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Transmission-Blocking Activities of Quinine, Primaquine, and Artesunate

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The infectivity of *Plasmodium falciparum* gametocytes after exposure in vitro to quinine, artesunate, and primaquine was assessed in *Anopheles dirus*, a major vector of malaria in Southeast Asia. Mature gametocytes (stage 5) of a Thai isolate of *P. falciparum* were exposed to the drugs for 24 h in vitro before membrane feeding to *A. dirus*. After 10 days, the mosquito midguts were dissected and the oocysts were counted. In this system, artesunate showed the most potent transmission-blocking activity; the mean (standard deviation [SD]) 50% and 90% effective concentrations (EC_{50}, and EC_{90}, respectively, in nanograms per milliliter) were 0.1 (0.02) and 0.4 (0.15), respectively. Transmission-blocking activity of quinine and primaquine was observed at relatively high concentrations (SDs): EC_{50} of quinine, 642 (111) ng/ml; EC_{50} of primaquine, 181 (23) ng/ml; EC_{90} of quinine, 816 (96) ng/ml; EC_{90} of primaquine, 543 (43) ng/ml. Artesunate both prevents the maturation of immature *P. falciparum* gametocytes and reduces the transmission potential of mature gametocytes. Both of these effects may contribute to reducing malaria transmission.

The sexual stages of *Plasmodium falciparum*, the gametocytes, are central to malaria transmission. Gametocytogenesis in falciparum malaria is delayed with respect to asexual stage development and is dependent on both asexual parasite densities and the duration of infection. In epidemiological studies, the development or persistence of gametocytemia has been associated with anemia, a prolonged history of illness, and inadequate responses to antimalarial treatment and subsequent recrudescence (18). Some drugs (e.g., sulfadoxine-pyrimethamine) appear to stimulate gametocyte production. The rapid emergence and spread of drug resistance in *P. falciparum* are major problems for malaria control. Reducing transmission is therefore important both to control malaria and to delay the spread of drug resistance. In falciparum malaria, antimalarial drugs can reduce transmission indirectly by reducing the number of asexual parasites that could subsequently develop into sexual stages. Most of the antimalarial drugs used for treating the asexual stages of *P. falciparum* have little or no direct effects on the already mature formed gametocytes of *P. falciparum*, whereas the activities against the asexual and sexual stages of the other human malaria pathogens (i.e., *P. vivax*, *P. malariae*, and *P. ovale*) are closely linked. Only primaquine (3, 10, 20) and artemisinin (2, 13, 19), derivatives of commonly used antimalarial drugs, have been shown to have significant activity against mature sexual stages in falciparum malaria. Other compounds may reduce transmission by interfering with parasite development in vector mosquitoes (sporontocidal activity) (4, 8, 15, 21, 25). We have studied the infectivity of mature gametocytes in *Anopheles dirus* after exposure to artesunate and compared this with infectivity after exposure to quinine and primaquine.

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MATERIALS AND METHODS

Parasites and cultivation of gametocytes. *P. falciparum* gametocyte-producing isolate AMB 47 was cultured continuously as described previously (16). One liter of culture medium (pH 7.4) consisted of RPMI 1640 medium (GIBCO) supplemented with 25 mM HEPES (Sigma), 2 g of $NaHCO_3$, 50 mg of hypoxanthine (Sigma), 40 mg of gentamicin (Sigma), and 10% human serum. Parasites which developed stage I and II gametocytes were cultured at 5% hematocrit and 5% parasitemia and maintained in a candle jar at 37°C. When the parasites had developed to stage III to stage IV gametocytes, they were transferred to a CO_2 incubator at 37°C and supplied with a gas mixture consisting of 5% O_2, 5% CO_2, and 90% N_2. The culture was maintained with daily change of medium. Gametocyte development (stages I to stage V) was assessed by microscopy of thick and thin blood films. Gametocytes were considered mature and therefore transmissible if they showed exflagellation on removal from culture and examination under ambient conditions at 25°C. These stage V gametocytes were used for testing of antimalarial drugs.

Assessment of gametocytocidal activities of antimalarial drugs. Cultures containing mature gametocytes were centrifuged and resuspended at 1% gametocytemia in hypoxanthine RPMI 1640 medium with 10% human AB serum at 5% hematocrit. Quinine dihydrochloride (300 mg of base/ml or 0.79 M; Government Pharmaceutical Organization, Bangkok, Thailand) and primaquine (diphosphate salt; Sigma catalog no. 62H0662; 1 mg of salt/ml or 570 μg of base/ml) were prepared as stock solutions in RPMI 1640 medium. The stock solution was then diluted with the red cell suspension to give final primaquine concentrations of 300, 600, and 1,200 salt ng/ml, respectively. Artesunate (60 mg/ml; Guilin Pharmaceutical Factory, Guilin, China) was prepared freshly as a stock solution at 1 mg/ml or 3.5 μM in $NaHCO_3$ and diluted in RPMI 1640 medium. The solution was then added to the red cell suspension in fivefold dilutions (range, 0.2 to 25 ng/ml). The red cell suspension was incubated with the antimalarial drugs at 5% CO_2 and 37°C for 24 h. Thin blood films were then taken for microscopic examination. Gametocytes cultured in medium without drugs were used as controls. After 24 h of incubation, gametocyte cultures with or without drug were centrifuged at 300 × *g* for 5 min to remove the supernatant, washed twice, and resuspended with RPMI 1640 medium to give a 50% hematocrit. Batches of *A. dirus* mosquitoes were then fed on each drug dilution by the membrane feeding method (5).

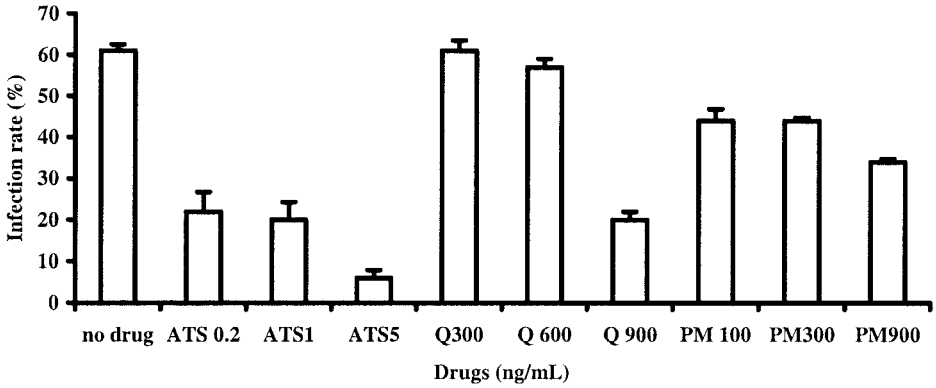

FIG. 1. Effects of artesunate (ATS), quinine (Q), and primaquine (PM) on IR. The IR is the ratio of mosquitoes with one or more oocysts to the number of dissected mosquitoes. Data are shown as mean percentages and SEs.

Oocyst formation. In each feeding experiment, 20 mosquitoes were sampled for examination of oocyst development 10 days after feeding. Midguts were stained with mercurochrome, and oocysts were counted under a phase-contrast microscope. The gametocytocidal activity of the drug was calculated as the percent reduction of oocyst development in comparison with the control values. Five replicate experiments were conducted, and results were pooled for each drug. The infection rate (IR) in the mosquitoes was calculated from the number of mosquitoes with ≥ 1 oocyst divided by the number of dissected mosquitoes. The concentrations that reduced the number of oocysts by 50% and 90% were calculated from the dose-response plots of percent inhibition against the drug concentration fitted by using the Table Curve 2 D program.

Statistical analysis. For each drug test, a paired *t* test was used to determine the significance of the difference in the number of oocysts between the drug-treated group and the control group. *P* values of <0.01 were accepted for statistical significance. All statistical analyses were performed with the statistical computing package SPSS version 11 for Windows (SPSS Inc.).

RESULTS

Cultures. After 14 days of in vitro cultivation, stage I gametocytes were present in the cultures. The time for gametocytes to develop from stage I to stage V gametocytes was a further 7 to 10 days. After 24 h, before membrane feeding to *A. dirus*, obvious changes in gametocyte morphology were observed in gametocyte cultures exposed to artesunate (pyknotic nuclei with incomplete cytoplasm), but no morphological changes were evident with quinine or primaquine.

Drug effects. The IR was defined as the ratio of mosquitoes with one or more oocysts to the number of dissected mosquitoes. The mean (standard deviation [SD]) IR was 61% (12%) in the control group (i.e., with no drug). The IR was decreased in all drug-treated groups. At the highest concentrations tested, it was reduced 90% by artesunate, 67% by quinine, and 44% by primaquine (Fig. 1).

The mean number of oocysts determined 14 days after mosquito feeding was decreased significantly ($P = 0.01$) by artesunate compared with the controls (Fig. 2). The number of oocysts was not affected significantly by quinine at concentrations below 600 ng of base/ml ($P = 0.5$), but a significant reduction in the number of oocysts was observed at higher concentrations of quinine ($P < 0.01$). Primaquine reduced oocyst production significantly at concentrations of greater than 170 ng of base/ml ($P < 0.01$). The estimated concentrations producing 50% and 90% of the maximum inhibitory effects on asexual development and transmission blocking (EC_{50} and EC_{90}, respectively) are shown in Table 1.

The effect on transmission potential (TP) was also assessed by calculating the ratio of the total number of oocysts to the total number of dissected mosquitoes ($n = 100$). The mean TP in the control group was 17 (SD, 2). The TP was decreased

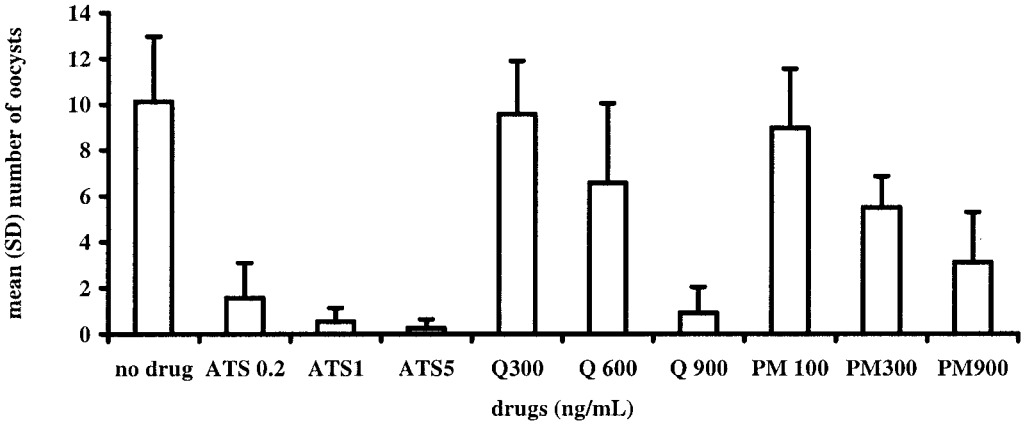

FIG. 2. Gametocytocidal activities of artesunate (ATS), quinine (Q), and primaquine (PM). Data are shown as mean numbers of oocysts ± SD ($n = 100$).

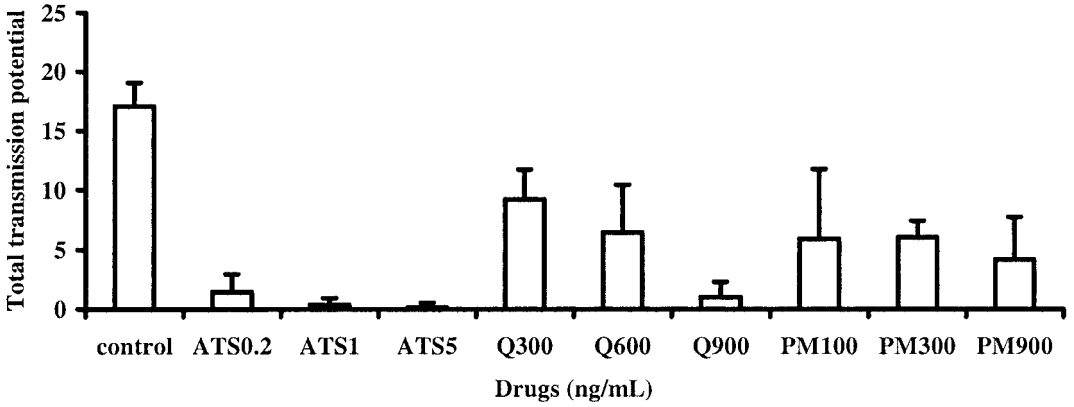

FIG. 3. Effects of artesunate (ATS), quinine (Q), and primaquine (PM) on TP. The TP is the ratio of the total number of oocysts to the total number of dissected mosquitoes ($n = 100$). Data are shown as means and SDs.

significantly in a dose-dependent manner when mature gametocytes were exposed to all drug-treated groups ($P < 0.1$). The most potent effects were observed in the artesunate-treated group (Fig. 3). The median (range) absolute reduction of TP was 98% (91 to 99%) in the artesunate group, 64% (47 to 94%) in the quinine group, and 64% (64 to 76%) in the primaquine group.

DISCUSSION

The transmission-blocking activity of antimalarial drugs is an important determinant of their public health impact. There have been several approaches to assessing the transmission-blocking activity of antimalarial drugs. In studies using volunteers, the infectivity of malaria parasites for mosquitoes is extremely variable and depends on a variety of factors. These include the gametocyte density in the blood of the human host, the stage of the infection, and the strain of the parasite. Alternatively, the transmission-blocking activity of antimalarial drugs can be assessed in vitro as presented here. All antimalarial drugs which are effective against asexual stages reduce TP by diminishing the production of gametocytes from the asexual forms. This is because gametocytogenesis in *P. falciparum* is delayed with respect to asexual stage expansion and the sexual stages are produced as a result of asexual stage multiplication (24). Quinine reduces the viability of young gametocytes (12). In this study, we found that quinine also had some inhibitory effect on mature gametocytes but only at relatively high concentrations. The EC_{50} for mature gametocytes was equal to the MIC of *P. falciparum* isolates considered highly resistant to quinine (23). Primaquine has been used as a hypnozoitocidal drug in the radical treatment of *P. vivax* malaria and as gametocytocidal drug against *P. falciparum* malaria (1). Primaquine accelerates gametocyte clearance (19) and potently reduces infectivity in volunteer studies. In this system, transmission-blocking activity of primaquine was achieved at concentrations which approximate the peak plasma concentrations reached during the treatment of malaria, i.e., 280 ± 180 ng/ml (11), but was less than expected from its well-documented activity in clinical studies.

Artesunate had a potent effect on gametocyte infectivity in *A. dirus*. At a concentration of 1.5 ng/ml, it produced 90% inhibition of infectivity. This concentration is lethal to both asexual parasites and gametocytes. However, as artesunate and it biologically active metabolite dihydroartemisinin are eliminated very rapidly, exposure in this experimental system represents a longer exposure than would occur in vivo and may overestimate the effects in vivo. Artesunate reduced the IR in the mosquitoes and the production of oocysts in those mosquitoes which were infected. This should translate into reduced transmissibility of falciparum malaria (5). Both in animal models (7) and in human malaria (6, 9, 11, 17, 22, 26, 27, 28), use of artemisinin derivative-based treatment does result in a significant reduction of gametocyte carriage. Widespread deployment of artemisinin-based combination treatments in low-transmission settings reduces gametocyte carriage and the incidence of malaria (14). The reduction of TP seen in malaria patients treated with artemisinin derivatives reflects activity against the full range of gametocyte development, including mature gametocytes. The resulting lower gametocyte prevalence and infectivity result in reduced transmissibility of the infection.

TABLE 1. Transmission-blocking effects of artesunate, quinine, and primaquine[a]

Drug	EC_{50}	EC_{90}
Artesunate[b]	0.1 ± 0.02	0.4 ± 0.15
Quinine[c]	642 ± 111	816 ± 32
Primaquine[c]	183 ± 23	543 ± 43

[a] The data are presented as means and SDs from five replicates for each drug.
[b] Values are in nanograms per milliliter.
[c] Values are in nanograms of base per milliliter.

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