In Vitro Antimalarial Activity of Azithromycin, Artesunate, and Quinine in Combination and Correlation with Clinical Outcome[▽]

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Azithromycin when used in combination with faster-acting antimalarials has proven efficacious in treating *Plasmodium falciparum* malaria in phase 2 clinical trials. The aim of this study was to establish optimal combination ratios for azithromycin in combination with either dihydroartemisinin or quinine, to determine the clinical correlates of in vitro drug sensitivity for these compounds, and to assess the cross-sensitivity patterns. Seventy-three fresh *P. falciparum* isolates originating from patients from the western border regions of Thailand were successfully tested for their drug susceptibility in a histidine-rich protein 2 (HRP2) assay. With overall mean fractional inhibitory concentrations of 0.84 (95% confidence interval [CI] = 0.77 to 1.08) and 0.78 (95% CI = 0.72 to 0.98), the interactions between azithromycin and dihydroartemisinin, as well as quinine, were classified as additive, with a tendency toward synergism. The strongest tendency toward synergy was seen with a combination ratio of 1:547 for the combination with dihydroartemisinin and 1:44 with quinine. The geometric mean 50% inhibitory concentration (IC₅₀) of azithromycin was 2,570.3 (95% CI = 2,175.58 to 3,036.58) ng/ml. The IC₅₀s for mefloquine, quinine, and chloroquine were 11.42, 64.4, and 54.4 ng/ml, respectively, suggesting a relatively high level of background resistance in this patient population. Distinct correlations ($R = 0.53$; $P = 0.001$) between quinine in vitro results and parasite clearance may indicate a compromised sensitivity to this drug. The correlation with dihydroartemisinin data was weaker ($R = 0.34$; $P = 0.038$), and no such correlation was observed for azithromycin. Our in vitro data confirm that azithromycin in combination with artemisinin derivatives or quinine exerts additive to synergistic interactions, shows no cross-sensitivity with traditional antimalarials, and has substantial antimalarial activity on its own.

With the recent introduction of artemisinin-based combination therapy as the first-line therapy in many countries where malaria is endemic, combination therapies have become the standard of care for the treatment of *Plasmodium falciparum* malaria. However, the spread of multidrug resistance from Southeast Asia to significant parts of the world where malaria is endemic calls for the development of antimalarial compounds with novel modes of action. Antibiotics with antimalarial activity may offer an interesting alternative for the treatment of multidrug-resistant *falciparum* malaria.

Azithromycin, a widely prescribed advanced-generation macrolide antibiotic with a favorable toxicological profile, has shown intrinsic activity against *Plasmodium* spp. both in vitro (11, 14) and in vivo for prophylaxis and treatment (1, 3, 4, 6, 8, 10, 15). Compared to other antibiotics used for malaria treatment (e.g., tetracyclines), azithromycin offers unique advantages

due to its safety in children and experience with use in pregnant subjects (5), the populations most affected by malaria. With an average terminal half-life of almost 3 days, azithromycin also has favorable pharmacokinetic properties, resulting in practical dosing regimens of as short as 3 days. Recent studies (8, 10) indicate good efficacy in phase 2 trials for the treatment of uncomplicated *falciparum* malaria when used in combination with artesunate or quinine.

Previous in vitro interaction studies done with clones and culture-adapted malaria parasites suggested that azithromycin combinations with quinine were additive to synergistic, whereas those with dihydroartemisinin were additive to antagonistic (14). The aim of the present study was to investigate drug interactions in clinical field isolates, to establish optimal combination ratios for azithromycin in combination with either dihydroartemisinin or quinine, to determine the clinical correlates of in vitro drug sensitivity for these compounds, and to assess cross-sensitivity patterns.

MATERIALS AND METHODS

Patient samples. Blood samples were obtained from adult men and nonpregnant women aged 20 to 65 years, recruited at the Hospital for Tropical Diseases, Mahidol University, Bangkok, Thailand, who presented with acute uncomplicated

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TABLE 1. Geometric mean 50, 90, and 99% ICs for azithromycin, dihydroartemisinin, quinine, mefloquine, and chloroquine

Drug	Inhibitory concn (ng/ml)		
	IC ₅₀ (95% CI)	IC ₉₀ (95% CI)	IC ₉₉ (95% CI)
AZ	2,570.28 (2,175.58–3,036.58)	13,311.41 (11,206.76–15,811.31)	23,950.53 (19,989.39–28,696.63)
DHA	0.53 (0.45–0.63)	1.43 (1.20–1.72)	2.28 (1.87–2.79)
QN	64.36 (55.92–74.09)	172.82 (149.74–199.47)	251.06 (219.71–286.88)
MQ	11.42 (9.43–13.84)	30.99 (25.86–37.14)	46.66 (39.29–55.41)
CQ	54.45 (48.11–61.62)	129.55 (112.98–148.55)	172.34 (149.80–198.28)

cated falciparum malaria, who were otherwise healthy, and who had parasite densities between 100 and 100,000 asexual *P. falciparum* parasites per μ l (equivalent to ca. 0.002 to 2% parasitemia). Parasite samples with more than 1% parasitemia were diluted with uninfected red blood cells. Patients were not admitted to the study if any of the following criteria were present: pregnant and nursing women; signs and symptoms of severe malaria; mixed malaria infection on admission; malaria drug therapy or blood transfusions in the preceding 30 days; laboratory evidence or history of significant cardiovascular, hepatic, or renal functional abnormality; severe vomiting; reported alcohol or drug abuse; any clinically significant illness; or likelihood of requiring treatment with drugs not permitted by the protocol. Written informed consent was obtained from all study participants and the protocol was approved by the appropriate ethical review boards.

All of the 97 fresh parasite isolates obtained before the initiation of treatment from patients with microscopically confirmed *P. falciparum* mono-infections were tested in duplicate for their drug sensitivity to azithromycin (AZ), dihydroartemisinin (DHA), mefloquine (MQ), quinine (QN), and chloroquine (CQ). Checkerboard drug interaction assays were performed on four randomly selected samples with the combination of AZ with either DHA or QN.

Drug susceptibility testing. All samples were tested in the histidine-rich protein 2 (HRP2) in vitro drug susceptibility assay (12). Fresh *P. falciparum* parasite isolates were cultured in the presence of twofold serial dilutions of the antimalarial drugs AZ (781.25 to 50,000 ng/ml), DHA (0.15 to 9.38 ng/ml), MQ (3.22 to 206.27 ng/ml), QN (24.11 to 1,543.21 ng/ml), and CQ (16.14 to 1,033.06 ng/ml) at 1.5% hematocrit in complete RPMI 1640 with 0.5% Albumax (Albumax I; Gibco, Bangkok, Thailand) and 25 mg of gentamicin/liter without freezing, washing, dilution, the addition of serum, or preculturing. Checkerboard assays with the drug combinations were performed by diluting AZ (97.7 to 12,500 ng/ml) vertically and either QN (3.01 to 385.80 ng/ml) or DHA (0.02 to 2.34 ng/ml) horizontally on an 8×8 checkerboard. Stock solutions of the test drugs at 1 mg/ml were prepared in 70% ethanol and then diluted with distilled water to the test concentration. Serial twofold dilutions (seven concentrations and one drug-free control well) of the drugs (25 μ l/well) were dispensed into standard 96-well microculture plates (Costar 3599) manually or by a semiautomated microdilution technique. The culture plates were then dried and stored at 4°C for up to 4 weeks. A total of 200 μ l of cell medium mixture was added to each well, and the plates were incubated for 72 h in a gas mixture (5% CO₂, 5% O₂, 90% N₂) at 37.5°C. The plates were subsequently freeze-thawed twice to obtain complete hemolysis.

Parasite growth inhibition was quantified by using a highly sensitive HRP2 enzyme-linked immunosorbent assay (ELISA) based on two commercially available monoclonal antibodies (Immunology Consultants Laboratory, Inc., Newberg, OR) directed against *P. falciparum*-specific HRP2: MPFM-55A, an immunoglobulin M antibody used as the capture antibody, and MPFG-55P, a horseradish peroxidase-conjugated immunoglobulin G antibody, which was used as the indicator antibody. The ELISA was performed as previously described and allows for the testing of parasitized blood samples down to ca. 0.002% parasitemia (13). Spectrophotometric analysis was performed with an ELISA plate reader (SpectraMAX 340 microplate spectrophotometer; Molecular Devices, Sunnyvale, CA) at an absorbance maximum of 450 nm.

Data analysis. The 50 and 90% inhibitory concentrations (IC₅₀s and IC₉₀s, respectively) were calculated from optical density readings by nonlinear regression analysis. ICs were used to calculate fractional inhibitory concentrations (FICs) as previously described (2). Isobolograms were plotted to demonstrate synergism and/or antagonism for drug combinations. Activity correlations were calculated by nonparametric correlation analysis (Spearman). The significance for differences between two groups of continuous data was calculated by using the Mann-Whitney U test.

RESULTS

Of 97 fresh isolates, 73 (75.2%; 95% confidence interval [CI] = 65.5 to 83.5) were successfully tested in the HRP2 drug sensitivity assay. For 69 (94.5%; 95% CI = 86.6 to 98.5) of 73 patients, 28-day follow-up data (cure rates, parasite, and fever clearance times) after treatment with azithromycin combination therapy were available for comparison. In four arms, patients were either treated with azithromycin (1 or 1.5 g per day as a single or split dose for 3 days) in combination with artesunate (200 mg/day either once daily or in split dose twice a day for 3 days) or quinine (20 or 30 mg/kg of body weight per day for 3 days). Six treatment failures were seen in the quinine arms, and five were seen in the artesunate-containing arms. The geometric mean parasite density for all samples tested in the in vitro assay was 4,634 per μ l (range, 95 to 134,404 per μ l).

The geometric mean IC₅₀ for azithromycin was 2,570.28 ng/ml (95% CI = 2,175.58 to 3,036.58); for dihydroartemisinin the corresponding value was 0.53 ng/ml (95% CI = 0.45 to 0.63), for quinine it was 64.36 ng/ml (95% CI = 55.92 to 74.09), for mefloquine it was 11.42 ng/ml (95% CI = 9.43 to 13.84), and for chloroquine it was 54.45 ng/ml (95% CI = 48.11 to 61.62). Detailed ICs with CIs are shown in Table 1. The IC₅₀s and FICs for the four samples tested in the checkerboard assays are presented in Table 2.

Drug interaction studies on fresh clinical isolates. Four clinical isolates were successfully tested in checkerboard interaction studies with azithromycin and either dihydroartemisinin or quinine. With an overall mean FIC of 0.84, the interaction between dihydroartemisinin and azithromycin was classified as additive, with a tendency toward synergism (Fig. 1). The mean

TABLE 2. Individual results for azithromycin, artesunate, and quinine, as well as the FICs for the samples tested in checkerboard assays

Drug combination	Parasite isolate	IC ₅₀ (ng/ml)			Sum FIC ₅₀ ^a
		DHA	QN	AZ	
AZ-DHA	AZ10011003	0.24		2,443.97	0.84
	AZ10011008	0.91		5,342.33	1.06
	AZ10011017	0.27		1,976.38	0.73
	AZ10011022	0.38		2,190.46	0.72
AZ-QN	AZ10011003		123.33	2,443.97	0.85
	AZ10011008		82.82	5,342.33	0.73
	AZ10011017		58.29	1,976.38	0.68
	AZ10011022		116.42	2,190.46	0.84

^a FIC₅₀, FIC at IC₅₀.

FIG. 1. Isobolograms for the checkerboard assays with azithromycin and dihydroartemisinin against clinical field isolates of *P. falciparum*, showing additive to synergistic interactions. The mean FICs for the clinical isolates were 0.84, 1.06, 0.73, and 0.72 for the samples AZ10011003, AZ10011008, AZ10011017, and AZ10011022, respectively.

FICs were 0.84, 1.06, 0.73, and 0.72, with individual datum points ranging from 0.47 to 1.20. This similarly applied to the combination of quinine with azithromycin (Fig. 2). Here, the mean FIC was slightly lower at 0.77. The FICs were 0.85, 0.73, 0.68, and 0.84, with individual datum points ranging from 0.37 to 1.05. Drug combination ratios between dihydroartemisinin and azithromycin of 1:34 to 1:1,198 (mean, 1:547) resulted in the highest levels of synergy (i.e., the lowest FICs). For azithromycin and quinine, the optimal combination ratios were 1:4 to 1:124 (mean, 1:44).

Clinical correlates. The IC_{50} s for individual patient isolates were compared to the treatment outcome in the corresponding patients. In the low-dose quinine arm three RIII failures (early treatment failures) were seen. These failures had QN IC_{50} s (116.4, 103.3, and 93.2 ng/ml) that were consistently greater than the geometric mean (64.4 ng/ml). At the same time two of these three isolates were classified as highly resistant to mefloquine (i.e., $IC_{50} > 30$ ng/ml), a drug that is widely used in Thailand and that is known for its cross-resistance with quinine ($R = 0.44$; $P < 0.001$ in the present study). In patients treated with quinine the quinine IC_{50} s were almost twice as high (114.8 ng/ml; $P = 0.01$) in isolates that later led to failures ($n = 6$; three RIs with 126.9 ng/ml and three RIIs with 103.9) than in those which did not (63.5 ng/ml). In patients treated with

artesunate combinations the IC_{50} in the five patients who had recrudescences within the 28-day follow-up was 0.81 ng/ml (compared to 0.45 ng/ml for the geometric mean in patients who were cured; $P > 0.05$). Azithromycin IC_{50} s in isolates from patients who later developed recrudescences (2,953 ng/ml) were similar to those in cured patients (2,627 ng/ml; $P > 0.05$), suggesting little influence of the azithromycin in vitro drug sensitivity on clinical outcome.

Individual parasite clearance times (PCTs), i.e., the time from administration of the first dose of the study drug until complete parasite clearance, in the quinine arms were significantly ($R = 0.53$; $P = 0.001$) correlated with quinine IC_{50} s. With a correlation coefficient of $R = 0.34$ ($P = 0.038$) the relation between PCTs and DHA IC_{50} s was less significant. No correlation was seen between azithromycin IC_{50} s and the PCT in the quinine ($R = -0.12$; $P > 0.05$) or artesunate ($R = -0.10$; $P > 0.05$) arms.

Correlations. Individual ICs calculated for all drugs tested in parallel were correlated by nonparametric correlation analysis to determine cross-sensitivity and/or cross-resistance patterns between the drugs at the IC_{50} level. Azithromycin IC_{50} s did not show any significant activity correlations with any of the other drugs, reflecting its unique chemical structure and mode of action among these drugs. Significant activity correlations were

FIG. 2. Isobolograms for the checkerboard assays with azithromycin and quinine against clinical field isolates of *P. falciparum*, showing additive to synergistic interactions. The mean FICs were 0.85, 0.73, 0.68, and 0.84 for the samples AZ10011003, AZ10011008, AZ10011017, and AZ10011022, respectively.

found between the IC_{50} s of DHA and MQ ($R = 0.34$; $P = 0.003$), as well as QN ($R = 0.50$; $P < 0.001$). Mefloquine activity at the IC_{50} level correlated with the IC_{50} s of QN ($R = 0.44$; $P < 0.001$).

DISCUSSION

Recently, azithromycin in combination with faster-acting antimalarials has demonstrated efficacy in phase 2 trials in treating *P. falciparum* malaria (3, 8, 10). As an antimalarial, azithromycin is relatively slow acting and therefore has to be combined with fast-acting compounds that will quickly reduce the initial parasite burden.

Analysis of activity correlations confirms a major advantage of many antibiotics over most traditional antimalarial drugs, namely, the fact that azithromycin as a macrolide antibiotic has a mechanism of action likely to be different from that of most other antimalarial drugs. In combination therapies this could greatly reduce the probability that drug resistance may develop and is likely to considerably extend the life span of this drug.

Our data suggest high levels of drug resistance in these isolates originating from Thailand, particularly to chloroquine

and mefloquine, and at least compromised sensitivity to quinine, which is also reflected in the correlation between elevated quinine IC_{50} s and clinical outcome.

The interaction studies and isobolograms suggest that both artesunate and quinine are promising combination partners for azithromycin. In our study both drugs showed additive interactions with a tendency toward synergism when used in combination with azithromycin. Both drugs have in common that they are relatively fast acting and that they have short half-lives. Despite the reduced in vitro drug sensitivity to quinine, the overall cure rates were similar in the artesunate and higher-dose quinine arms (10).

Both quinine and artesunate are considered to be relatively safe, with quinine considered safe for use even in pregnant subjects. One of the reasons azithromycin is particularly attractive as an antimalarial is its safety in children and past experience with its use in pregnancy. In this case the combination of quinine with azithromycin could also overcome the known toxicity (cinchonism) and compliance issues associated with longer quinine therapy by reducing the duration of quinine treatment from 7 to 3 days. The combination of these compounds could therefore provide an alternative for the treat-

ment of falciparum malaria in populations particularly affected by malaria, such as children and pregnant women.

Previous in vitro studies performed with culture-adapted parasites (14) provide similar findings for the combination with quinine. The three isolates tested by Ohrt et al. with quinine showed IC_{50} s that were slightly lower and revealed similar FICs with the two more resistant isolates. Of the two DHA isolates reported in the same publication FIC_{50} s were on average higher, similar to one of the four isolates reported in our study. For culture-adapted parasite strains Ohrt and coworkers (8) suggested an additive to antagonistic interaction for the combination of azithromycin with artesunate and indicated that this could be one of the reasons for the limited success of this combination in early clinical trials, in addition to the fact that, in previous studies, the doses were much lower than those used in our parallel clinical trial (7, 9). Our in vitro data indicate an additive to synergistic interaction between artesunate and azithromycin in clinical field isolates. The different results may largely be attributable to differences in methodology, i.e., the fact that our study was done in clinical field isolates using a checkerboard design as opposed to the use of culture-adapted parasite strains tested in fixed combinations by Ohrt and coworkers. Based on the experiences from our study, as well as the one by Ohrt and coworkers, and considering the different chemical structures and their likely modes of action, the interaction between these drugs can be expected to be largely indifferent (i.e., additive).

The ability of in vitro drug susceptibility results to predict clinical failure in malaria patients is traditionally limited. Thus far, few studies have managed to demonstrate a close correlation between in vitro data and clinical outcome (16). In part this may have to do with the methodology used in some of these studies. In particular, polyclonal samples tend to lose their intrinsic drug susceptibility pattern when adapted to culture, thereby leading to poor correlations between in vitro and clinical results. The use of highly sensitive ELISA-based drug susceptibility assays that allow for the testing of clinical samples directly from the patient irrespective of the parasite density and without preculturing may be an important step in predicting clinical outcome. Minor variations in drug sensitivity that remain below a certain threshold can be expected to have little influence on treatment response and will therefore not lead to significant correlations between IC_{50} s and PCT. Once the drug sensitivity is compromised the variation tends to exceed this threshold and start having an impact on treatment response, particularly PCT. A distinct correlation between in vivo and in vitro results could therefore possibly be an early indication of developing drug resistance. Moreover, drug resistance is not the only parameter leading to treatment failures. Numerous other factors, such as the bioavailability of individual drugs, the patient's immune system, etc., can influence treatment response. Only when drug sensitivity is severely compromised can intrinsic drug sensitivity become a dominant cause of failures and inadequate treatment response.

In conclusion, our in vitro data confirm the promising results from recent clinical trials with azithromycin-based combinations for the treatment of falciparum malaria. Azithromycin in combination with artemisinin derivatives or quinine exerts additive to synergistic interactions, shows no cross-sensitivity with

traditional antimalarials, and has substantial antimalarial activity on its own.

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