Differential Effects of Quinoline Antimalarials on Endocytosis in *Plasmodium falciparum* ▼

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Received 14 November 2007/Returned for modification 18 December 2007/Accepted 26 February 2008

The effects of quinoline antimalarials on endocytosis by *Plasmodium falciparum* was investigated by measuring parasite hemoglobin levels, peroxidase uptake, and transport vesicle content. Mefloquine, quinine, and halofantrine inhibited endocytosis, and chloroquine inhibited vesicle trafficking, while amodiaquine shared both effects. Protease inhibitors moderated hemoglobin perturbations, suggesting a common role for heme binding.

Despite problems associated with drug resistance and side effects, quinolines remain widely used for the treatment of severe malaria and malaria prophylaxis, in artemisinin combination therapy regimens, and for the development of novel quinoline drug candidates (1, 2, 11, 16, 19). The mechanism of action of the 4-aminoquinoline chloroquine (CQ) has been extensively investigated. Intraerythrocytic malarial parasites ingest the erythrocytic cytosol by endocytosis and deliver it to the parasit food vacuole via hemoglobin (Hb) transport vesicles. Hb digestion in the vacuole releases ferrisoporphyrin IX (FP), which is detoxified by incorporation into inert hemozoin crystals (6). CQ concentrates in the food vacuole and is thought to dimerize with FP to cause an inhibition of hemozoin formation and the lethal accumulation of toxic FP or FP-CQ complexes (5, 9, 14, 21, 22). Other quinolines have also been found to inhibit hemozoin formation in vitro at concentrations that correlate with their parasite-inhibitory concentrations (4, 5, 7–9, 13, 14), which supplies a strong argument that they share the mechanism of action of CQ and produce toxic levels of FP by disrupting hemozoin formation. This conclusion is confounded, however, by their disparate effects on the parasite Hb endocytic pathway (10). We have previously found that CQ inhibits Hb transport vesicle trafficking, with a resultant accumulation of Hb and vesicles, while mefloquine (MQ) inhibits Hb endocytosis (15). In this study, we investigated the extent to which these differential effects extend to other therapeutically relevant quinolines, i.e., quinine (Q), halofantrine (H), and amodiaquine (AQ).

Early trophozoite-stage cultures of *Plasmodium falciparum* (strain 3D7) were incubated for 8 h with the quinolines at concentrations 5 times their 50% inhibitory concentration values, the parasites were released from the infected erythrocytes by saponin lysis, and the parasite Hb content was determined by Western blotting (15, 20) (Fig. 1A). Consistent with the notion that MQ inhibits fluid phase endocytosis in malaria parasites (10, 15), the Hb content in MQ-treated parasites was reduced by 83%. The structurally related quinoline-methanols Q and H also reduced Hb content, by 64% and 84%, respectively. By contrast, the Hb content in CQ-treated parasites increased to 283% compared to that in the untreated controls. Being a 4-aminoquinoline related to CQ, AQ was expected to act similarly (10). However, the Hb levels in AQ-treated parasites were comparable to that in the controls or slightly reduced (statistically insignificant; *P* = 0.37). In parallel cultures, parasites were incubated with the quinolines in combination with 40 μM of the protease inhibitors (PIs) ALLN and E64 to inhibit Hb digestion (12) and were subjected to Hb Western blotting (20) (Fig. 1B). Predictably, the addition of the PIs increased the Hb levels in the control parasites more than threefold. CQ or AQ did not significantly affect Hb levels in parasites in the presence of PIs. Although a reduction in Hb levels was still found with Q and H, it was less profound than that obtained in the absence of PIs (31% and 30% versus 64% and 84% for Q and H, respectively). Hb uptake inhibition by MQ was also alleviated by the PIs (66% versus 83% inhibition). This modification of quinoline effects on Hb levels in the presence of PIs agrees with published reports that Hb antagonize quinoline action (18). It supports the notion that quinolines share FP binding as a mode of action and suggests that their differential effects on the Hb endocytic pathway may be manifestations of differences in the physicochemical properties of the individual quinoline-FP complexes. The residual endocytosis inhibition by quinoline-methanols in the presence of PIs may be due to their additional propensity to bind phospholipid membranes (3, 13, 23), which could disrupt the plasma membrane properties required for endocytic vesicle formation. The Hb dependence of quinoline action may potentially be explored by using erythrocyte-free parasites (15). However, reevaluation of the procedure suggests that the removal of parasites from their intraerythrocytic environment usually terminates endocytic activity, precluding the reproducible application of this technique.

To further investigate the effects of the quinolines on endocytosis, an assay was performed using an exogenous indigest-
Confidence interval (CI).

**Hemoglobin levels in drug-treated parasites.** (A) Parasite cultures were untreated (control [Con]) or incubated with amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ), or quinine (Q) at concentrations 5 times their 50% inhibitory concentrations for 8 h. The concentrations used were 137 nM CQ, 156 nM MQ, 665 nM Q, 102 nM AQ, and 27 nM H. (B) In parallel cultures, the quinoline drugs were added in combination with the protease inhibitors ALLN and E64. Following treatment, parasite hemoglobin levels were detected by Western blotting. The intensities of the hemoglobin bands on the blots were determined using the histogram function of Adobe Photoshop (version 7.0) and normalized to control intensities. Error bars indicate standard deviations. An asterisk (*) indicates a significant change from the results for the controls ($P < 0.05; 95\% \text{ CI}$).

**FIG. 2.** Effect of quinoline drugs on horseradish peroxidase endocytosis by malaria parasites. Parasites were allowed to invade erythrocytes preloaded with HRP by hypotonic lysis and infected by incubation with enriched schizont-infected erythrocytes. Parasites in the HRP-loaded erythrocytes were exposed to quinolines for 10 h, released with saponin, and lysed with Triton X-100, and their HRP content was measured by a colorimetric peroxidase assay (20). Consistent with the Hb Western blotting results, MQ, Q, and H inhibited HRP uptake by the parasites (Fig. 2). Surprisingly, AQ produced a comparable inhibition of HRP uptake, while CQ had a weak inhibitory effect. The apparent incongruity between the inhibition of HRP endocytosis by AQ and the lack of a significant effect on Hb levels by this compound was clarified by a subsequent Hb immunofluorescence microscopy assay. Following quinoline treatment, parasites were released with saponin and fixed on polylysine-coated coverslips, using paraformaldehyde and glutaraldehyde. After being permeated in Triton X-100, the parasites were incubated with rabbit anti-Hb antiserum followed by tetramethyl rhodamine isocyanate-labeled secondary antibodies, and examined by fluorescence microscopy. The panels on the left are fluorescence micrographs, and the panels on the right are the corresponding phase-contrast light micrographs. The large arrows in panels A and B indicate the position of the parasite food vacuole, identifiable by the presence of the prominent hemozoin crystal in the phase-contrast images. The smaller arrows in panel A denote individual hemoglobin transport vesicles. The mean number of hemoglobin transport vesicles per parasite ($\pm$ the standard error of the means) in the control and treated samples, determined by counting 100 randomly selected parasites per sample, is tabulated below the micrographs. All values for the treated samples were significantly different from that for the control ($P < 0.05; 95\% \text{ CI}$).

**FIG. 3.** Effect of quinoline drugs on hemoglobin transport vesicle numbers in malaria parasites, as determined by immunofluorescence microscopy. Parasite cultures were left untreated (A) or incubated with chloroquine (B), amodiaquine (C), quinine (D), halofantrine (E), or mefloquine (F) for 8 h. Following saponin lysis, parasites were immobilized, fixed onto glass coverslips, incubated with anti-hemoglobin antiserum followed by tetramethyl rhodamine isocyanate-labeled secondary antibodies, and examined by fluorescence microscopy. The panels on the left are fluorescence micrographs, and the panels on the right are the corresponding phase-contrast light micrographs. The large arrows in panels A and B indicate the position of the parasite food vacuole, identifiable by the presence of the prominent hemozoin crystal in the phase-contrast images. The smaller arrows in panel A denote individual hemoglobin transport vesicles. The mean number of hemoglobin transport vesicles per parasite ($\pm$ the standard error of the means) in the control and treated samples, determined by counting 100 randomly selected parasites per sample, is tabulated below the micrographs. All values for the treated samples were significantly different from that for the control ($P < 0.05; 95\% \text{ CI}$).
the net content of Hb in AQ-treated parasites remains largely unaffected. Further investigation of the molecular mechanisms underlying quinoline effects is required to determine if the endocytosis perturbations result from upstream primary effects or are directly related to the parasiticidal mechanisms of the drugs.

This work was supported by a Wellcome Trust Senior International Research Fellowship to H.C.H. and a Medical Research Council postgraduate scholarship to L.R.

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