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Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes

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Abstract

Several reports suggest that the antimalarial mode of action of quinoline drugs may differ in their mechanistic details. The malaria parasite *Plasmodium falciparum* was treated in culture with chloroquine, amodiaquine, quinine and mefloquine in a dose- and time-dependent fashion. After removal of the drug, the viability of the parasites and their hemoglobin content were determined. Whereas in the presence of chloroquine and amodiaquine, there was a correlation between parasite killing and accumulation of hemoglobin, with quinine and mefloquine parasite killing was not associated with the accumulation of hemoglobin. Mefloquine inhibited the chloroquine-dependent accumulation of hemoglobin. It is suggested that whereas chloroquine and amodiaquine inhibit the digestion of hemoglobin, mefloquine and possibly quinine inhibit the ingestion of host cell hemoglobin by interfering with the ingestion process. These results may explain the demonstrable antagonism between chloroquine and mefloquine and their antipodal sensitivity to these drugs.

Keywords: Malaria; *Plasmodium falciparum*; Chloroquine; Mefloquine; Mode of action

1. Introduction

When malaria parasites propagate inside the erythrocytes of their host, they ingest the cytosol of the infected cell by endocytosis into their food vacuole [1]. The ingested cytosol which consists of ~95% hemoglobin is then digested by a concerted action of several acidic proteases [2]. The resulting short peptides egress from the food vacuole [3] whereas the ferriprotoporphyrin IX (FPIX) that is toxic to the parasite [4–6] is either polymerized *in situ* into an insoluble FPIX polymer (hemozoin, or malaria pigment, [7], or is degraded by glutathione after exiting from the food vacuole into the parasite cytosol [8]. It is surmised that 4-aminoquinolines exert their antimalarial action by interfering with some of these detoxification processes (see [9] for review). Whereas, the accumulation of hemozoin in intact infected cells is inhibited by chloroquine (CQ), amodiaquine (AQ), quinine (Q) and mefloquine (MQ), the glutathione-dependent destruction of FPIX is inhibited only by CQ and AQ but not by Q or MQ [10,11]. Several other reports have hinted that the detailed antimalarial modes of action of CQ are different. Thus, MQ has an antagonistic effect to the inhibitory effect of CQ on parasite growth [12] and parasite resistance to CQ-parallels MQ sensitivity and *vice versa* [13–17].

In the present investigation, we have measured the accumulation of undigested hemoglobin in drug-treated infected cells in conjunction with inhibition of parasite growth in order to get a deeper insight into the differential mode of action of 4-aminoquinoline antimalarial drugs.

2. Materials and methods

2.1. Materials

Fresh O^+ or A^+ blood and human O^+ or A^+ plasma were kindly donated by the Hadassah Hospital Blood Bank. RPMI-1640 was purchased from Biological Industries,

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Abbreviations: CQ, chloroquine; AQ, amodiaquine; Q, quinine; MQ, mefloquine; FPIX, ferriprotoporphyrin IX

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Kibbutz Bet Haemek, Israel. Chloroquine diphosphate was obtained from Serva. Amodiaquine dihydrochloride, quinine hydrochloride and *O*-dianisidine were purchased from Sigma Chemicals Co. Mefloquine was generously provided by A.F. Cowman. [^{3}H]Hypoxanthine (43 Ci/mmol) was procured from Amersham. All other chemicals were of the best available grade.

2.2. Parasite cultivation

The *FCR3* strain of *P. falciparum* was cultivated as previously described [8] in RPMI-1640 medium supplemented with 10 mM glucose, 25 mM $NaHCO_3$, 25 mM Hepes and 10% pooled human heat-inactivated plasma. Cultures were synchronized by the sorbitol technique [18] using an iso-osmotic solution (300 mOsM) of the less toxic alanine.

2.3. Effect of drug treatment on parasite viability

Synchronized cultures were seeded at the ring stage and allowed to grow for another 20 hr until most parasites reached the trophozoite stage. Cultures at 1% hematocrit and 15–20% parasitemia were exposed to drugs under culture conditions for periods of time that are indicated in Section 3. One milliliter of culture was washed twice in wash medium (culture medium without plasma, 37°) to remove the drug [19], and cells were seeded in 24-well culture plates in full culture medium supplemented with 5 μCi/mL [^{3}H]Hypoxanthine (43 Ci/mmol). After 4 hr of further cultivation, triplicate samples of 200 μL were aliquoted into 96-well culture plates and parasite associated radioactivity was determined using the Filtermate/Matrix 96 Direct Beta counter. Inhibition of parasite growth was calculated compared to untreated control.

2.4. Preparation of free parasites from cultures

Trophozoites were released from infected RBC by saponin lysis (0.003% saponin (w/v) in PBS; [10]) followed with repeated washes in PBS buffer (1000 g for 5 min) until no hemoglobin could be detected in the wash solution (usually four washes were needed). The free parasites were resuspended in an equal volume of buffer content 0.1 M KCl in 20 mM Na-phosphate buffer at pH 7.4. Free parasites were disrupted by five cycles of freezing in liquid nitrogen and thawing at 37°. The cell debris and hemozoin were spun down by centrifugation at 10400 g for 15 min, and the supernatant consisting of parasite cytosol was recovered for further experiments.

2.5. Gel electrophoresis of parasite extracts and detection of FPIX

The method of Francis and Becker [20] was used. Samples of parasite cytoplasm were run on SDS-PAGE (10% polyacrylamide) omitting dithiotreitol from the running buffer in order to avoid destruction of FPIX. Gels were fixed for 20 min with 12.5% trichloracetic acid and washed for 20 min in DDW. Gels were then stained with a mixture of 0.1% (w/v) *O*-dianisidine chloride and 20 mM hydrogen peroxide in 40 mM citric acid until appearance of stained FPIX.

2.6. Determination of hemoglobin level in lysates of free parasites

Fifty microliters of lysates were dissolved in 550 μL 0.2 M Hepes buffer pH 7.0. The absorption spectrum was determined in the 300–650 nm range by means of a Milton Roy Spectronic 3000 spectrophotometer. Since hemoglobin has a very high molar absorption coefficient, its presence could be ascertained. The changes in hemoglobin level were monitored by changes in absorbance of the Soret band at 412 nm and were normalized to parasite cell number in the culture.

3. Results

3.1. Drug treatment results in differential accumulation of undigested hemgoglobin

Treatment of trophozoite-infected cells with 10 μM of different drugs resulted in differential response to the drugs. As seen in Fig. 1, in the presence of CQ or AQ it is clearly seen that most of the FPIX is associated with a protein that has a molecular mass of 16.5–17 kDa, that is, identical to monomers of hemoglobin, as evidenced by the same band appearing in the lane that contains the erythrocyte lysate. Also seen are some free FPIX (bands in the gel front) and some additional bands of higher molecular weight due to binding of FPIX to specific proteins. No FPIX-stainable bands could be observed in untreated controls. The densities of stainable bands in the Q-treated sample were much lower and in the MQ-treated sample they were barely distinguishable. These results clearly indicate that CQ and AQ inhibit the digestion of hemoglobin as well as the destruction of free FPIX [8,11].

3.2. Effect of drug treatment on the levels of hemoglobin in parasite compartment and on parasite viability

Parasite cultures at the trophozoite stage were exposed under culture conditions to different drugs, either at fixed concentration for variable length of time, or for a fixed incubation time at increasing drug concentrations. After removing the drug, the cells were manipulated to estimate the level of hemoglobin in parasite extracts and parallel samples were returned to culture conditions to test parasite

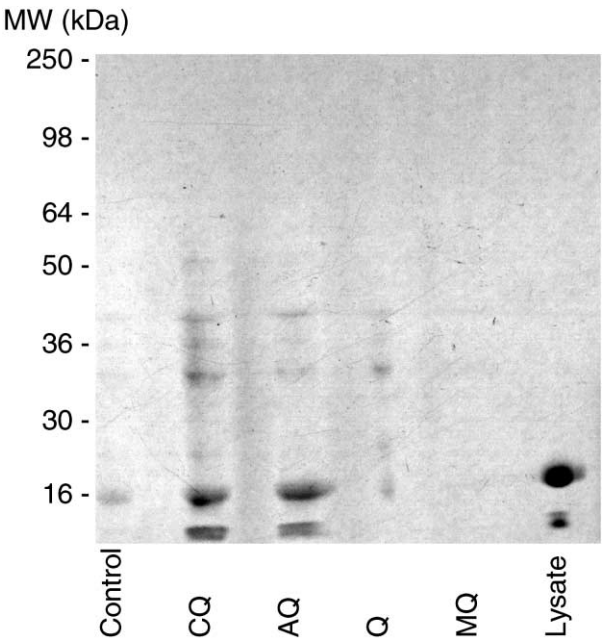

Fig. 1. Effect of drug treatment on the accumulation of hemoglobin in parasite lysate. Cultures were treated with 10 μM of different drugs for 3 hr and free parasites were obtained as described in Materials and Methods. Samples of parasite cytoplasm and of lysate of normal erythrocytes were run on SDS-PAGE (10% polyacrylamide) omitting dithiotreitol from the running buffer in order to avoid destruction of FPIX. After electrophoresis, gels were fixed for 20 min with 12.5% trichloracetic acid and washed for 20 min in DDW and stained for FPIX [20]. The band at the running front is FPIX.

viability. The use of relatively high drug concentrations in these experiments deserves justification. The antimalarial effect of the drugs is both time- and dose-dependent [19]. As shown in Fig. 2, at 1 μM only 20% inhibition of parasite growth is achieved, and the effect was time-dependent, giving a maximal inhibition of about 80% after 4 hr of exposure. These results indicate that the IC_{50} of drug action under the present experimental conditions of 1–2% hematocrit and 15–20% parasitemia has been only slightly surpassed at the highest concentrations used. This conclusion is based on our previous demonstration that with the same hematocrit but only 1% parasitemia, after 4 hr of exposure to the same drugs the IC_{50} was in the sub-micromolar range for CQ and MQ and ~10 μM for Q [19]. For the time-dependent effect, 10 μM drug concentrations have been chosen because there the build-up of undigested hemoglobin was easily detectable.

Hemoglobin level and inhibition of parasite growth are shown in Fig. 2 for the dose-dependent effect, and in Fig. 3 for the time-dependent effect at fixed drug concentration. CQ is always used as a reference since the parasites are not always at the same precise stage. Treatment of trophozoite-infected cells with CQ or AQ resulted in the accumulation of a 412 nm-absorbing substance in the lysates of disrupted parasites as a function of drug concentration (Fig. 2A) and the time of incubation (Fig. 3A). This substance was

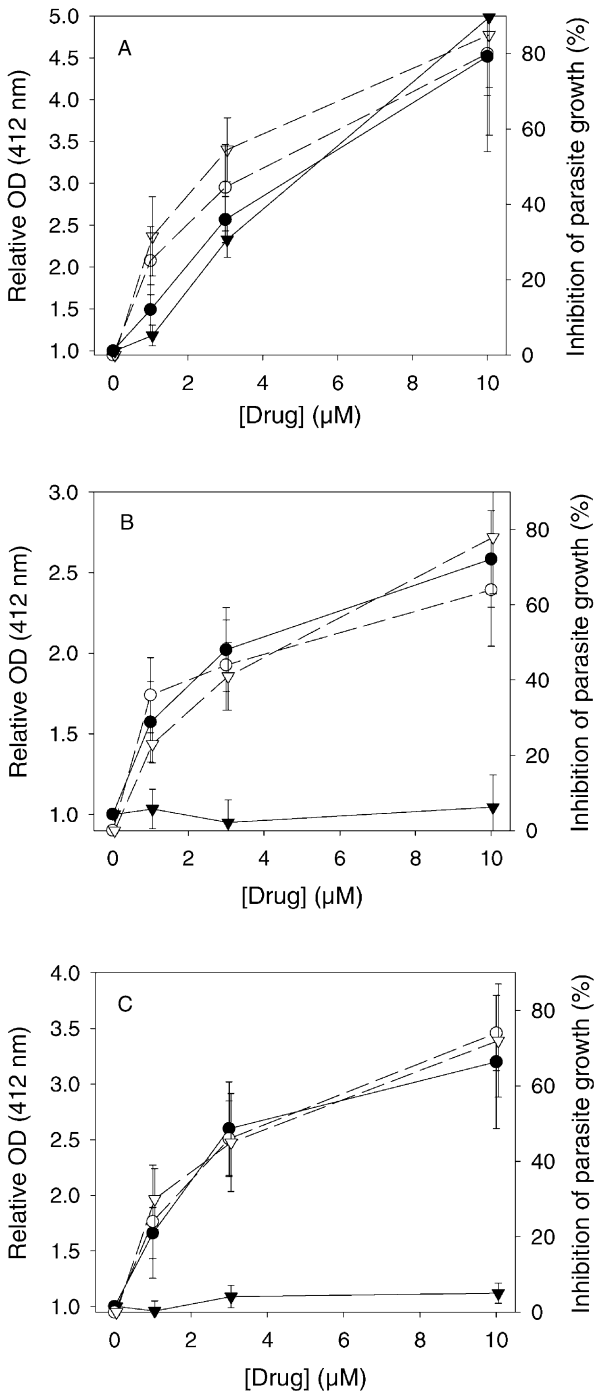

Fig. 2. Dose-dependent effect of drugs on hemoglobin accumulation in parasite fraction. Parasite cultures at the trophozoite stage were incubated in wash medium (culture medium without plasma) at 1% hematocrit and 15–20% parasitemia, in the absence or the presence of the indicated drug concentrations for 4 hr under culture conditions. Samples were taken at time 0 and at the end of incubation, lysed with saponin to obtain free parasites, and the amount of hemoglobin was determined by the absorbance of the Soret peak (412 nm). Parallel sample were taken at the end of the incubation, washed extensively to remove the drug and returned to culture conditions in the presence of [^{3}H]Hypoxanthine for 4 hr to assess parasite viability. Full lines and filled symbols describe relative hemoglobin concentration (in absorbance units compared to zero drug concentration); broken lines and empty symbols depict % inhibition of parasite growth. (A) CQ (circles) and AQ (inverted triangles); (B) CQ (circles) and Q (inverted triangles); (C) CQ (circles) and MQ (inverted triangles).

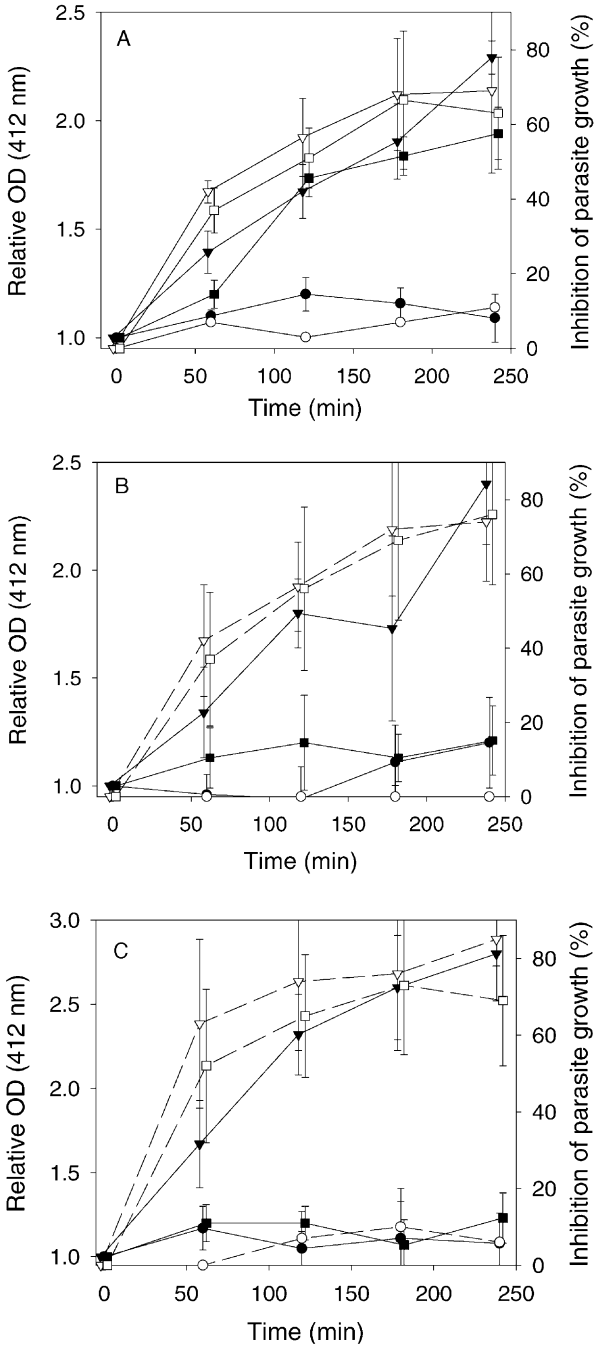

Fig. 3. Time dependence of drug action on hemoglobin accumulation. Conditions of experiments were as described in the legend to Fig. 2, except that drugs were added at 10 μM and samples were taken at different time intervals. Full lines and filled symbols describe relative hemoglobin concentration (in absorbance units compared to time zero); broken lines and empty symbols depict % inhibition of parasite growth. (A) Control (circles), CQ (inverted triangles) and AQ (squares); (B) Control (circles), CQ (inverted triangles) and Q (squares); (C) Control (circles), CQ (inverted triangles) and MQ (squares).

judged from its absorption spectrum to be hemoglobin, suggesting that drug treatment inhibited hemoglobin digestion. With CQ and AQ, the accumulation of hemoglobin paralleled the inhibition of parasite growth (Figs. 2A and 3A). In contrast, cells treated with Q (Figs. 2B and 3B) or

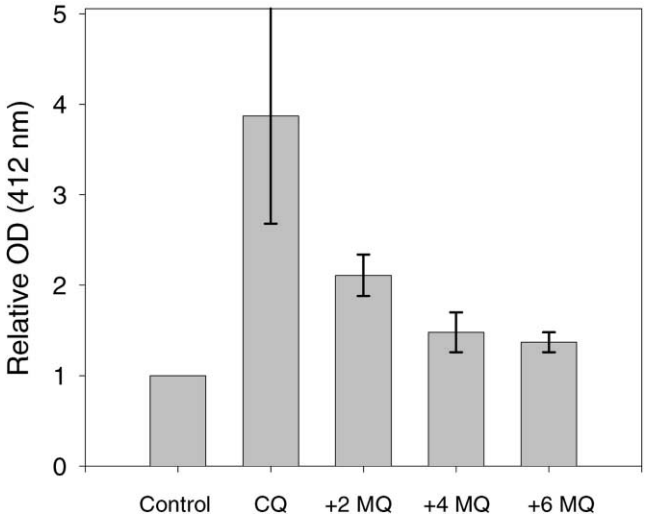

Fig. 4. Antagonistic effect of MQ on CQ-induced accumulation of hemoglobin. Experiments were performed as described in the legend to Fig. 2. Cells were cultivated in the presence of 6 μM CQ with none or increasing concentrations of MQ.

MQ (Figs. 2C and 3C) did not show such accumulation although parasite growth was inhibited to the same extent. Moreover, MQ reversed the effect of CQ on hemoglobin accumulation (Fig. 4).

4. Discussion

We found that CQ inhibits hemoglobin degradation. This is in accordance with previous results [1,21,22] showing by electron microscopy the accumulation of undegraded host cell cytosol inside the food vacuole. We show here that AQ behaves similarly. In contrast, Q and MQ did not affect this process and MQ even antagonized the inhibitory action of CQ. These results suggest that the antimalarial mode of action may differ in its details among the various 4-aminoquinolines. CQ, Q and MQ are known to inhibit *in vitro* acid proteases of *P. falciparum* that are demonstrably involved in the digestion of ingested host cell cytosol inside the acidic food vacuole of the parasite [23,24], but they do so only at very high drug concentrations. Such concentrations may probably be reached inside the acid food vacuole of the parasite by the diprotic CQ but not by the monoprotic MQ or Q [25]. FPIX, a product of hemoglobin digestion inhibits proteolytic activity and this is not diminished in the presence of CQ, suggesting that complex formation between FPIX and CQ [26] does not interfere with FPIX action on vacuolar protease(s). AQ [27,28] and Q [29] can also form complexes with FPIX, but Q probably does not reach the necessary concentrations inside the food vacuole. These considerations may explain the observed differential effect of the drugs on hemoglobin degradation. Since all drugs equally inhibited parasite growth, it may be safe to

conclude that either the detailed mode of action of the drugs is different, and/or that inhibition of hemoglobin degradation is not an essential component of drug action in the cases of Q and MQ.

Inhibition of proteolysis *in situ* may be exacerbated by a drug-dependent increase of the vacuolar pH, although this has been shown to occur only at supra-pharmacological concentration [25]. Drug concentrations that are higher than the respective IC_{50} have been used in this investigation, for reasons explained above in Section 3. Most importantly, the viability tests that resulted in much less than 100% inhibition of parasite growth are the relevant attributes that indicate that the pharmacological range has not been surpassed. Hence, it seems safe to conclude that the possible drug-dependent alkalinization of the food vacuole can be excluded. This presumption is further supported by the ability of MQ to reverse the effect of CQ. An additive effect of CQ and MQ on vacuolar alkalinization is expected [30]. Thus, if increase in vacuolar pH is the major factor affecting proteolytic activity, one would have expected an additive effect in hemoglobin accumulation in the presence of both CQ and MQ, contrary to what has been observed.

The antagonistic effect of MQ on CQ-dependent accumulation of hemoglobin (Fig. 3) could be due to a hitherto unknown mechanism, that is, the inhibition of endocytosis *per se* by MQ and possibly also by Q. MQ has been shown to inhibit the phagocytic activity of human peripheral blood leucocytes [31] and of human polymorphonuclear neutrophils [32]. This may be due to the intricate interaction of MQ and Q with membrane phospholipids (discussed in details in [11]). Thus, whereas CQ and AQ inhibit the proteolysis of hemoglobin and the polymerization of FPIX due to their high capacity accumulation in the food vacuole and their ability to complex with FPIX, MQ and possibly Q inhibit the endocytic process. These differential activities will nevertheless all lead to the observed inhibition of hemozoin formation [10]. These conclusions should be confirmed by additional direct investigation of the endocytotic process. It is not suggested that the sole antimalarial action of MQ and possibly Q are due to the inhibition of endocytosis. Quinoline containing antimalarials are known to have pleiotropic effects [9], and inhibition of endocytosis by MQ or Q may be one of them. The relative importance of this effect is not known and may be very difficult to assess.

It has been known for a long time that MQ inhibits the uptake of CQ in infected cells [33,34]. The recent demonstration that saturable uptake of CQ into infected cells is mediated by binding to FPIX [35], suggests an interpretation to this inhibition: if MQ inhibits the ingestion of hemoglobin, there could not be any generation of FPIX for the binding of CQ. This mechanism explains the antagonistic effect of CQ and MQ on parasite growth and the general phenomenon showing that increased resistance of parasites to CQ parallels an increased sensitivity to MQ and *vice versa*, as mentioned in Section 1.

Acknowledgments

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