DIFFERENTIAL EFFECTS OF QUINOLINE ANTI-MALARIALS ON ENDOCYTOSIS IN *PLASMODIUM FALCIPARUM*.

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ABSTRACT

The effects of quinoline antimalarials on endocytosis by *Plasmodium falciparum* was investigated by measuring parasite haemoglobin, peroxidase uptake and transport vesicle content. Mefloquine, quinine and halofantrine inhibited endocytosis, chloroquine inhibited vesicle trafficking, while amodiaquine shared both effects. Protease inhibitors moderated haemoglobin perturbations, suggesting a common role for haem binding.
Despite problems associated with drug resistance and side-effects, quinolines remain widely used for the treatment of severe malaria, for 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. Intra-erythrocytic malaria parasites ingest erythrocyte cytosol by endocytosis and deliver it to the parasite food vacuole via haemoglobin (Hb) transport vesicles. Hb digestion in the vacuole releases ferrirhodoporphyrin IX (FP), which is detoxified by incorporation into inert haemozoin crystals (6). CQ concentrates in the food vacuole and is thought to dimerize with FP to cause an inhibition of haemozoin formation and lethal accumulation of toxic FP or FP-CQ complexes (5, 9, 14, 21, 22). Other quinolines have also been found to inhibit haemozoin formation in vitro at concentrations which correlate with their parasite-inhibitory concentrations (4, 5, 7-9, 13, 14). It supplies a strong argument that they share the mechanism of action of CQ and produce toxic levels of FP by disrupting haemozoin 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 have 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 hours with the quinolines at concentrations 5x their IC_{50} values, the parasites released from the infected erythrocytes by saponin lysis, and parasite Hb content determined by Western blotting (15, 20; Fig. 1A). Consistent with the notion that MQ inhibits fluid-phase endocytosis in malaria parasites (10, 15), 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, Hb content in CQ-treated parasites increased to 283% compared to untreated controls. Being a 4-aminoquinoline related to CQ, AQ would have been expected to act similarly (10). However, Hb levels in AQ-treated parasites were comparable to 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 subjected to Hb Western blotting (20; Fig. 1B). Predictably, addition of the PIs increased the Hb levels in control parasites by more than 3-fold (Fig. 1B vs. 1A, control bar). 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% vs. 64% and 84% for Q and H, respectively). Hb uptake inhibition by MQ was also alleviated by the PIs (66% vs. 83% inhibition). This moderation of quinoline effects on Hb levels in the presence of the PIs agrees with published reports that Hb protease inhibitors 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 physiochemical 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, re-evaluation of the procedure suggests that removal of parasites from their intra-erythrocytic 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 indigestible endocytic tracer, horseradish peroxidase (HRP) (17). Erythrocytes were 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 hours, released with saponin, lysed with Triton X-100, and their HRP content 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 poly-lysine-coated coverslips using paraformaldehyde and glutaraldehyde. After permeation in Triton X-100, the parasites were incubated with rabbit anti-Hb antiserum followed by TRITC-conjugated goat anti-rabbit antibodies and viewed by fluorescence microscopy (15, 20). The majority of the Hb co-localized with the haemoglobin crystal in the parasite food vacuole (Fig. 3A and B, large arrows). Additional fluorescent puncta represented Hb transport vesicles in transit from the plasma membrane to the food vacuole (Fig. 3A, small arrows). Enumeration of the vesicles showed that Q, H and MQ caused a reduction in endocytic vesicle content per parasite, supporting the conclusion that they inhibit endocytosis and, consequently, endocytic vesicle formation (Fig. 3D-F; table). CQ-treatment markedly increased vesicle content, consistent with a disruption of normal vesicle trafficking (Fig. 3B; table). Unexpectedly, AQ also caused an increase in vesicle content, often strikingly in individual parasites (Fig. 3C; table). This suggests that AQ produces a combination of the cellular effects of CQ on the one hand, and of Q, H and MQ on the other – by inhibiting Hb trafficking and endocytosis simultaneously, 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.
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REFERENCES


FIGURE LEGENDS

Figure 1. Haemoglobin levels in drug-treated parasites. 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 IC\textsubscript{50} values for 8 hours (A). Concentrations used were 137 nM CQ, 156 nM MQ, 665 nM Q, 102 nM AQ and 27 nM H. In parallel cultures, the quinoline drugs were added in combination with the protease inhibitors ALLN and E64 (B). Following treatment, parasite haemoglobin levels were detected by Western blotting. Intensities of the haemoglobin 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 deviation. * significant change from the controls (p < 0.05; 95% CI).

Figure 2. Effect of quinoline drugs on horseradish peroxidase endocytosis by malaria parasites. Parasites were allowed to invade erythrocytes preloaded with HRP. They were then left untreated (control), or incubated with amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) or quinine (Q) for 10 hours. Following incubation, parasites were released from erythrocytes by saponin treatment, washed repeatedly, lysed with Triton X-100 and HRP levels measured by enzymatic activity following addition of o-phenylenediamine (absorbance of the product at 450 nm). Error bars indicate standard error. * significant difference from controls (p < 0.05; 95% CI).

Figure 3. Effect of quinoline drugs on haemoglobin 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 hours. Following saponin lysis, parasites were immobilized and fixed onto glass coverslips and incubated with anti-hemoglobin antiserum followed by TRITC-labeled secondary antibodies, and examined by fluorescence microscopy. The left-hand panels are fluorescence micrographs, and the right-hand panels the corresponding phase-contrast light micrographs. The large arrows in A and B indicate the position of the parasite food vacuole, identifiable by the presence
of the prominent haemozoin crystal in the phase-contrast images. Smaller arrows in A denote individual haemoglobin transport vesicles. The mean number of haemoglobin transport vesicles per parasite (± SEM) in 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 the control (p < 0.05; 95% CI).
Mean # transport vesicles ± SEM:

C   : 1.2 ± 0.12   Q   : 0.66 ± 0.09
CQ  : 6.1 ± 0.10   H   : 0.67 ± 0.09
AQ  : 2.1 ± 0.23   MQ  : 0.34 ± 0.06