaria parasites and washing them after concentration through saponin hemolysis (Orjih, 1996). It was prepared and supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium bicarbonate and 10% human serum (blood group O-positive) according to Jensen and Trager (1977). Each liter was supplemented with 2 g of glucose, 50 mg of hypoxanthine and 19.2 mg of gentamicin sulfate (1.62 mg/ml concentration of doubly distilled water). The malaria parasite studied was the chloroquine-susceptible HB-3 strain of *P. falciparum*. The parasites were grown in stored blood group O-positive human erythrocytes by the conventional method, with incubation in a gas mixture containing 3% O₂, 3% CO₂ and 94% N₂.

Hemozoin synthesis in hemolysates. Infected erythrocytes concentrated through saponin hemolysis were used in preparing hemolysates at both ring and trophozoite stages of parasite development. After concentration, a suspension of the ring-infected erythrocytes was prepared in culture medium, and 2-ml aliquots, each containing a total of 2.7×10^7 parasitized erythrocytes, were prepared in sterilized high-speed Oak Ridge centrifuge tubes. For he-

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ABBREVIATION: FP, ferriprotoporphyrin IX.

mozoin synthesis at the ring stage, the ring-infected erythrocytes were hemolyzed immediately, whereas for synthesis at the trophozoite stage, the aliquots of concentrated rings were incubated for 20 hr before they were hemolyzed. To prepare hemolysates, each aliquot was centrifuged for 30 min at $27,000 \times g$, and the supernatant was discarded. The pellet was resuspended in 2 ml of modified Krebs-Ringer phosphate buffer solution, pH 7.4, containing 68 mM NaCl, 50 mM NaHPO_4 , 4.8 mM KCl and 1.2 mM MgSO_4 , and the pH was adjusted by adding HCl. The suspension was centrifuged for 30 min at $27,000 \times g$, and the supernatant was discarded. The pellet was vortexed vigorously before being frozen for 10 min in liquid nitrogen. It was then thawed at room temperature; 2 ml of a 500 mM sodium acetate buffer solution, pH 5, was added to the lysate, and the tube was vortexed vigorously and sonicated briefly. The tube was placed on a slowly rotating mechanical mixer in a 37°C incubator. Hemolysates of rings were incubated for 18 to 22 hr, and those of trophozoites were incubated for 4 to 6 hr. After incubation, each sample was centrifuged for 30 min at $27,000 \times g$, and the supernatant was discarded. The pellet was resuspended in 2.5 ml of sodium acetate buffer and centrifuged, and the supernatant was discarded. This washing procedure was repeated with 2.5 ml of modified Krebs-Ringer phosphate buffer solution.

To extract hemozoin, the crude pellet (largely a mixture of cell membranes and β -hematin) was suspended in 2 ml of 2.5% sodium dodecyl sulfate buffered with 25 mM Tris to pH 7.8 and left overnight (16 hr) at room temperature. It was then centrifuged for 60 min at $27,000 \times g$, the supernatant was discarded and the pellet was washed once with 2 ml of sodium dodecyl sulfate buffer solution. The hemozoin was then analyzed as described previously (Orjih and Fitch, 1993).

Measurement of chloroquine accumulation. Infected erythrocytes, concentrated through saponin hemolysis, were used for measurement of chloroquine accumulation at the ring and trophozoite stages of parasite development. Ring-labeled [^{14}C]chloroquine (specific activity, 2.36 mCi/mmol; New England Nuclear Research Products, Boston, MA) was added to each culture, and the mixture was incubated at 37°C for 60 min. In every case, the infected erythrocytes were suspended in fresh culture medium immediately before the addition of [^{14}C]chloroquine to ensure that the pH was adequately maintained. At the end of incubation, the suspensions were centrifuged at $27,000 \times g$ at 4°C for 30 min, and the supernatant fluid was separated from the pellet. Both supernatant fluid and pellet were assayed radiochemically for chloroquine with the use of Opti-Fluor-O scintillation cocktail (Packard Instruments, Meriden, CT).

Antimalarial action of hemozoin inhibition. Normal synchronized cultures containing a mean of 3.5% parasitemia (± 1 S.E.M. of 4 experiments) were used to study the effect of chloroquine on hemozoin production and parasite viability. The cultures were exposed to various concentrations of chloroquine at the ring or trophozoite stage of parasite development and incubated for ≥ 20 hr. Control cultures were not exposed to chloroquine. Hemozoin was extracted and measured according to the standard method (Orjih and Fitch, 1993).

The therapeutic effect of hemozoin inhibition by chloroquine on *P. falciparum* rings was evaluated morphologically by counting parasites that grew to schizont stage, whereas the effect on trophozoites was evaluated by counting new ring-infected erythrocytes that appeared in the culture after trophozoites have been exposed to chloroquine for 20 hr.

Results

In this study, the parasitemia levels in unconcentrated cultures of *P. falciparum* rings were 2% to 8%, and after concentration by saponin hemolysis, the levels were 90% to 99%. The concentration procedure was necessary for heme polymerase activity and chloroquine accumulation experi-

ments to avoid uninfected erythrocytes complicating the results.

Heme polymerase activity. The total quantities of hemozoin produced in ring and trophozoite lysates are shown in figure 1. Values represent mean \pm S.E.M. of triplicate experiments. Before hemolysate preparation, the quantity of hemozoin was 2 ± 0.2 nmol of FP in ring-infected erythrocytes and 13 ± 1.5 nmol of FP in trophozoites; after incubation, the values increased to 3.6 ± 0.1 and 16.4 ± 1.6 nmol of FP, respectively. The difference between the mean quantities of FP incorporated into β -hematin in the two hemolysates is statistically significant ($P < .03$ by the paired *t* test).

Chloroquine accumulation. The total number of infected erythrocytes used in the chloroquine accumulation study was $\sim 2.6 \times 10^7$ in both ring and trophozoite cultures. The experiments were done three times. At a steady state of accumulation, after 1 hr of incubation, extracellular (*i.e.*, unbound) chloroquine concentrations decreased, depending on the parasite stage and initial drug concentration added to the medium (fig. 2). For example, when 53 nM chloroquine was added to the medium, the concentration decreased by 28% in ring cultures and 66% in trophozoite cultures; when 318 nM chloroquine was added to the medium, the decrease was 6% in the ring and 70% in the trophozoite cultures.

Generally, chloroquine accumulation by *P. falciparum*-infected erythrocytes was a saturable process, reaching a maximum at ~ 0.8 nmol/ 10^6 ring-infected erythrocytes and 7 nmol/ 10^6 trophozoite-infected erythrocytes (fig. 3). In cultures containing 106 nM of added chloroquine, ring-infected erythrocytes accumulated 80% less than that accumulated by trophozoites, and this difference did not change much when the added chloroquine was 318 nM. As shown in figure 3, the difference between chloroquine accumulation by uninfected erythrocytes and ring-infected erythrocytes was not as noticeable as it was with trophozoites; nevertheless, it was statistically significant ($P = .0001$ by the two-tailed paired *t* test). The use of highly concentrated infected erythrocytes improved the sensitivity of drug accumulation measurement.

Antimalarial action of hemozoin inhibition by chloroquine. Various studies by other investigators have shown that chloroquine inhibits β -hematin synthesis in cell-free

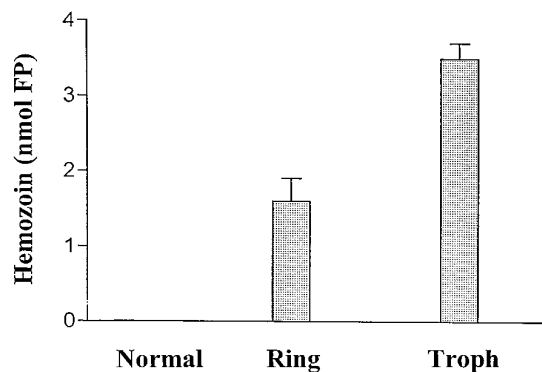


Fig. 1. Heme polymerase activity (incorporation of FP into hemozoin) in cell-free preparations. Hemolysates of normal and *P. falciparum* ring-infected erythrocytes were incubated in sodium acetate at pH 5 for 18 to 22 hr, whereas those of trophozoites were incubated at the same conditions for 4 to 6 hr. Each lysate was prepared from 2.7×10^7 erythrocytes. The total amounts of hemozoin produced were calculated in nM of FP, and mean \pm S.E.M. values of three experiments are shown.

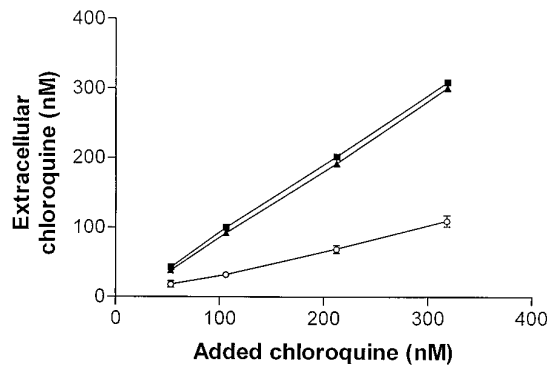


Fig. 2. Chloroquine accumulation by normal and *P. falciparum*-infected erythrocytes: steady state concentrations of unbound (extracellular) drug. Mean ± S.E.M. values of three experiments are shown. ○, Trophozoite-infected erythrocytes; ▲, ring-infected erythrocytes; ■, normal erythrocytes.

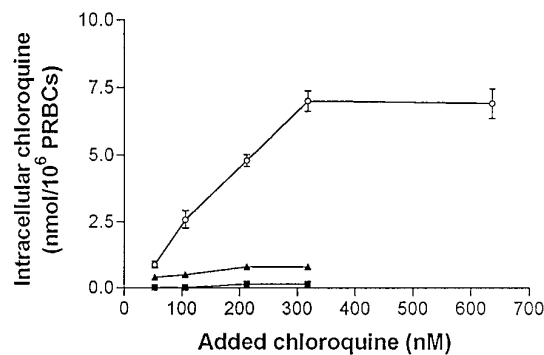


Fig. 3. Chloroquine accumulation by normal and *P. falciparum*-infected erythrocytes: steady state concentrations of bound (intracellular) drug. Mean ± S.E.M. values of three experiments are shown. ○, Trophozoite-infected erythrocytes; ▲, ring-infected erythrocytes; ■, normal erythrocytes.

preparations. However, to demonstrate the therapeutic significance of this inhibition, the study must be carried out with viable parasites (Asawamahasakda *et al.*, 1994; Orjih *et al.*, 1994; Orjih and Fitch, 1993). When ring-infected erythrocytes were incubated for 20 hr in 106 nM nonradioactive chloroquine, producing an intracellular drug concentration of 0.5 nmol/10⁶ ring-infected erythrocytes, hemozoin synthesis was inhibited by 90% (fig. 4), and parasite growth was arrested. After an additional 20 hr of exposure, the drug was washed away, and the cells were maintained for an additional 3 days in normal medium to see whether the parasites would recover. They did not, indicating that the parasites had died.

Antimalarial action of hemozoin inhibition on trophozoites was evaluated somewhat differently from that on the ring stage. The question here was whether the trophozoites would complete their life cycle and produce infective merozoites. For this purpose, parasite growth was synchronized at the ring stage, and the culture was incubated for 20 hr to produce trophozoites. The mean parasitemia in the culture was 3.5%, leaving sufficient uninfected erythrocytes in the culture for invasion by newly released merozoites. The trophozoites were then incubated for 20 hr in culture medium containing 106, 212, 318 or 636 nM nonradiolabeled chloroquine. The steady state amounts of chloroquine accumulated by trophozoites from these drug concentrations (fig. 3) have been compared with the antimalarial action of chloroquine (table 1). It

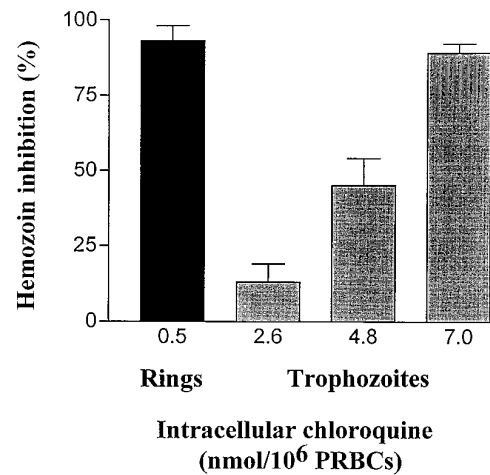


Fig. 4. Intracellular concentrations of chloroquine and inhibition of hemozoin production in *P. falciparum*-infected erythrocytes. To obtain the intracellular drug concentrations indicated from left to right, ring-infected erythrocytes were exposed to 106 and trophozoite-infected erythrocytes were exposed to 106, 212 and 636 nM chloroquine, respectively. Hemozoin in drug-treated cultures was subtracted from that in untreated controls, and the results were expressed as percentage of the controls. Mean ± S.E.M. values of three experiments are shown.

TABLE 1

Viability of *P. falciparum* trophozoites exposed to chloroquine

Appearance of parasite rings in the culture after 20 hr of incubation indicates viability.

Added chloroquine nM	Intracellular chloroquine nmol/10 ⁶ trophozoites	Stages of parasite		
		Ring	Trophozoite	Schizont
None	None	89 ± 6	3 ± 2	8 ± 3
106	0.5	85 ± 7	3 ± 1	13 ± 6
212	2.6	85 ± 3	6 ± 1	10 ± 3
316	4.8	70 ± 4	14 ± 2	14 ± 2
636	7.0	70 ± 3	20 ± 4	13 ± 1

Values are mean ± S.E.M. of three experiments.

shows that in all the chloroquine concentrations tested, trophozoites were able to complete their life cycle and infect new erythrocytes. Total parasitemia was, however, highest in the culture without chloroquine, indicating that some of the parasites have been killed or inhibited in growth by the drug. The lowest total parasitemia (20–40% of the control; data not shown) was observed in cultures that contained the highest amount of chloroquine (636 nM in medium or 7 nmol/10⁶ trophozoites).

Discussion

In this study, some differences between heme polymerase activity in rings and trophozoites of *P. falciparum* have been detected. Although equal numbers (2.7 × 10⁷) of infected erythrocytes were used, mean hemozoin synthesis in ring hemolysate was only 50% of that in trophozoites. The initial quantity of hemozoin in the lysates was also lower in the rings than in the trophozoites, but Slater and Cerami (1992) have shown that hemozoin by itself does not have heme polymerase activity. They showed that heme polymerase activity increased linearly with protein concentration in parasite lysate. Obviously, hemoglobin-free *P. falciparum* para-

sites are expected to contain less protein at the ring than at the trophozoite stage, and this may account for the differences in hemozoin synthesis observed in the present study. However, Dorn *et al.* (1995) have shown that protein-free hemozoin can promote formation of additional hemozoin. In the present study, there was more preexisting hemozoin in the trophozoite lysates than in the ring lysates, and this may have contributed to the differences in the results.

The nature of heme polymerase is still unresolved. Sullivan *et al.* (1996a) reported that recombinant or native histidine-rich proteins of *P. falciparum* promoted the formation of hemozoin in cell-free preparations, but Bendrat *et al.* (1996) found that phospholipids caused rapid FP polymerization. Future studies with histidine-rich proteins and lipids from rings and trophozoites of malaria parasites may help clarify the role of these molecules in hemozoin synthesis.

To demonstrate the pharmacological importance of heme polymerase, Slater and Cerami (1992) added various antimalarial drugs to hemolysates of *P. falciparum* trophozoites and found that ~1 mM chloroquine was necessary to cause ~90% inhibition of hemozoin production. This drug concentration is on the high side and carries the risk of not acting selectively. High concentrations of chloroquine inhibit other metabolic processes, such as DNA replication and protein synthesis, and kill bacteria (Ciak and Hahn, 1966). Excess chloroquine may also delay, rather than promote, the toxicity of FP (Orjih *et al.* (1981), the effector molecule that is expected to kill malaria parasites when hemozoin production is inhibited.

For the inhibition by chloroquine of heme polymerase activity to be biologically relevant, drug concentrations in the test system should be comparable to those that have been found to inhibit intracellular hemozoin production. In the present study, ~90% inhibition of hemozoin production was observed when intracellular chloroquine concentration was 0.5 nmol/10⁶ ring-infected erythrocytes or 7 nmol/10⁶ trophozoite-infected erythrocytes, suggesting that the mechanism, possibly heme polymerase activity, that is responsible for hemozoin formation is more sensitive to chloroquine in rings than in trophozoites. Chloroquine accumulates selectively in the food vacuoles of malaria parasites, and Yayon *et al.* (1985) assumed that the amount accumulated was directly proportional to the volume of the food vacuole. However, it has recently been demonstrated that chloroquine accumulation in *P. falciparum*-infected erythrocytes is dependent on the rate of FP generation (*i.e.*, hemoglobin catabolism) by the parasites (Orjih *et al.*, 1994). The volume of a food vacuole increases with parasite maturation, reaching a maximum at late schizont stage (Langreth *et al.*, 1978); in contrast, hemoglobin catabolism and chloroquine accumulation reach their peaks at the trophozoite stage and decrease dramatically thereafter (Orjih *et al.*, 1994). It has been recently reported that quinoline drugs bind to hemozoin, possibly in complex with FP (Sullivan *et al.*, 1996b); nevertheless, hemozoin by itself may not account for drug accumulation in parasitized erythrocytes. Schizonts contain more hemozoin than do trophozoites, whereas trophozoites accumulate more chloroquine than do schizonts (Orjih *et al.*, 1994).

This study has also shown that the therapeutic effect of hemozoin inhibition is dependent on the developmental stage of *P. falciparum*, being more lethal to rings than to trophozoites. Although some dead parasites were observed when hemozoin production in trophozoites was inhibited by 90%,

the surviving trophozoites were able to complete their life cycle and infect other erythrocytes. When a comparable level of inhibition of hemozoin production was induced at the ring stage, none of the parasites completed the life cycle. How intracellular FP kills malaria parasites in the presence of chloroquine has not yet been established, but it is becoming evident that some metabolic processes taking place in the food vacuole, as well as this organelle itself, are susceptible (Ginsburg, 1996; Olliaro and Goldberg, 1995). When added externally, FP and FP/chloroquine complex lyse cells (Chou and Fitch, 1980; Orjih, *et al.*, 1981), and in cell-free preparations, they have been shown to inhibit proteases (Gluzman *et al.*, 1994; Vander Jagt *et al.*, 1987).

The above observations suggest the following explanations for the stage-specificity of antimalarial action of chloroquine. Hemozoin formation, a requirement for continued utilization of hemoglobin as nutrient source, is readily inhibited by chloroquine at the ring stage of intraerythrocytic malaria parasite development. The excessive accumulation of chloroquine in mature parasites may be in part due to diversion of drug/FP complexes into hemozoin (Sullivan *et al.*, 1996b), away from the actual targets of antimalarial action. Also, at later stages of parasite development, excess nutrients have accumulated in the host erythrocyte (Zarchin *et al.*, 1986), and there are parasitophorous ducts through which extracellular macromolecules may be imported (Pouvelle *et al.*, 1991).

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