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Ultrastructural and Real-time Microscopic Changes in *P. falciparum*-infected Red Blood Cells Following Treatment with Antimalarial Drugs

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Abstract

Ultrastructural changes to *P. falciparum*-infected red blood cells were examined in vitro after treatment with antimalarial drugs. Artesunate had the most rapid parasitocidal effect. All three drugs caused structural changes within the parasite, including dilatation of the parasitophorous vacuole membrane, depletion of ribosomes, mitochondrial swelling, and decreased formation of hemozoin crystals. The structure of surface knobs and Maurer's clefts were similar to controls but reduced in number. Only depletion of free ribosomes correlated with antimalarial drug exposure. Drug treatment decreased movement of hemozoin granules within parasites on real-time microscopy, before recognizable morphological changes of parasite death.

Keywords

drug treatment; malaria; *Plasmodium falciparum*; real-time microscopy; ultrastructural changes

Plasmodium falciparum, remains a major global health issue, causing 1–2 million deaths each year worldwide, of which 95% were in the African region, followed by the Southeast Asia (3%) and Eastern Mediterranean region (1%) in 2010 [1]. The parasite undergoes a complex life cycle in the mosquito vector and the human host, including cyclical intraerythrocytic development during clinical disease in the human. Electron microscopy can be used to characterize the complex architectural ultrastructure of the *Plasmodium* parasite, reflecting the numerous biological activities of the developing intraerythrocytic parasite, its functional specialization for intracellular existence, and interactions with host cells [2]. Pathological studies of severe malaria indicate that membrane knobs, which are electron-dense protrusions on the surface of *P. falciparum*-infected red blood cells (iRBC), allow

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cytoadhesion to endothelial cells, resulting in microvascular obstruction [3]. This contributes to vital organ dysfunction, particularly in the brain in cerebral malaria [4].

Effective antimalarial drug treatment is required to ameliorate the effects of infection and prevent death. Artesunate, quinine, and piperazine are three drugs currently used for treatment of severe malaria. Artesunate is the fastest-acting drug and shows the highest paracitocidal effects in patients [5]. Quinine is still active in chloroquine-resistant areas and remains a mainstay of treatment, but there are increasing problems with drug resistance and studies have proven the advantages of artesunate-based combination treatment [5]. Local parasite adhesion-dependent or adhesion-independent effects may activate and damage the microvasculature, such as cerebral endothelial cells, contributing to decreased blood flow, hypoxia, or the effects of parasite toxins, such as hemozoin pigment. Quinine is thought to cause death of the intraerythrocytic malaria parasite by inhibiting hemozoin formation. The combination of dihydroartemisinin and piperazine was developed in China (Artekin, Holleykin Pharmaceuticals) and is registered in China and Cambodia. It has been evaluated extensively in clinical trials in Thailand, Vietnam, Cambodia, and China. It is a promising and inexpensive option for antimalarial therapy, but efficacy and pharmacokinetic studies are needed, especially for its use in children [6]. Of prime importance in combating the enormous health burden of malaria is increased understanding of parasite response to antimalarial drugs.

To delineate their mechanism of action on the parasite, the targets of drug action in different organelles of the parasite and changes to the morphology of *P. falciparum*-infected red blood cells need to be studied. Although there are reports detailing the ultrastructural changes to *Plasmodium* spp. caused by antimalarial drugs, there is still controversy over their respective mechanism of actions and the effects on the parasite. In the present study, electron microscopy and real-time microscopy were used to examine the changes observed in *P. falciparum*-infected red blood cells exposed to therapeutic plasma concentrations of artesunate, quinine, and piperazine in vitro.

MATERIALS AND METHODS

In Vitro Culture of *Plasmodium falciparum*

Plasmodium falciparum wild-type isolates, both knob-positive (K^+) and knob-negative (K^-) phenotypes (AK121), obtained from a Thai malaria patient as previously described [7], were maintained in culture according to standard methods [8]. Strains were isolated then cultured to give K^+ phenotype, which was used in these experiments, where the presence or absence of membrane knobs could be used as a morphological criterion for maturation to the trophozoite stage or inhibition of protein export in drug treatment groups. Parasite cultures were synchronized using sorbitol lysis and gradient centrifugation to obtain a synchronized culture of ring-form infected red blood cells (iRBCs) [9]. Trophozoite stage iRBCs were harvested approximately 6–10 h after synchronization, and parasitemia was determined by light microscopy on Giemsa-stained smear preparations.

Antimalarial Drug Preparation and Treatment of in vitro Cultures

Concentrations of the three different drugs used in this study were designed to mimic the peak plasma concentration of each antimalarial drug in treated patients: artesunate (20,000 nM) [10], quinine (20 mg/L) [11], and piperazine (300 μ g/L) [12]. Each drug was dissolved and diluted to a stock solution (1 mg/mL) immediately before use; artesunate (60 mg/mL; Guilin Pharmaceutical Factory, Guilin, China) in NaHCO_3 and diluted in RPMI 1640 medium; quinine dihydrochloride (300 mg of base/mL; Government Pharmaceutical Organization, Bangkok, Thailand) in RPMI 1640 medium; and piperazine phosphate (300

µg/L; Guangzhou University of Traditional Chinese Medicine, Guangzhou, China) in 0.1 M phosphoric acid and further diluted in RPMI 1640.

Before each experiment, the culture was expanded in 200-cm² sealed flasks (Falcon, Becton Dickinson, UK), maintaining less than 5% parasitemia in 5% hematocrit (Hct). The *Plasmodium falciparum*-infected RBC (iRBC) were exposed to drugs for differing periods of 1, 2, 4, and 8 h, then washed and fixed in order to observe the temporal morphological changes associated with drug treatment.

A control (without drug) was maintained in a separate flask and samples were taken at the same time points as for the drug-treated aliquots. Flasks were incubated at 37°C, 5% CO₂ and 0.5 mL of the culture for each drug concentration and control flask was collected and the cell pellets were used to prepare Field-stained thin films and also processed for transmission electron microscopy to assess parasite morphology.

Preparation for light Microscopy

A 20-µl aliquot of each iRBC sample, control and drug-treated, was taken at serial time points after drug exposure, dropped onto a glass microscope slide, and smeared to make a blood film. This was fixed and Field stained prior to examination under light microscopy.

Preparation for Transmission electron Microscopy

iRBC samples were immediately fixed in 2.5% glutaraldehyde in 0.1 M sucrose-cacodylate buffer, pH 7.2 at 4°C for 2 h, washed, postfixed with 2% osmium tetroxide in 0.1 M sucrose-cacodylate buffer for 1 h, washed with distilled water, treated with 2% uranyl acetate in water for 1 h, dehydrated in graded ethanols, infiltrated with propylene oxide, and embedded in Spurr's epoxy resin. Thin sections were cut with glass knives on the ultramicrotomes (Ultracut E, Austria). Copper grids (200 mesh square) were used to collect the sections and stained with lead citrate and uranyl acetate prior to examine by a transmission electron microscope (Jeol-1200ExII, Japan).

Assessment of Parasite Morphology

A qualitative and quantitative examination of the ultrastructural changes was performed. The nature and severity of the following changes were recorded: parasitemia, changes in red blood cell structure associated with infection, including expression of surface knobs, cytoplasmic Maurer's clefts, and the tubulovesicular network, and changes in parasite organelle structure, including the membrane of the parasite and the parasitophorous vacuole, nucleus, mitochondria, apicoplast, free ribosomes, and the food vacuole. To prevent bias in the quantitation, all red cells (infected and uninfected) in each grid square were counted and this was continued until 100 infected cells (irrespective of parasite stage) had been counted. The quantitation of changes within the infected red cells was based on this random sample of 100 infected cells.

Statistical Analysis

Quantitative differences in parasitemia, stage, and amount of parasite death were compared between groups using analysis of variance (ANOVA), performed using SPSS 11.5 statistical software (IBM, New York, USA).

Investigating effects of Antimalarial Drug exposure on Parasites Using Real-time Microscopy

For real-time microscopy studies, iRBC were cultured in 200-cm² sealed flasks (Falcon, Becton Dickinson, UK), maintaining less than 5% parasitemia in 5% Hct, and then exposed

to drugs using the same concentrations as described previously. The specimens were prepared for real-time microscopic assessment at 12, 18, 24, and 30 h after exposure. The standard procedure used to prepare specimens for viewing under real-time microscope (RTM) was wet mounting. The nucleus of malaria parasite was stained with Hoechst 33342 dye (Invitrogen, USA), 5 µg/mL in RPMI 1640 for 5 min at room temperature in the dark, then 4.8 µL of parasite culture (25% Hct) was placed in the middle of a microscopic glass slide and covered with a standard 22 × 22-mm glass coverslip that allowed fluid to spread out evenly. These steps provided a 10-µm-thick fluid layer, which is the ideal thickness for blood suspension. The samples were observed immediately using a real-time microscope (RTM-3 Richardson Technologies Inc, Canada). The parasites were observed in both RTM native mode and fluorescent mode with 100× oil-immersion objective. With Hoechst 33342 staining, the nucleus of parasite was presented as blue using filter cubes position 1, UV excitation. The images were captured as still images as TIFF files and time-lapse sequences from the microscope using Openlab version 3.1.2 software (Improvision, Coventry, UK).

Assessment of Trophozoite Movement

Fifty parasites were counted and divided into those that showed movement of the parasite within the iRBC or those in which there was no movement (stationary). Video clips were taken for quantitative assessment. Parasites were observed with 100× oil immersion objective lens for 5 s. The movement of parasites was evaluated only in the trophozoite stages, which have 1-2 nuclei/parasite and pigment in the food vacuole, and schizont stages, where pigment granules fuse together and form a single large mass. The movement of ring forms could not be determined because they lacked pigment.

RESULTS

Effect on Stage Development and Parasitemia

The criteria used for determining parasite stage in this study were similar for both light and electron microscopy. The parasites were graded for stage development into 3 groups: ring, trophozoite, and schizont. The assessment of parasite development was evaluated by light microscopy and the results are presented in Figure 1 and Table 1. In the control group at 0 h, 75% of parasites were ring stage and about a quarter (23%) trophozoites, with very few pyknotic parasites noted (2%). After 8 h, the control group had undergone stage development from ring (26.0%) to trophozoite (72.3%) with pyknosis (1.7%). In the artesunate-treated group at 1, 2, 4, and 8 h after exposure, the parasites showed progressively decreased viability (5.2, 4.5, 2.8, and 1.7%, respectively) and an increasing number of pyknotic parasites (2.0, 1.7, 15.7, and 31.3%, respectively). Parasites in the quinine- and piperazine-treated groups showed a slight decrease in viability from 1 h (5.2 and 5.4%) to 8 h (4.3 and 4.0%), and fewer rings had developed into trophozoite at 8 h (39.3 and 35.7%) compared to the control (72.3%).

When evaluated by electron microscopy, the results showed a similar distribution of parasite stages, with only slight variations in the percentages compared to that observed by light microscopy (Table 2). In the control group at time zero (0 h), half of parasites were ring stage and the other half were trophozoites with no pyknotic parasites observed. By 8 h after exposure in the matched drug-treatment groups, the control group showed parasite stage progression with an increased proportion of trophozoites (78%), many fewer ring stages (17%), and a few pyknotic parasites (4%). In the artesunate group from 1, 2, 4, and 8 h after exposure, the parasites showed marked and progressive decrease in % parasitemia (4.0, 3.5, 2.5, and 1.0) and increasing number of pyknotic parasites at 4 and 8 h (0, 0, 25, and 39), respectively. Parasites in quinine- and the piperazine-treated groups, compared to the artesunate group, showed a much smaller decrease in the parasitemia from 1 h (4.5 and

4.5%) to 8 h (3.0 and 3.5%) with a significant number of rings progressing to the trophozoite stage (55 and 55%, respectively).

It was difficult to assess parasite changes at the light microscope level. In the control group, examples are shown of maturation of the parasite from late ring stage through early and mid-trophozoite stage, with increased cytoplasmic volume and parasite size, and deposition of hemozoin within the food vacuole to form the characteristic pigment granule (Figure 1). In contrast, the artesunate-treated parasite group failed to develop at all and became small, shrunken, and pyknotic, with decreased parasite size and no maturation of the pigment granule, cytoplasmic, or nuclear features within the parasite. These changes are clear within 1-2 h after drug exposure. Changes in the quinine- and piperazine-treated groups are less marked but again show a failure of maturation of the parasite by 8 h (Figure 1). However, the increased resolution of the electron microscope allowed details of parasite structure and organelles to be evaluated. Expression of surface knob proteins is a sign of parasite maturation to trophozoite stages. The proportion of knob-positive infected red blood cells (K + iRBC) increased in the control group, 67% at 8 h compared to 7% at 1 h. There was also a marked increase in the average number of knobs and Maurer's clefts per iRBC, 42.1 and 4.2, respectively. In drug-exposed groups, quinine and piperazine showed a very similar pattern to the control group in that K+ iRBC increased at 8 h (43 and 44%) compared to 1 h (8 and 13%), with average number of knob and Maurer's clefts at 34.2, 3.0 and 20.7, 2.7, respectively. The artesunate-treated group showed markedly fewer K+ iRBC (29%) with a lower average number of knobs (27.0) and Maurer's clefts (2.0).

Using statistical analysis, the only ultrastructural abnormality that showed a significant difference ($p < .01$) from control non-drug-treated iRBC was the number of parasites that showed depletion of free cytoplasmic ribosomes in all drug-exposure groups.

Morphological effects of Drug Treatment on Parasite Ultrastructure

To identify ultrastructural changes associated with drug-treatment, fixation and preservation of the parasites in control (untreated) parasites was optimized and confirmed in the present study (Figures 2 and 3). As the parasite developed into the trophozoite stage, it became more spherical in shape and often contained a large spherical or subspherical food vacuole (Figure 3A). At this stage, it was possible to identify the acristate mitochondria, the apicoplast, characterized by its enclosure by multiple membranes, and the single membrane vacuole forming the Golgi body (Figure 3A-C). The food vacuole was limited by a unit membrane but showed marked variation in size and shape between parasites (Figures 2B, E, 3A, D). The food vacuoles possessed a homogenous, moderately electron-dense ground substance but the number and distribution of the hemozoin crystals were variable (Figure 3A, D). Trophozoite development was also associated with characteristic changes to the host red cell cytoplasm and plasmalemma. The red cell infected by trophozoite stage was seen to contain a number of flattened membrane-bound structures, termed Maurer's clefts (Figure 3D), and small circular (70 nm) electron-dense deposits beneath the red cell plasmalemma, termed knobs (Figure 3A, D).

In the drug-treated iRBC, a number of morphological changes were noted, which were most marked in the artesunate-exposed group. Artesunate affected the exposed parasites by retarding parasite development and causing a reduction in size of parasite cytoplasm and nucleus by light microscopic assessment. When examined by electron microscopy, the parasites showed changes in their limiting membranes together with alterations in ribosomal organization (Figure 4A, B). The space of the parasitophorous vacuole membrane (PV) appeared dilated (Figure 3A). There were also lucent areas in the parasite cytoplasm with focal loss of ribosome (Figure 4A, B). Food vacuoles were rare, relatively small with limited hemozoin formation (Figure 4B). Further degenerative changes with dense amorphous

material replacing recognizable organelles and degenerate parasites were observed from 4 h postexposure (Figure 4C, D). The altered parasites exhibited total disorganization with remnants of hemozoin crystals. Only a few host erythrocytes displayed the formation of knobs, Maurer's cleft, and tubulovesicular network and, where formed, these structures appear relatively normal, even in cells containing degenerate parasites (Figure 4C, D).

In the quinine-exposed group, ultrastructural examination provided evidence of some parasite development in comparison to the artesunate group. However, it was observed that there was extensive depletion of free ribosomes in the cytoplasm (Figure 5A, B). It was possible to find parasites with hemozoin in food vacuoles but these were very few in comparison to the control group, although more than in the artesunate-treated group (Figure 5D, F). The mitochondria showed marked swelling with a more electron-lucent matrix (Figure 5C-E). The iRBC containing more intact parasites also showed formation of knobs and Maurer's clefts (Figure 5D).

Following exposure to piperazine, ring-stage parasites showed extensive depletion of free ribosomes in their cytoplasm (Figure 6A, B). There was evidence of hemozoin formation in certain food vacuoles (Figure 6C). However, the unique observation was that in this group many of the trophozoites appear to possess large food vacuoles containing a number of membrane-bound packets of undigested hemoglobin (Figure 6C, D). The parasites that had developed to the trophozoite stage were observed to have formed knobs and Maurer's clefts in a similar manner to the controls (Figure 6C, D).

The effects of Antimalarial Drugs on *Plasmodium falciparum*, Studied Using Real-time Microscopy

Real-time microscopy was used to examine hemozoin-inhibiting effects and viability of *Plasmodium falciparum* trophozoite and schizont stages after exposure to quinine, artesunate, and piperazine at peak plasma concentrations in this study. Due to the optics of real-time microscopic assessment (Tables 3 and 4, Figure 7), only hemozoin in trophozoite and schizont stage iRBC can be detected, so movement in ring-stage parasites, which do not have hemozoin, could not be measured. Because antimalarial drugs delayed parasite stage transition, the total number of parasites determined by real-time microscopy was less than in controls. In the control group at 0 h, 100% of parasites were ring stage. By 30 h, the control group showed parasite stage development from ring (0%) to trophozoites (96%) and schizonts (4%).

In the artesunate-treated group from 12, 18, 24, and 30 h after exposure, the parasites showed a significant decrease in hemozoin movement in the trophozoite stage compared to the quinine- and piperazine-exposed groups. In quinine- and piperazine-exposed groups, hemozoin movement in trophozoites showed similar results. Only the artesunate-treated group showed a significant difference in hemozoin movement in trophozoites ($p < .01$) compared to controls.

Comparing the percentage of parasites with no hemozoin movement observed by real-time microscopy (Figure 7) and the percentage of pyknotic parasites observed on light microscopy (Figure 8), both graphs showed the same pattern of increase with drug treatment, but the levels of no hemozoin movement showed up to twice the value compared to the percentage of pyknotic parasites assessed by light microscopy.

DISCUSSION

Previous studies of the effects of antimalarial drugs have been performed in human and animal malaria parasites both in vivo and in vitro. This present study investigated the effects

of quinine, artesunate, and piperaquine on parasitemia, parasite stage development, and the morphology of *P. falciparum* as observed using real-time and light microscopy and transmission electron microscopy. The main finding was that artesunate had the fastest and most marked antimalarial effects, compared to quinine and piperaquine, but that individual ultrastructural correlates could not be distinguished between the individual drugs tested.

It is critically important in any ultrastructural study to identify changes associated with drug treatment that great care be taken to ensure that the parasites are optimally preserved. Many of the changes described as being associated with drug treatment can be seen in suboptimally fixed parasites. Therefore, great care was taken in the present study (using large numbers of individual parasites) to ensure that any changes could be directly related to the treatment of the parasite. All of the normal ultrastructural findings in our study conform to the previous studies reviewed [2, 3]. However, the ultrastructural appearances are based on dynamic biological processes and it is unlikely that drug treatment will appear as a single effect. This is especially true for the rapid-acting drugs whose action will result in a cascade of abnormal appearances, such as artesunate, which showed early changes in parasite morphology. The loss of efficient protein processing due to ribosome loss in artesunate-treated parasites will have inhibitory affects on all aspects of development.

Using real-time microscopic assessment, only artesunate-treated iRBC showed a significant difference from controls in loss of hemozoin movement in the trophozoite. This result reflects the observation from light and electron microscopic studies that artesunate exposure has both a faster and greater paracitocidal effect than the other drugs. Therefore, loss of hemozoin movement may be one of the first indicators of an adverse parasite response to external stimuli, before distinct morphological changes occur. Further experiments are required to determine the correlation between loss of hemozoin movement and pyknosis as measures of parasite death. This may be important biologically, because (in a similar way to concentrations of antimicrobial compounds used against bacteria) serum concentrations of antimalarial drugs may only cause parasite stasis. This could be a mechanism of recrudescence of infection and allow development of drug-resistant clones.

Statistical analysis of the ultrastructural changes induced by different drug groups using TEM showed that only the incidence of ribosome depletion was significantly different from that of controls. However, the descriptive findings of other morphological changes (to unit membranes and food vacuoles) may reflect differing mechanisms of drug action. Thus, formation of a normal food vacuole or pigment could be inhibited by drugs effecting specific pathways necessary for formation of this organelle, but could also be inhibited by a drug that preferentially affected ring-stage parasites, before such structures had a chance to develop.

In quinine-exposed parasites, there was extensive depletion of free ribosomes in the cytoplasm. Hemozoin in food vacuoles was decreased or almost absent and the mitochondria showed marked swelling with more electron-lucent matrix. These results are similar to other reports [13,14]. The proposed mechanisms of action of quinine include inhibition of hemozoin formation by direct interaction with ferriprotoporphyrin IX without mediation of a catalyst or enzyme [15, 16] or quinine capping the growing hemozoin chain, thus preventing further sequestration of ferriprotoporphyrin IX [17]. Previous studies [14] have reported ultrastructural changes in the parasite nucleus and membranes in quinine treatment that were not observed in this study.

In artesunate-treated parasites, stage development was significantly retarded, with reduction in the size of the parasite, pyknotic changes, and death in direct proportion to exposure time. On TEM, there was dilation of the parasitophorous vacuole membrane together with

alterations in parasite ribosomal organization. This is similar to other reports [18, 19]. Some authors also reported changes in mitochondria [20, 21] but this was not observed in the current study. After 4 h exposure, parasites showed degenerative changes with dense amorphous material replacing recognizable organelles and death, although some preformed structures, such as membrane knobs and Maurer's clefts, remained. This implies that at least one primary effect of artesunate was on membrane integrity.

In vitro studies of the mechanism of action of artesunate have emphasized the importance of the endoperoxide bridge in its chemical structure. Since peroxides are a known source of reactive oxygen species, free radicals might be involved in its mechanism of action [22] as they can cause lipid peroxidation, a typical end product of free radical damage. Some studies [23, 24] suggested that artemisinins act by heme-dependent activation of an endoperoxide bridge occurring within the parasite's food vacuole. However, localization of artemisinins to the parasite rather than the food vacuole membranes, and killing of small ring-stage forms lacking hemozoin argue against the food vacuole being a major site for drug action. Artemisinins contain a structure that is a highly specific inhibitor of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) and acts by inhibiting PfATP6 outside the food vacuole after activation by iron [25]. There is also evidence that only one amino acid change in PfATP6 can reduce the sensitivity of artemisinin [26]. So, the mechanism of action of artesunate is still controversial; it may act via several different pathways, but membrane lipid peroxidation by free radical action may interfere with parasite membrane integrity.

In piperazine-treated parasites, the ring stages showed extensive depletion of free ribosomes in their cytoplasm. The most significant morphological feature was the presence of enlarged food vacuoles packed with small membrane-bound, undigested hemoglobin, which were also observed within the parasite cytoplasm. The mechanism of action of piperazine is still unclear. Chen et al. [27] studied the ultrastructure of erythrocytic stages of *Plasmodium berghei* ANKA strain treated with Artekin (combination of dehydroartemisinin and piperazine), and concluded that this drug primarily interfered with the physiological function of the food vacuole membrane of trophozoites. Other researchers [28, 29] reported that its antimalarial activity is similar to other 4-aminoquinolines, by interference with the heme polymerization. In our study, the parasites showed undigested contents surrounded by unit membrane within the food vacuole, suggesting that piperazine's mechanism of action inhibits early hemoglobin digestion and hemozoin formation.

Studying the specific mechanisms of action of different drugs morphologically is limited by the disruption of parasite energy or protein synthesis, which may have more global effects on ultrastructure that are not specific to a particular drug, especially given the complex biology of stage development in *Plasmodium* parasites. Optimal fixation and processing for electron microscopy is important to avoid the overinterpretation of morphological changes. We have shown a variety of ultrastructural changes to parasites and iRBC that did not quantitatively differ between drug types. However, correlation with real-time microscopy shows that functional parasite death precedes morphological changes as determined by light and electron microscopy.

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REFERENCES

1. WHO World Malaria Report. World Health Organization; 2010.
2. Bannister L, Hopkins J, Fowler R, Krishna S, Mitchell G. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. *Parasitol Today*. 2000; 16:427–433. [PubMed: 11006474]
3. Aikawa, M.; Seed, T. Morphology of plasmodia. In: Kreier, JP., editor. *Malaria*. Academic Press; 1980. p. 285–344.
4. Pongponratn E, Turner G, Day N, et al. An ultrastructural study of the brain in fatal *Plasmodium falciparum* malaria. *Am J Trop Med Hyg*. 2003; 69:345–359. [PubMed: 14640492]
5. White NJ. Qinghaosu (artemisinin): the price of success. *Science*. 2008; 320:330–334. [PubMed: 18420924]
6. Denis M, Davis T, Hewitt S, et al. Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated falciparum malaria. *Clin Infect Dis*. 2002; 35:1469–1476. [PubMed: 12471565]
7. Chotivanich K, Sattabongkot J, Udomsangpetch R, et al. Transmission-blocking activities of quinine, primaquine and artesunate. *Antimicrob Agents Chemother*. 2006; 50:1927–1930. [PubMed: 16723547]
8. Trager W, Jensen J. Human malaria parasites in continuous culture. *Science*. 1976; 193:673–675. [PubMed: 781840]
9. Lambros C, Vanderberg J. Synchronization of *Plasmodium falciparum* Erythrocytic stages in culture. *J Parasitol*. 1979; 65:418–420. [PubMed: 383936]
10. Davis T, Phuong H, Ilett K, et al. Pharmacokinetics and pharmacodynamics of intravenous artesunate in severe falciparum malaria. *Antimicrob Agents Chemother*. 2001; 45:181–186. [PubMed: 11120963]
11. White NJ. The treatment of malaria. *N Engl J Med*. 1996; 335:800–806. [PubMed: 8703186]
12. Hung T, Davis T, Ilett K, et al. Population pharmacokinetics of piperaquine in adults and children with uncomplicated falciparum or vivax malaria. *Br J Clin Pharm*. 2003; 57:253–262.
13. Ladda R. Morphologic observation on the effect of antimalarial agents on the erythrocytic forms of *Plasmodium berghei* *in vitro*. *Military Med J*. 1966; 131(Suppl):993–1008.
14. Davies E, Warhurst D, Peters W. The chemotherapy of rodent malaria, XXI: action of quinine and WR122 (a 9-phenanthrenemethanol) on the fine structure of *Plasmodium berghei* in mouse blood. *Ann Trop Med Parasitol*. 1975; 69:147–153. [PubMed: 1098583]
15. Egan T, Ross D, Adams P. Quinoline anti-malarial drugs inhibit spontaneous formation of beta-haematin (malaria pigment). *FEBS Lett*. 1994; 352:54–57. [PubMed: 7925942]
16. Egan T, Combrinck J, Egan J, et al. Fate of heme iron in the malaria parasite *Plasmodium falciparum*. *Biochem J*. 2002; 365:343–347. [PubMed: 12033986]
17. Sullivan D, Gluzman I, Russel D, Goldberg D. On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci USA*. 1996; 93:11865–11870. [PubMed: 8876229]
18. Ellis D, Li Z, Gu H, et al. The chemotherapy of rodent malaria. *Ann Trop Med Parasitol*. 1985; 79:367–374. [PubMed: 3907556]
19. Maeno Y, Toyoshima T, Fujioka H, et al. Morphologic effects of artemisinin in *Plasmodium falciparum*. *Am J Trop Med Hyg*. 1993; 49:485–491. [PubMed: 8214279]
20. Jiang J, Jacobs G, Liang D, Aikawa M. Qinghaosu induced changes in the morphology of *Plasmodium inui*. *Am J Trop Med Hyg*. 1985; 34:424–428. [PubMed: 3890573]
21. Kawai S, Kano S, Suzuki M. Morphologic effects of artemether on *Plasmodium falciparum* in *Aotus trivirgatus*. *Am J Trop Med Hyg*. 1993; 49:812–818. [PubMed: 8279648]
22. Meshnick S. Artemisinin: mechanisms of action, resistance and toxicity. *Int J Parasitol*. 2002; 32:1655–1660. [PubMed: 12435450]
23. Hong Y, Yang Y, Meshnick S. The interaction of artemisinin with malarial hemozoin. *Mol Biochem Parasitol*. 1994; 63:121–128. [PubMed: 8183310]

24. Cazelles J, Robert A, Meunier B. Alkylating capacity and reaction products of antimalarial trioxanes after activation by a heme model. *Org Chem J.* 2002; 67:609–619.
25. Eckstein-Ludwig U, Webb R, Van Goethem I, et al. Artemisinin targets the SERCA of *Plasmodium falciparum*. *Nature.* 2003; 424:957–961. [PubMed: 12931192]
26. Uhlemann A, Cameron A, Eckstein-Ludwig U, et al. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat Struct Biol.* 2005; 12:628–629.
27. Chen P, Chen L, Li G, Huang Z, Yang D. Effects of new antimalarial drugs in combination, Artekin, on ultrastructure of erythrocytic stages of *Plasmodium berghei* ANKA strain. *Chin Med J.* 2002; 115:129–131. [PubMed: 11930647]
28. Vennerstrom J, Ellis W, Ager A, et al. Bisquinolones. 1. *N, N*-bis (7-chloroquinolin-4-yl) alkanediamines with potential against chloroquine-resistant malaria. *J Med Chem.* 1992; 35:2129–2134. [PubMed: 1597862]
29. Slater A. Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum*. *Pharm Therapeut J.* 1993; 57:203–235.

FIGURE 1.

Light microscopy illustrating typical parasitized red blood cell morphology following antimalaria drug exposure. The changes to infected red cell morphology were observed using Field staining under light microscopy (magnification $\times 1000$ oil). The figure shows sequential changes with time after exposure to the different drugs, compared to control untreated parasites.

FIGURE 2.

Transmission electron micrographs of the ring stage of *Plasmodium falciparum* in the control sample. (A) Low-power transmission electron micrograph of an early ring showing the cytoplasm with numerous ribosomes (R) and a nucleus (N). Bar is 200 nm. (B) Later ring stage showing a nucleus (N) and small food vacuole (FV) within the cytoplasm. Bar is 200 nm. (C) Detail of the enclosed area in A showing the limiting membrane of the parasitophorous vacuole (PVM) and the plasmalemma of the parasite (PM). Note the membrane of the endoplasmic reticulum (ER). Bar is 100 nm. (D) Detail of the lower enclosed area in B showing protrusion of the PVM into the red cell cytoplasm (arrow), representing the initiation of the tubular vesicular network. Bar is 100 nm. (E) Detail of the upper enclosed area in B illustrating the small electron-dense food vacuole with hemozoin crystals (H) and the nucleus (N). Bar is 100 nm.

FIGURE 3.

Transmission electron micrographs of the trophozoite stage of *Plasmodium falciparum* in the control sample. (A) Low-power micrograph of a trophozoite showing the cytoplasm, a nucleus (N) and food vacuole (FV). K, knob; H, Haemozoin. Bar is 100 nm. (B) Detail of the enclosed area in A showing two small acristate mitochondria (M). Bar is 100 nm. (C) Detail of the single flattened vacuole representing a Golgi body (G) and associated vesicles (V). Bar is 100 nm. (D) Low-power micrograph of part of an iRBC showing a trophozoite with a food vacuole (FV) with tightly packed hemozoin crystals (H). M, mitochondrion; MC, Maurer's cleft; K, knob. Bar is 200 nm.

FIGURE 4.

Transmission electron micrographs of *Plasmodium falciparum* in the artesunate-treated group. (A) Low-power micrograph of trophozoite 1 h after exposure, showing the dilatation of the parasitophorous vacuole (PV) and ribosomal loss (*). FV, food vacuole. Bar is 200 nm. (B) Micrograph from 2 h postexposure showing the ring stage with patchy ribosomal loss (*) and small food vacuoles (FV) within the cytoplasm. Bar is 200 nm. (C) At 4 h post drug exposure the parasite (P) shows degenerative changes with dense amorphous material replacing recognizable organelles. Note the presence of knobs (K) and Maurer's cleft (Mc). Bar is 200 nm. (D) A parasite (P) 8 h after exposure showing total disorganization, with remnants of hemozoin crystals. Note the infected RBC retains the Maurer's clefts (Mc) and tubulovesicular network (TVN). Bar is 200 nm.

FIGURE 5.

Transmission electron micrographs of *Plasmodium falciparum* in the quinine-treated group. (A) Low-power micrograph of an early ring 1 h after exposure, showing the depletion of ribosomes (*) in cytoplasm and a small food vacuole (FV). Bar is 200 nm. (B) Two hours after exposure, a ring stage parasite showing extensive depletion of ribosomes (*) in the cytoplasm. Bar is 200 nm. (C) Four hours postexposure the parasite showed swollen mitochondria (M). Bar is 200 nm. (D) Parasite 4 h after exposure showing swollen mitochondria (M) and a food vacuole (FV) containing a few hemozoin crystals. MC, Maurer's cleft. Bar is 200 nm. (E) Detail of the enclosed area in C showing the electron-lucent in matrix of the swollen mitochondrion. Bar is 100 nm. (F) At 8 h after exposure the parasite is showing a food vacuole (FV) containing abnormally few hemozoin crystals. Bar is 200 nm.

FIGURE 6.

Transmission electron micrographs of *Plasmodium falciparum* in the piperaquine-treated group. (A) Low-power micrograph of an early ring, 1 h after exposure, showing the depletion of ribosomes (*) in cytoplasm. Bar is 500 nm. (B) Two hours postexposure, the ring stage shows extensive depletion of ribosomes (*) in cytoplasm. Bar is 500 nm. (C) Four hours postexposure, the trophozoite shows a large food vacuole (FV) with undigested hemoglobin vesicles (*) and a few hemozoin crystals (H). Note the Maurer's cleft (MC) and knobs (K) at the surface of the infected RBC. Bar is 200 nm. (D) Eight hours after exposure trophozoite showing the undigested vesicles (*) within a distended food vacuole. K, knobs; MC, Maurer's cleft. Bar is 200 nm.

FIGURE 7.

Graph showing the percentage of parasites with no hemozoin movement after drug treatment using real-time microscopy.

FIGURE 8.

Graph showing the percentage of pyknotic parasites after exposure to drug treatment using light microscopy.

Table 1

The effects of antimalarial drugs on *Plasmodium falciparum* viability *in vitro*.

Drugs	Exposure time (hr)	% Parasitemia	Total parasite count	Parasite stage (%)			
				Ring	Trophozoite	Schizont	Pyknosis
Control	0	5.0	300	75.0	23.0	0.0	2.0
Control	1	5.0	300	87.7	11.0	0.0	1.3
	2	5.1	300	60.7	39.3	0.0	0.0
	4	4.8	300	49.7	47.3	0.0	3.0
	8	4.9	300	26.0	72.3	0.0	1.7
Artesunate	1	5.2	300	84.7	13.3	0.0	2.0
	2	4.5	300	86.3	12.0	0.0	1.7
	4	2.8	300	62.0	22.3	0.0	15.7
	8	1.7	300	54.0	14.7	0.0	31.3
Quinine	1	5.2	300	86.0	12.3	0.0	1.7
	2	5.0	300	76.0	22.3	0.0	1.7
	4	4.5	300	65.0	32.0	0.0	3.0
	8	4.3	300	55.3	39.3	0.0	5.3
Piperaquine	1	5.4	300	71.7	24.3	0.0	4.0
	2	5.1	300	79.7	17.3	0.0	3.0
	4	4.2	300	75.0	21.0	0.0	4.0
	8	4.0	300	47.0	35.7	0.0	17.3

The effects of antimalarial drugs on Plasmodium falciparum ultrastructure using transmission electron microscopy.

Table 2

Drugs	Expo sure time (h)	Total parasite count	% Parasi temia	Parasite stage (%)				Number of abnormal organelles														
				Ring	Troph ozoite	Schizont	Pyknosis	No. of K+	No. of knob/ K+	Average no. of Mc/K+	Average no. of Mc/K- parasite	Average no. of TVN/ K+	Average no. of TVN/K- parasite	PVM	PPM	PV	Nuclear mem- brane	Food vacuole membrane	Food vacuole content	Mitoc hondria	Apico plast	Ribo some
Control	0	100	4.9	50	50	0	0	8	92	30.7	2.2	1.1	0.0	0.0	0	0	0	0	0	0	0	0
Control	1	100	4.5	51	49	0	0	7	93	32.7	2.3	1.3	0.0	0.0	0	0	0	0	0	0	0	1
	2	100	4.5	39	61	0	0	23	77	38.9	2.7	0.9	0.2	0.0	0	0	0	0	0	0	0	0
	4	100	4.0	35	65	0	0	30	70	36.8	3.8	1.5	0.1	0.1	0	0	0	0	0	0	0	0
	8	100	4.0	17	78	1	4	67	33	42.1	4.2	0.5	0.2	0.0	0	0	0	0	0	0	0	0
Artesunate	1	100	4.0	50	50	0	0	8	92	35.6	2.0	0.9	0.3	0.0	0	0	0	0	0	0	0	43*
	2	100	3.5	47	53	0	0	12	88	43.8	1.3	1.3	0.2	0.0	0	0	4	0	0	0	0	64*
	4	100	2.5	45	30	0	25	13	87	24.5	2.9	1.4	0.6	0.1	1	1	1	0	0	1	0	42*
	8	100	1.0	35	26	0	39	29	71	27.0	2.0	1.2	0.5	0.1	7	7	11	0	0	1	0	39*
Quinine	1	100	4.5	50	49	0	1	8	92	46.6	1.0	0.8	0.3	0.0	0	0	0	0	1	0	0	41*
	2	100	3.5	52	42	0	6	18	82	32.3	2.6	0.9	0.1	0.0	0	0	0	0	0	0	0	55*
	4	100	3.5	39	57	0	4	18	82	25.7	1.7	1.2	0.1	0.0	0	0	1	0	0	1	0	49*
	8	100	3.0	41	55	0	4	43	57	34.2	3.0	0.7	0.3	0.1	0	0	0	0	2	3	2	45*
Piperaquine	1	100	4.5	60	40	0	0	13	87	47.5	2.7	1.1	0.2	0.0	0	0	0	0	0	0	0	27*
	2	100	3.5	47	51	2	0	11	89	30.1	3.0	1.0	0.0	0.0	0	0	2	0	0	0	0	39*
	4	100	3.5	47	49	0	4	30	70	32.6	3.1	1.5	0.1	0.0	0	0	4	0	0	2	0	53*
	8	100	3.5	38	55	0	7	44	56	20.7	2.7	0.4	0.1	0.1	0	0	6	0	0	1	0	56*

* Significant difference ($p < 0.1$) from control for the average time point.

Table 3

The effects of antimalarial drugs on *Plasmodium falciparum* using light microscopy to evaluate samples processed for real-time microscopy.

Drugs	Exposure time (h)	Total parasite count	% Parasitemia	Parasite stage (%)			
				Ring	Trophozoite	Schizont	Pyknosis
Control	0	300	5.4	100.0	0.0	0.0	0.0
Control	12	300	4.6	91.3	4.3	0.0	4.3
	18	300	4.5	14.7	78.3	0.0	7.0
	24	300	5.2	3.3	90.3	0.0	6.3
	30	300	4.8	4.0	89.7	0.0	6.3
Artesunate	12	300	4.0	86.3	1.3	0.0	12.3
	18	300	3.8	64.0	2.0	0.0	34.0
	24	300	1.9	60.7	0.0	0.0	39.3
	30	300	1.9	38.0	0.0	0.0	62.0
Quinine	12	300	4.5	95.0	1.0	0.0	4.0
	18	300	4.6	94.0	1.0	0.0	5.0
	24	300	4.8	91.0	4.0	0.0	5.0
	30	300	3.7	87.0	3.0	0.0	10.0
Piperaquine	12	300	4.5	90.3	1.0	0.0	8.7
	18	300	4.4	87.7	2.0	0.0	10.3
	24	300	3.4	79.3	2.7	0.0	18.0
	30	300	2.6	67.7	1.7	0.0	30.7

Table 4

The effects of antimalarial drugs on *Plasmodium falciparum* stage development, parasitemia, and hemozoin movement in vitro using real-time microscopy.

Drugs	Exposure time (hrs)	Total parasite count	Parasite stage (%)			
			Trophozoite		Schizont	
			Hemozoin movement		Hemozoin movement	
			Ring	Positive	Negative	Positive
Control	0	50	100	0	0	0
Control	12	50	0	96	0	4
	18	50	6	90	0	2
	24	50	4	90	2	4
	30	50	0	96	0	4
Artesunate	12	50	68	4	10*	10
	18	50	70	18	6*	6
	24	50	84	12	4*	0
	30	50	66	6	22*	4
Quinine	12	50	84	10	4	0
	18	50	78	20	0	0
	24	50	76	14	6	4
	30	50	96	2	2	0
Piperaquine	12	50	62	22	6	8
	18	50	50	38	6	6
	24	50	76	12	10	2
	30	50	86	4	10	0

* Significant difference ($p < .01$) from control for the average time point.