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Kinetics of Inhibition of Glutathione-Mediated Degradation of Ferriprotoporphyrin IX by Antimalarial Drugs

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ABSTRACT. We have shown previously that chloroquine and amodiaquine inhibit the glutathione-dependent degradation of ferriprotoporphyrin IX (FP). We have also demonstrated that treatment of human erythrocytes infected with *Plasmodium falciparum* with chloroquine or amodiaquine results in a dose- and time-dependent accumulation of FP in the membrane fraction of these cells in correlation with parasite killing. High levels of membrane FP are known to perturb the barrier properties of cellular membranes, and could thereby irreversibly disturb the ion homeostasis of the parasite and cause parasite death. We here report on the effect of various 4-aminoquinolines, as well as pyronaridine, halofantrine and some bis-quinolines, on glutathione-mediated destruction of FP in aqueous solution, when FP was bound non-specifically to a protein, and when it was dissolved in human erythrocyte ghost membranes. We showed that all drugs were capable of inhibiting FP degradation in solution. The inhibitory efficacy of some drugs declined when FP was bound non-specifically to protein. Quinine and mefloquine were unable to inhibit the degradation of membrane-associated FP, in line with their inability to increase membrane-associated FP levels in malaria-infected cells following drug treatment. The discrepancy between chloroquine and amodiaquine on the one hand, and quinine and mefloquine on the other, is discussed in terms of the particular location of drugs and FP in the phospholipid membrane, and may suggest differences in the mechanistic details of the antimalarial action of these drugs. BIOCHEM PHARMACOL **58**;1: 59–68, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. antimalarial drugs; 4-aminoquinolines; ferriprotoporphyrin IX degradation; glutathione; mode of action

Malaria-infected RBC† are distinguished from the cells of the infected host by their high levels of non-hemoglobin FP, produced during the ingestion and digestion of the hemoglobin-rich cytosol of the host erythrocyte [1]. FP must be neutralized since it is toxic to the parasite [2, 3]. FP detoxification in parasitized RBC is understood to be accomplished by the sequestration of FP into the insoluble FP polymer HZ [4, 5]. Although HZ formation is thought to be an efficient process [6–9], we have recently shown that in *Plasmodium falciparum* at the trophozoite stage only ca. 30% of the FP is converted into HZ [10]. Hence, substantial amounts of FP escape polymerization. To allow normal parasite development, the FP must leave the food vacuole since it demonstrably inhibits vacuolar proteases [11, 12]. As FP can dissolve into, and translocate across, membranes [13–16], it could, once it reaches the cytosol of the parasite, be degraded by FP oxygenase, whose activity has been reported in *P. berghei* and *P. knowlesi* [17]. However, as *P. falciparum* does not display such activity ([5, 18] and our own unpublished observations), it could be surmised that FP is decomposed by GSH, as we have recently demonstrated [19].

Owing to their ability to form complexes with FP, aminoquinoline-containing antimalarials inhibit FP polymerization *in vitro* either in solution [6, 8, 20, 21], in the presence of parasite extract at the acid pH that prevails inside the food vacuole [6, 9, 22, 23], or in intact infected cells [24, 25]. FP:drug complexation does not prevent FP dissolution in phospholipid membranes [15, 16], and in all likelihood, the eventual translocation across it. Obviously then, FP can be rendered toxic to the parasite only if its decomposition by GSH is inhibited. Increased levels of free FP have been observed in CQ- and AQ-treated *P. berghei*-infected mouse cells, but not when cells were treated with MQ or Q [24]. We have recently shown that CQ and AQ inhibit GSH-dependent FP degradation in drug-treated cultured *P. falciparum*, resulting in the dose- and time-dependent accumulation of FP in the membrane fraction of

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† *Abbreviations:* 8AC6, *N*,*N*′-bis[4-((4-(diethylamino)-1-methylbutyl) amino)-quinolin-8-yl]suberimide; AQ, amodiaquine; CQ, chloroquine; FP, ferriprotoporphyrin IX; HZ, hemozoin; MQ, mefloquine; Q, quinine; Q1-17, N^1,N^6-bis(7-chloroquinolin-4-yl)hexane-1,6-diamine; Q2-93, (±)-*trans*-N^1,N^2-bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine; and RBC, red blood cells.

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trophozoite-infected RBC. We have also shown that this accumulation correlates with the cytotoxic effect of these drugs [10]. In the present work, we studied in detail the effect of CQ and AQ on GSH-dependent FP degradation, and extended this investigation to other antimalarial drugs.

MATERIALS AND METHODS

GSH, Q hydrochloride, verapamil hydrochloride, AQ dihydrochloride, and defatted BSA were purchased from Sigma Chemical Co. RPMI-1640 was bought from Biological Industries. CQ diphosphate was obtained from Serva. FP was obtained from Porphyrin Products. MQ and artemisinin were generously provided by A. F. Cowman. Dehydroartemisinin was a gift from S. A. Ward. Artemether and halofantrine were generously donated by S. L. Croft. 8AC6 was kindly provided by L. W. Deady [26]. Q1-17 and Q2-93 [27] were generously supplied by Dr. J. L. Vennerstrom. All other chemicals were of the best available grade. Fresh human blood was kindly donated by the Hadassah Hospital Blood Bank.

Inhibition of GSH-Dependent FP Degradation

FP and GSH stock solutions were prepared fresh prior to experiment and maintained on ice in the dark until use. FP (3 μM) and GSH (2 mM, or as otherwise indicated were made up in 0.2 M HEPES (pH 7), and incubated at 37°. FP degradation was observed by measuring the decline in absorbance at 396 nm in a diode-array spectrophotometer at 10–60 sec time intervals (Milton Roy, Spectronic 3000). Drugs were added from stock solutions to the desired concentration, and degradation of heme was similarly followed in the spectrophotometer, with equal drug concentration in the reference cuvette. Drugs did not interact with the final degradation product, as ascertained by the following control experiment: 3 μM heme was degraded with 2 mM GSH, and drugs were added at the end of 1 hr incubation; no spectral changes could be observed between 400 and 700 nm (data not shown). The degradation of FP by GSH and its inhibition by drugs was also investigated under conditions where most of the FP is bound non-specifically to defatted BSA: 3 μM FP was added premixed with 0.3 μM BSA.

Effect of Drugs on FP Levels in FP-loaded Ghosts

White ghosts were prepared from normal washed RBC by hypotonic lysis in ice-cold solution of 5 mM Na-phosphate, pH 8 (5P8) and extensive washing in the same buffer. Ghosts (10^8/mL) were incubated with 10 μM FP in 0.2 M HEPES, pH 7 for 7 min at 37°. Under these conditions, full equilibration of FP was achieved (data not shown). Membranes were then centrifuged (27,000 *g*; 20 min at 4°) and washed once in the same buffer. FP-loaded ghosts were incubated at 37° in the same buffer ±2 mM GSH, ±6 μM of each drug. At different time intervals, aliquots of 0.8 mL were withdrawn, and membranes were pelleted at 18,000 *g* for 6 min and dissolved in 0.8 mL of SDS 1% (w/v). The absorbance of FP was determined spectrophotometrically at 400 nm.

Data Analysis

When data were acquired at short time intervals, the rate of FP degradation was determined by linear regression of the initial rate of the decrease in absorbance. When the decrease in absorbance was assayed for longer times, essentially until completion of FP degradation, the rate of degradation was calculated from the best fit of non-linear regression using the equation $O.D. = A \cdot \exp(-k \cdot t) + Offset$, where A is the initial absorbance, k is the rate constant (in sec^{-1}), t is time (in sec), and Offset is absorbance at infinite time, due to presence of non-identified products of FP degradation [19]. In the case of Q, there was a delay in the onset of FP degradation, and data analysis was performed only from the time point where absorbance decline versus time became linear.

In order to determine the apparent inhibitory constant of drug on GSH-dependent FP degradation, the rate at each drug concentration was derived from the time-dependent course of FP degradation ($=k_d$), and divided by the rate with no drug ($=k_{con}$). The ratios were then plotted against drug concentration and the K_i (the apparent inhibitory constant) was calculated by non-linear least square analysis fitting $k_d/k_{con} = K_i(K_i + [I])$, where [I] is the drug concentration.

RESULTS

Inhibition of GSH-Dependent Degradation by Antimalarial Drugs in Aqueous Solution

GSH degrades FP with a K_m of 0.64 ± 0.07 mM, i.e. well below the prevalent GSH concentration (2.4 mM) found in the parasite compartment or the host cell compartment (1.1 mM) of *P. falciparum* trophozoite-infected RBC [28]. The degradation of FP in the presence of 2 mM GSH and drugs was tested spectrophotometrically in 0.2 M HEPES buffer pH 7 at 37°. Results are shown in Fig. 1A. The absorbance of FP alone declined to some extent with incubation time (with a rate constant $k = [2.48 \pm 1.25] \times 10^{-4}\ sec^{-1}$; N = 8), probably due to partial precipitation of FP at pH 7, or to spontaneous self-destruction. The addition of GSH considerably accelerated the decline in absorbance ($k = [2.73 \pm 0.19] \times 10^{-3}\ sec^{-1}$; N = 8) as previously observed, due to the degradation of FP [19]. Absorbance did not fall to zero as the degradation product also absorbed to some extent at 400 nm; drugs did not alter the spectroscopic properties of the end product. Various antimalarial drugs were added at different concentrations, and the rates of FP degradation were similarly measured (the effect of CQ is shown as an example). Inhibition of GSH-dependent degradation of FP was competitive, as shown in Fig. 2. The relative rates of FP degradation as a

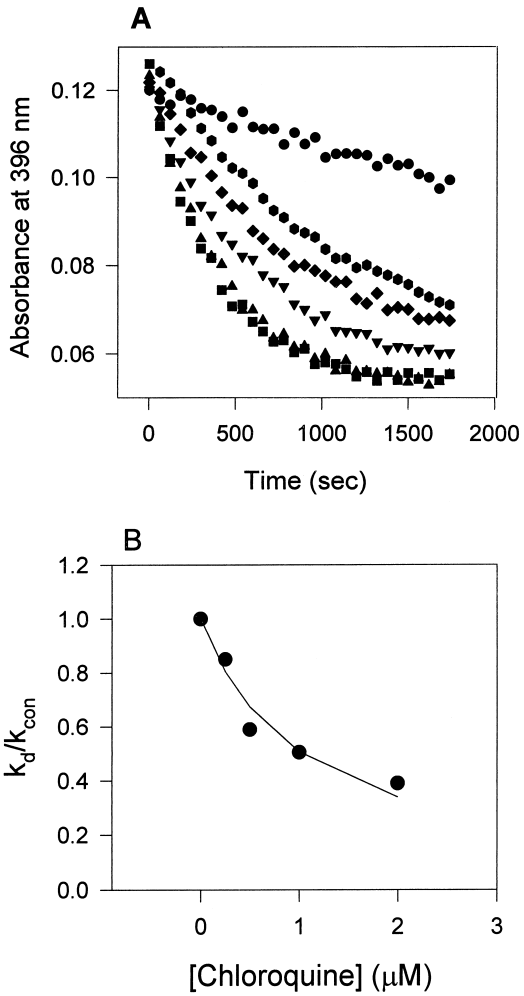

FIG. 1. Effect of chloroquine on GSH-mediated degradation of heme in aqueous solution. Heme (3 μM) was incubated in the presence of GSH (2 mM) and of increasing concentrations of CQ. Heme degradation was observed spectrophotometrically (upper panel). The uppermost curve depicts heme with no GSH, while the lowest one depicts heme + GSH, and curves from the latter up were obtained with increasing concentrations of CQ. The rate constant of degradation was calculated by best fit to a first order exponential decay. The dependence of k_d/k_{con} on [CQ] is shown in the lower panel, and the line describes the best fit to competitive inhibition. In this example, K_i = 1.03 ± 0.3 μM.

function of drug concentration are shown in Fig. 1B. The inhibitory constant was deduced from the best fit of k_d/k_{con} versus [CQ]. All other drugs tested in this investigation similarly inhibited the degradation of FP by GSH, as shown in Fig. 3, and the inhibitory constants were correspondingly determined. From data presented in Table 1, it can be seen that the rank order of inhibition potency was Q2-93 > pyronaridine > AQ > halofantrine ≈ Q1-17 > MQ > CQ > Q > 8AC6.

As FP is known to bind non-specifically to proteins and can bind to cytoplasmic proteins on its way out of the food vacuole, its degradation by GSH as affected by drugs was also tested. The molecular ratio of 1 BSA to 10 FP was chosen because BSA has previously been shown to bind 1 FP molecule with high affinity and 10 molecules of FP with relatively lower affinity [29]. As FP binds to the fatty acid binding sites, defatted BSA was used. Results obtained with AQ as an example are shown in Fig. 4, and the K_i values for all drugs are displayed in Table 1. The spontaneous decline in the absorbance of FP alone was slower than that of free FP, probably because the self-aggregation of FP was expected to be considerably reduced. However, the rate of degradation of BSA-bound FP by GSH was very similar to that observed in solution (Fig. 1A). When the ratio of FP to BSA was reduced to 1:1, i.e. when all FP is presumed to be bound to the high-affinity binding site, no FP degradation could be observed (data not shown). The rank order of inhibitory strength was: Q2-93 > AQ > Q1-17 ≈ halofantrine > CQ > pyronaridine > Q > 8AC6. The K_i for CQ, AQ, and halofantrine were somewhat reduced, whereas those for Q, MQ, pyronaridine, and 8AC6 were significantly increased compared to their values in solution.

FP readily dissolves in membranes due to its amphipathic properties. RBC leaky white ghosts were used as model membranes. The kinetics of FP dissolution in RBC ghosts

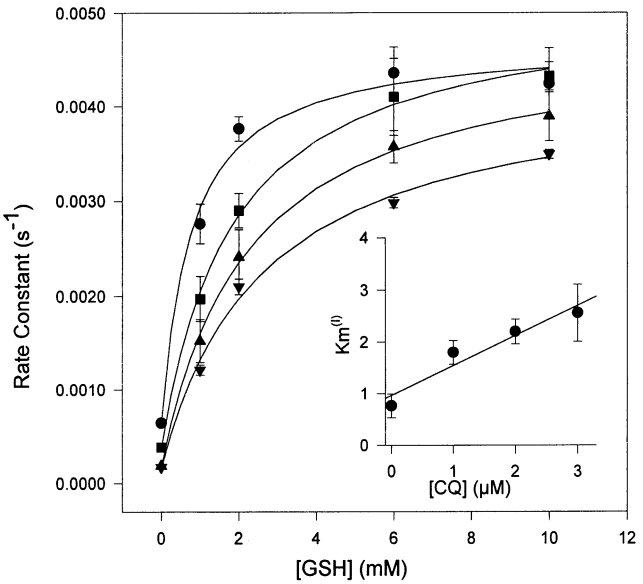

FIG. 2. The inhibition of GSH-dependent degradation of heme by chloroquine is competitive. The rate constants of heme degradation by GSH ± different concentrations of CQ were determined as described in Materials and Methods. Rate constants were plotted against [GSH] and data were fitted to the Michaelis–Menten equation. The continuous lines depict the best fit, and the parameters obtained (means ± SE of K_m in mM and V_{max} in sec^{-1}) were: no drug (circles), K_m = 0.76 ± 0.23; V_{max} = (4.07 ± 0.33) × 10^{-3}; [CQ] = 1 μM (squares), K_m = 1.80 ± 0.23; V_{max} = (4.7 ± 0.17) × 10^{-3}; [CQ] = 2 μM (triangles), K_m = 2.2 ± 0.24; V_{max} = (4.6 ± 0.15) × 10^{-3}; [CQ] = 3 μM (inverted triangles), K_m = 2.56 ± 0.55; V_{max} = (4.15 ± 0.27) × 10^{-3}. Inset: The K_m values were fitted to the equation $K_m^{(I)} = K_m (1 + [I]/K_i)$, where $K_m^{(I)}$ is the K_m in presence of drug, K_m is the Michaelis constant for heme degradation by GSH, [I] is the drug concentration, and K_i is the inhibitory constant for CQ. The following values for these parameters were derived: K_m = 0.96 ± 0.21 mM; K_i = 1.65 ± 0.66 μM. r = 0.961.

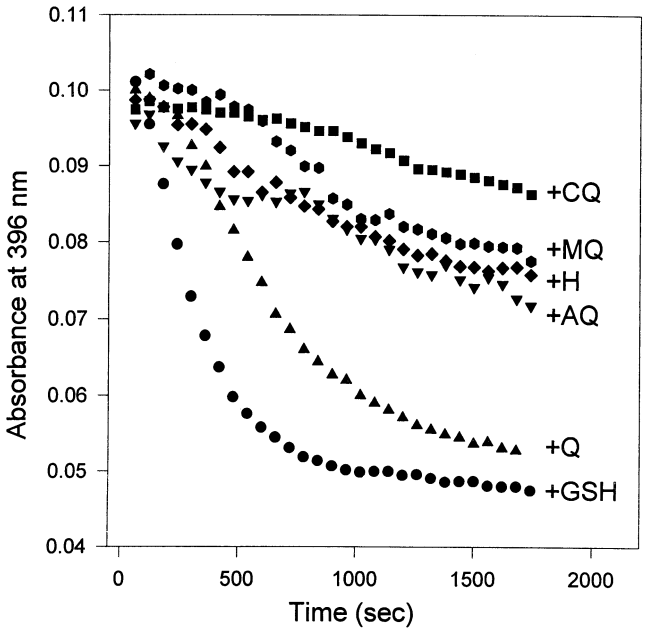

FIG. 3. Comparative inhibition of GSH-mediated degradation of heme in aqueous solution by various drugs. H, heme (3 μM) alone; + GSH, heme + GSH (2 mM); CQ, heme + GSH + CQ; AQ, heme + GSH + AQ; Q, heme + GSH + Q; MQ, heme + GSH + MQ. All drugs were at 6 μM.

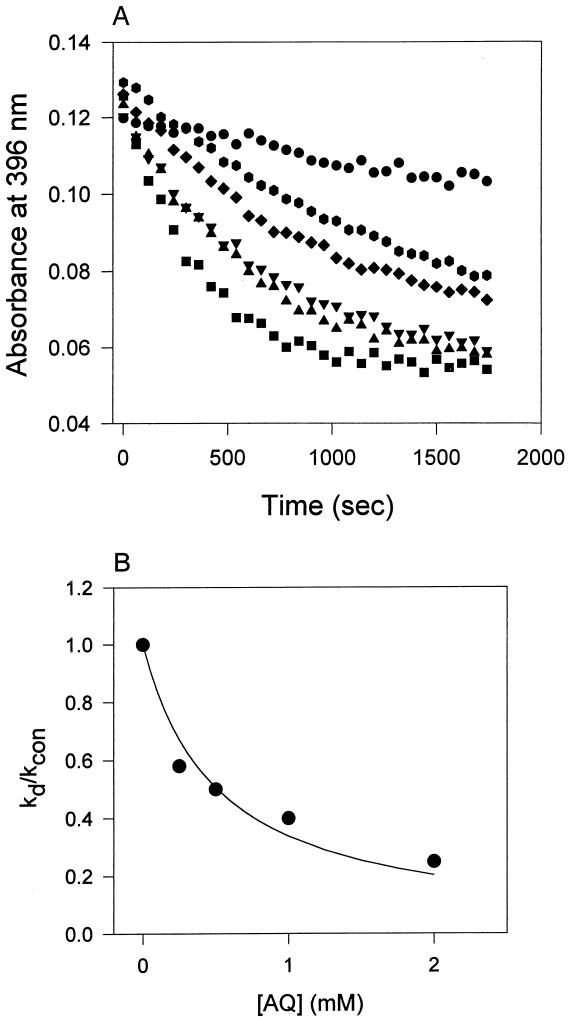

FIG. 4. Effect of AQ on GSH-mediated degradation of heme in aqueous solution in the presence of BSA. Heme (3 μM) was incubated in the presence of BSA (0.3 μM) and GSH (2 mM) and in the presence of increasing concentrations of AQ. All details are identical to those described in the legend to Fig. 1. In this example K_i = 0.51 ± 0.075 μM.

were investigated and from the results shown in Fig. 5, the half-time of equilibration and the FP partition coefficients are displayed in Table 2. The details of the calculations are described in the legend to this table. The maximal FP content was ≈2.44 nmol/10^8 ghosts, compared to the 3 nmol/10^8 ghosts found to be the maximal capacity for FP binding [30]. Hence, the concentrations of FP used in the present experiments seemed to be in the right order of magnitude. The partition coefficient of FP ($4.8 \cdot 10^5$) was only slightly increased in the presence of Q (4%) and MQ (8.3%), but appreciably so in the presence of CQ (21%) and AQ (27%). The kinetics of solubilization of FP alone or in the presence of Q and MQ were biphasic. This may indicate partial disintegration of membranes [31] and loss of the fragments during the centrifugation step preceding the determination of membrane-associated FP. The half-times of FP dissolution in ghost membranes are somewhat reduced by drugs, probably because the drugs titrate the negative charges of the phospholipid head groups.

TABLE 1. Constants of inhibition (K_i) of GSH-mediated destruction of heme by various drugs in different media

Drug	HEPES	HEPES-BSA	Ghost
Chloroquine	1.66 ± 0.31	1.13 ± 0.07	0.85 ± 0.08
Amodiaquine	0.88 ± 0.16	0.55 ± 0.08	0.68 ± 0.13
Quinine	7.32 ± 0.47	14.41 ± 1.60	No inhibition
Mefloquine	1.44 ± 0.02	7.26 ± 0.57	>50
Halofantrine	0.95 ± 0.11	0.79 ± 0.14	0.95 ± 0.15
Pyronaridine	0.72 ± 0.05	2.69 ± 0.37	0.89 ± 0.13
Q2-93	0.3 ± 0.1	0.3 ± 0.1	ND
Q1-17	1.0 ± 0.15	0.77 ± 0.08	ND
8AC6	9.0 ± 0.5	16.7 ± 2.4	ND

The degradation of heme by GSH was determined in HEPES buffer, in the presence of BSA, and when heme was dissolved in ghost membranes. The K_i values (in μM) for each drug were determined as described in Materials and Methods. ND, not determined.

Unlike the inhibition of GSH-mediated FP degradation in aqueous solution, which is similar in the presence or absence of BSA, significant differential effects were seen when FP was dissolved in membranes (Fig. 6). Here, Q and MQ were not inhibiting at all. AQ was the most effective inhibitor, followed by CQ, pyronaridine, and halofantrine (Table 1). For the bis-quinolines, only one drug concentration was tested (0.5 μM), and results are shown in Table 3. The rank order of potency was Q1-17 > Q2-93 > CQ > 8AC6.

Artemisinin, artemether, and dehydroartemisinin, the active antimalarial form of this group of compounds, had no effect on GSH-mediated FP degradation (data not shown).

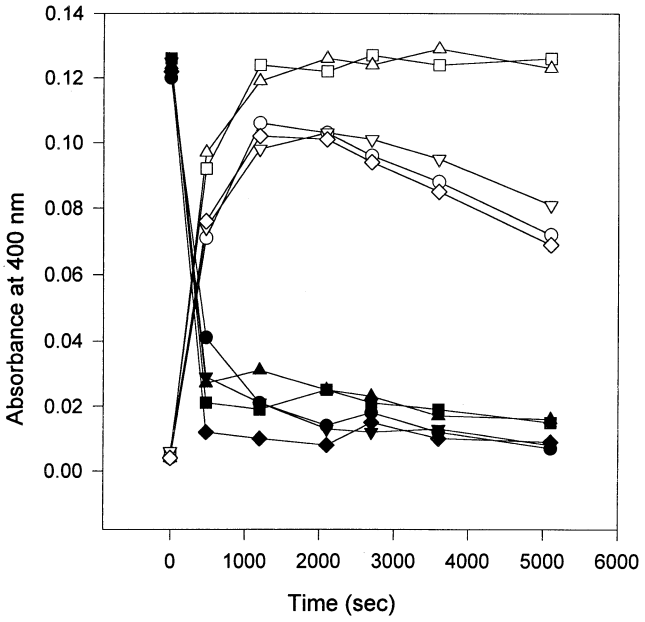

FIG. 5. Kinetics of dissolution of heme in ghost membranes as affected by drugs. Ghosts (10^8/mL) were incubated with 3 μM heme ± 6 μM drug in 0.2 M HEPES, pH 7, at 37°. Samples were taken at various time intervals, membranes were centrifuged (27,000 g for 20 min at 4°), and the supernatant was collected for heme determination. The pellet was washed once in the same buffer, dissolved in 0.8 mL of SDS 1% (w/v), and the absorbance of heme was determined spectrophotometrically at 400 nm. Results from several experiments (N = 3–5) varied, with an SD ≤ 10% of the means. All filled symbols represent heme in bathing solution; empty symbols represent heme in membrane. Control - ●, ○; CQ - ▲, △; AQ - ■, □; Q - ◆, ◇; MQ - ▼, ▽.

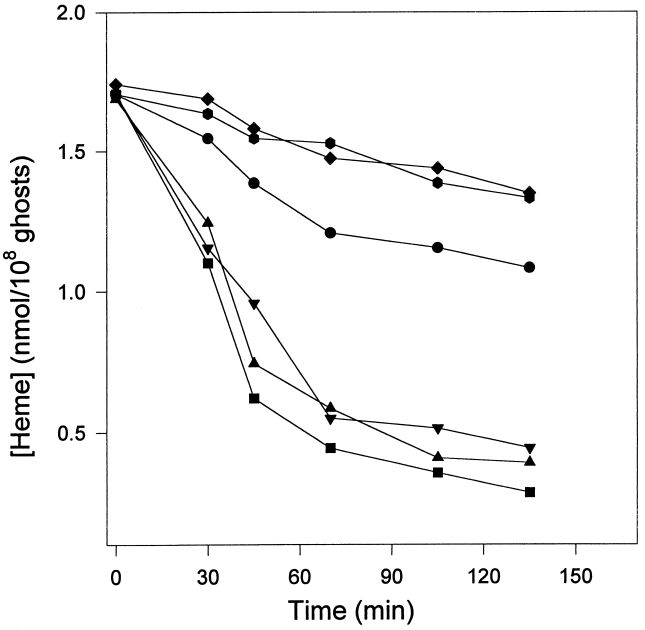

FIG. 6. Comparative inhibition of GSH-mediated degradation of heme dissolved in ghost membranes by various drugs. Designations are as described in the legend to Fig. 3. Heme was at 3 μM, GSH at 2 mM, and all drugs were at 6 μM.

Verapamil, a compound known to enhance the antimalarial action of CQ [32], had no effect on FP degradation either in presence or absence of CQ (data not shown). This observation implies that verapamil exerts its enhancing antimalarial action elsewhere.

TABLE 2. Effect of drugs on the partition of heme into erythrocyte ghosts

Drug	Partition coefficient	$t_{1/2}$ of heme solubilization (sec)
Control	$4.80 \cdot 10^5$	299
Chloroquine	$5.83 \cdot 10^5$	219
Amodiaquine	$6.08 \cdot 10^5$	243
Quinine	$5.00 \cdot 10^5$	244
Mefloquine	$5.2 \cdot 10^5$	266

Experimental data were taken from Fig. 5. The partition coefficients were calculated from the 1200-sec samples as follows: In 10^8 ghosts there are 0.03 mg phospholipid or 4×10^{-8} moles. As 1 mol lipids is equivalent to 1 liter, the volume of the lipid phase in 10^8 ghosts is equal to 4×10^{-8} liter. The concentration of membrane-associated heme could thus be calculated by dividing the heme content by the volume. The partition coefficient was obtained by dividing the concentration of heme in the membrane by its concentration in the bathing medium at this time point. The $t_{1/2}$ of heme solubilization was calculated by fitting the measured membrane-associated heme to the equation: Membrane heme = Ax(1-exp(-kxt)) and $t_{1/2} = 0.693/k$.

DISCUSSION

Malaria-infected erythrocytes are characterized by a high rate of production of FP as a result of the ingestion and digestion of host cell hemoglobin. FP is demonstrably toxic to the parasite, and it is generally held that the polymerization of FP into hemozoin provides the necessary protection for the parasite against the toxicity of FP. However, we have recently demonstrated that at the mid-term trophozoite stage, as much as 70–75% of the FP evades polymerization and therefore must exit from the food vacuole. Where could this FP reside? It can be either free in solution, bound non-specifically to cytosolic proteins, or dissolved in the parasite membranes. In this investigation, we have confirmed our previous observations [19] that FP is degraded by GSH in all three forms. We suggest, therefore,

TABLE 3. Effect of bis-quinolines on GSH-mediated degradation of membrane-associated heme

System	Ki (sec^{-1}) ± SE × 10^4	% inhibition
Heme	1.76 ± 0.63	—
Heme + GSH	7.9 ± 1.8	—
Heme + GSH + CQ	5.26 ± 2.02	33
Heme + GSH + 8AC6	7.2 ± 0.14	9
Heme + GSH + Q1-17	1.18 ± 0.21	85
Heme + GSH + Q2-93	2.9 ± 0.59	63

The degradation of membrane-associated heme in the presence of 2 mM GSH was determined as described in Materials and Methods. Only one concentration (0.5 μM) of each drug was used, and the % of inhibition of heme degradation was calculated compared to the rate of degradation in the presence of GSH alone.

that such degradation protects the parasite from unpolymerized FP.

The current opinion on the antimalarial mode of action of 4-aminoquinoline drugs is that they inhibit the polymerization of FP into the harmless hemozoin inside the food vacuole of the parasite, thus allowing free FP to exit this organelle and intoxicate the parasite. However, since most of the FP already exits the food vacuole, this inhibition, even if total, would add at most 50% to the FP load probably an extent that does not overwhelm the GSH-dependent detoxification mechanism. In order for FP to exert its toxic effect, its degradation must be inhibited as well.

We here demonstrate that the 4-aminoquinoline drugs, some bis-quinolines, and pyronaridine and halofantrine competitively inhibit the GSH-dependent degradation of FP in aqueous solution. Drugs also inhibit the spontaneous degradation of heme (data not shown). All these drugs are known to form complexes with FP and inhibit the polymerization of FP to β-hematin [6, 20, 21, 33–37]. Such complex formation evidently protects FP from both spontaneous and GSH-mediated degradation, although the mechanism of neither process is known. The rank order of inhibitory potency is slightly different from the affinity of the various drugs for FP (as deduced from FP:drug binding constants), but so is the potency of inhibition of FP polymerization as well [20]. Such variations indicate that the affinity of the drug for FP is not the sole factor that determines the inhibition of either FP degradation or FP polymerization.

FP binds rapidly, non-specifically, and with low affinity to various proteins. As such binding may intercept FP that exits the food vacuole, we have tested the ability of GSH to degrade FP bound to the low-affinity sites of BSA. We found that the rate of degradation is only slightly slower than in aqueous solution and is also inhibited by drugs. The rank order of inhibitory potency is different from that observed in aqueous solution of free FP. Compared to their effect in aqueous solution, CQ, AQ, Q1-17, and halofantrine are more inhibitory in this system, while Q, MQ, 8AC6, and pyronaridine are significantly less so. The differential potency is probably not related to binding of drugs themselves to albumin, as the K_d of binding of CQ and Q are almost identical and equal to ≈1.5 mM, and unaffected by the presence of fatty acids [38, 39], which means that under the conditions of the present experiments less than 0.1% of the drug will be bound to the protein. It seems that when FP resides in a less polar environment or in a different aggregation state, it is either less exposed to the latter drugs or its affinity for these drugs decreases, as recently suggested [40]. Hence, non-specific binding to proteins may render FP more susceptible to degradation by GSH in the presence of the less effective drugs, while conceivable binding to putative high-affinity sites will protect FP from degradation by GSH altogether, even in the absence of drug (FP bound to the high-affinity site is fully protected from GSH; data not shown), but such binding will obviously be of little concern to parasite viability. The demonstration that photoreactive analogs of CQ [41] and MQ [42] can bind to some specific parasite and host cell proteins suggests that such binding occurs subsequent to the binding of FP, a notion warranting further investigation. It remains to be shown if such binding of FP can interfere with the activity of these proteins and thereby affect parasite viability.

We now proceed to discuss the effect of drugs on the GSH-mediated destruction of membrane-associated FP. FP dissolves very rapidly into the lipid phase of membranes with a partition coefficient of $5 \cdot 10^5$. For egg lecithin liposomes, the $t_{1/2}$ of uptake of CO-FP has been estimated to be 690 sec [43] and for hemin it can be some 4 times shorter [13], i.e. in the same order of magnitude as the 300 sec found in the present work.

The concentration of FP in the present membrane preparation (assuming that all FP is dissolved in the lipid phase) is 61 mM (2.44 nmol/10^8 cells), compared to the maximal capacity of FP binding to normal erythrocytes, which amounts to 75 mM (3 nmol/10^8 cells) and causes the disruption of normal erythrocytes [30]. In *P. falciparum*-infected cells, we find at the trophozoite stage that the concentration of membrane-associated FP is ≈ 1.5 nmol/10^8 cells and can increase to 6 nmol/10^8 cells in the presence of toxic CQ or AQ concentrations [10]. It should be mentioned, however, that infected cells may contain as much as 5-fold more membrane material compared to non-infected cells [44]. Hence, the concentrations of membrane-associated FP used in the present studies are relevant to the physiology of the malaria-infected erythrocyte and the pharmacology of antimalarial drugs.

Owing to the asymmetrical structure of the FP molecule, it can be surmised that the carboxylic side chains most probably interact with the polar heads of the phospholipids, while the vinyl group side of the molecule is buried in the hydrophobic interior of the membrane [13, 45]. The iron may be near the surface of the membrane [46]. This assertion explains the ability of the polar GSH to degrade FP when it is dissolved in the membrane, as shown previously [10, 19]. The rate of degradation of membrane-associated FP by GSH is much slower than that in aqueous solution, and the rate-determining step remains to be elucidated. As Q and MQ inhibit the degradation of FP in aqueous solution but not when it is dissolved in membranes, it can be concluded that the degradation of FP by GSH occurs while it is still bound to the membrane and not in the aqueous medium following the dissociation of FP from the membrane.

All drugs tested in this study, except Q and MQ, were found to inhibit the degradation of membrane-associated FP by GSH in a competitive manner. Such competition is completely in agreement with our previous report showing that increasing the cellular level of GSH results in a decreased sensitivity (increased IC_{50}) to CQ, while depleting the cells from GSH increased the susceptibility to the drug [10].

Let us now proceed to interpret the differential effect of CQ and AQ on the one hand, and that of Q and MQ on the other, on the GSH-dependent degradation of membrane-associated FP. This differential effect may be related to the respective localization of FP and the drugs within membranes. The di-protonated form of CQ (prevalent at neutral pH) is known to react with the negatively charged phosphates of phospholipids (reviewed in [47]), but not to intercalate into them [15, 48–50]. Hence, CQ most likely resides mainly in the head group region of the lipid bilayer where it would have the greatest chance to interact with FP and protect it from degradation by GSH. AQ probably shares similar characteristics, although it is much more hydrophobic as its partition coefficient in octanol is 407 compared to 1.13 for CQ [51]. As positively charged phospholipids demonstrably increase the partitioning of FP into liposomes [14, 43], the neutralization of the phospholipids' negative charges by CQ and AQ explains their ability to enhance the solubilization of FP in the membrane as observed here. In contrast, the interactions of Q and MQ with phospholipid bilayers are quite different. Q has a partition coefficient in octanol of 68 according to one report [51] and 2000 according to another, in which the partition into dimyristoylphosphatidyl choline liposomes is larger by one order of magnitude [52]; the partition coefficient of MQ in these liposomes is 10^6, while in erythrocyte membranes it ranges between 1500 [53] and 3000 [54]. Both Q and MQ induce micellar lipid conformation and MQ is even able to generate hexagonal phases [49, 50], thus accounting for their inability to increase the dissolution of FP in membranes. In addition, Q and MQ are monoprotic at physiological pH and would be expected to be less efficient in titrating the negative charges on the phospholipids, thus accounting for their inability to increase the dissolution of FP in membrane, as observed here. Q and MQ are most likely deeply buried inside the lipid bilayer of the membrane and are less accessible to interact with FP [16, 49, 50]. The interaction of Q and MQ with FP is 30- and 42-fold weaker, respectively, than that of CQ or AQ in an environment (40% DMSO in aqueous buffer) which simulates the polarity of the phospholipid head groups [40], probably rendering them less inhibitory to FP degradation by GSH.

All these deliberations explain reasonably well why Q and MQ are unable to protect FP from degradation by GSH. The differential effects of CQ and AQ versus those of Q and MQ may underlie the fact that MQ-sensitive parasite strains are CQ-resistant, and vice versa, that there exists a degree of cross-resistance between CQ and AQ, but not with Q or MQ [55], and that verapamil, which enhances the sensitivity to CQ (but notably not to AQ) of some strains [32], has no effect on the susceptibility to MQ [56]. Likewise, halofantrine is a potent inhibitor of degradation of membrane-associated FP, but shows cross-resistance with MQ and Q [57, 58]. The message from such discrepancies is that analogous chemical structures do not bestow an identical mode of action. Further investigations are needed to dissect the details of the antimalarial mode of action of Q and MQ. It must be emphasized that the drug action of 4-aminoquinolines is probably pleiotropic (acting on different targets), and it is therefore possible that additional mechanism(s) of action may prevail as could be the mechanisms of drug resistance. In the present work only one plausible, and possibly dominant, mode of action for CQ and AQ has been elucidated. Those observations which do not completely conform with the suggested mechanism must be acting by somewhat different rate-limiting and specific steps.

Let us now consider some phenomenological corollaries of our observations. FP is known to lyse normal RBC and isolated malaria parasites [2, 3]. Lysis was found to be inhibited by β-mercaptoethanol, dithiothreitol [59], and GSH, and is enhanced by sulf-hydryl-oxidizing agents [30] and by CQ [60]. These observations are in full agreement with the ability of GSH (as well as β-mercaptoethanol and dithithreitol, data not shown) to degrade FP, and the inhibition of this process by CQ.

Identical effects most probably occur in the drug-treated malaria-infected erythrocyte. The source of FP here is that which escapes polymerization into HZ, and its quantity increases in the presence of drugs which inhibit its sequestration into HZ. The inhibition of the degradation of membrane-associated FP by GSH allows FP to accumulate in the membrane fraction of infected cells in a dose-dependent manner at therapeutically relevant concentrations. This effect accounts for the observed accumulation of FP in the membrane fraction of drug-treated infected cells: while CQ and AQ increase this level, Q and MQ do not, either in *P. berghei*-infected mouse cells [24] or in *P. falciparum*-infected human erythrocytes [10, 61]. The lysis of erythrocytes by FP is preceded by a massive loss of cellular potassium and cell swelling, which is indicative of increased permeability to sodium [59, 60], consistent with the structural defects caused by FP in phospholipid membranes [62]. At the present time, it is not known in which of the various membranes of the infected cell FP accumulates to disrupt their barrier properties. As the treatment of *P. falciparum*-infected cells with CQ results in a total depletion of potassium from the parasite cytosol [63], the parasite plasma membrane is a likely primary target. The impairment of ion homeostasis would result in an irreversible inhibition of cellular metabolism and parasite death, thus accounting for the cytotoxic effect of the drugs.

The K_i values derived in this study for the inhibition of GSH-mediated degradation of protein- or membrane-bound FP are greater than the IC_{50} values for inhibition of parasite growth. One has to consider that at the trophozoite stage, 10 nmol of FP is generated every hour in 10^8 infected cells, but only 30% of this amount is polymerized. In order to reach the level of membrane FP that correlates with almost total parasite killing in 4 hr (6 nmols/10^8 cells), even partial inhibition of FP degradation, i.e. at lower [CQ] than the K_i for this drug, would suffice to allow for the accumulation of FP to toxic levels. Indeed, we have shown

previously that treatment of trophozoite-infected cells with [CQ] equal to the IC_{50} results in an increase in membrane-associated FP that is related to the extent of parasite killing [10]. Recent investigations suggest that the accumulation of CQ in malaria-infected cells involves a saturable component whose k_d is directly proportional to the IC_{50}, while the maximal binding is identical in different strains of *P. falciparum* [64]. As the reduction of the generation of FP resulted in a decreased binding of CQ, it has been inferred that CQ binds to FP, in full accord with the model presented here. The higher k_d of CQ binding found in resistant strains hints at the possibility that they have a higher concentration of GSH. The identical maximal binding obviously implies that the accumulation of FP is finite and instrumental in the mechanism of drug toxicity.

Although the K_i values of the various antimalarial drugs are not directly correlated with their relative antimalarial efficacy, this discrepancy does not seem to invalidate the mechanistic interpretation of their action presented here. In comparing different drugs, one also has to consider their ability to concentrate inside the food vacuole, their relative efficacy in inhibiting FP polymerization and thereby affecting the amounts of FP that are released from the food vacuole, their possible interference with the translocation of FP across the vacuolar membrane or its interception (whether free or bound to proteins) on the way from the vacuole to the membranes, and their effect on the solubilization of FP in membranes. Each of these distinct steps may be differently affected by each drug. Using the various drugs for comprehensive studies of all these effects, it may be possible to identify which of the effects determines the pharmacological outcome. It may well be that for different drugs the definitive effect may be different, and ways could be found to integrate the partial effects into a predictive therapeutic potency. Such an approach may also reveal the factor(s) which may variably account for drug resistance, and explain the invariable potency of the bis-quinolines, which have been attracting much interest as of late.

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