

RESEARCH NOTE

ASSESSMENT OF *IN VITRO* ANTIMALARIAL INTERACTIONS BETWEEN DIHYDROARTEMISININ AND FOSMIDOMYCIN

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Abstract. Malaria remains one of the leading causes of morbidity and mortality in the tropics with an annual estimate of 500 million clinical cases and 2 million deaths. The treatment and control of malaria is becoming increasingly difficult due to *Plasmodium falciparum* resistance to commonly used antimalarials. Combination therapy is currently the strategy for combating multi-drug resistant falciparum malaria, through exploiting pharmacodynamic synergistic effects and delaying the emergence of drug resistance. The combination of artemisinin derivatives with fosmidomycin, which have different modes of action, appears to be one of the most promising combinations. The objective of the present study was to investigate the antimalarial interactions between dihydroartemisinin and fosmidomycin *in vitro*, against chloroquine-resistant (K1) and chloroquine-sensitive (G112) *P. falciparum* strains. Concentration-response analysis was performed based on an *in vitro* schizont maturation inhibition test. The fixed concentration ratios of dihydroartemisinin: fosmidomycin used were 0:5,000, 2:4,500, 6:3,500, 10:2,500, 14:1,500, 18:500 and 20:0 nM. The highest final concentrations of dihydroartemisinin and fosmidomycin were 20 and 5,000 nM, respectively. Results showed IC₅₀ (drug concentration which produced 50% schizont maturation inhibition) medians (range) for dihydroartemisinin against K1 and G112 strains to be 1.6 (1.2-2.0) and 2.5 (2.4-2.6) nM, respectively. The IC₅₀ medians (range) for fosmidomycin against K1 and G112 strains were 1,347 (1,068-1,625) and 786 (737-834) nM, respectively. An isobologram revealed an increasing trend for the fraction IC₅₀ (FIC), which indicates marked antagonism of this drug combination against both chloroquine resistant and chloroquine sensitive strains.

INTRODUCTION

Fosmidomycin is a potent inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, an essential enzyme of the nonmevalonate pathway in the plastid-like organelle (apicoplast) of malaria parasites. It blocks the biosynthesis of isopentenyl diphosphate that subsequently develops into isoprenoids in

Plasmodium falciparum, thereby effectively inhibiting parasite growth, including multidrug-resistant strains *in vitro* and in murine malaria (Jomaa *et al*, 1999). *In vitro* experiments have shown that fosmidomycin exhibits its full antimalarial potency when the parasites are exposed to the agent for a full replication cycle, leading to an arrest in development of the late schizont stage (Lell *et al*, 2003). Results of previous studies indicate that the drug is well tolerated by humans (Murakawa *et al*, 1982; Kuemmerle *et al*, 1985). In a recent clinical study conducted in Gabon and Thailand, 20 patients with acute uncomplicated *P. falciparum* malaria were treated with fosmidomycin admin-

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istered orally (Lell *et al*, 2003). The treatment regimen was well tolerated and resulted in rapid parasite and fever clearance times, comparable to those obtained with conventional quinoline antimalarial agents. All patients were clinically and parasitologically cured by day 7. However, reappearance of parasitemia was observed in 9 out of 18 patients by day 28 (recrudescence). To deal with this problem, fosmidomycin combination therapy with other drugs is recommended.

In searching for potential fosmidomycin combinations, Wiesner and colleagues (2002) performed *in vitro* antimalarial activity with fosmidomycin and currently used antimalarials. Synergistic interactions were observed between fosmidomycin and the lincosamides, lincomycin and clindamycin. Interestingly, the interaction between fosmidomycin and artemisinin was antagonistic. However, a subsequent study by Borrmann and colleagues (2005) in phase II clinical trials of an artesunate-fosmidomycin combination showed controversial results. A total of 50 Gabon children with acute uncomplicated *P. falciparum* malaria were treated with a short-course regimen (3 days) of artesunate-fosmidomycin (1-2 mg/kg body weight and 30 mg/kg of body weight, given every 12 hours). The cure rate was virtually 100% with a good safety profile. This result provided evidence for artesunate-fosmidomycin as a promising short course combination regimen for the treatment of multidrug resistant *P. falciparum* malaria (Borrmann *et al*, 2005). Due to the discrepancy between the *in vitro* and *in vivo* data, the present study aimed to reexamine the antimalarial interactions between dihydroartemisinin, an artemisinin derivative, and fosmidomycin, in both chloroquine-resistant and chloroquine-sensitive strains.

MATERIALS AND METHODS

Parasite isolates

The *P. falciparum* laboratory strains used

were K1 (chloroquine-resistant strain from Thailand) and G112 (chloroquine-sensitive strain from Gambia), which were kindly provided by the Malaria Research Unit, Institute of Health Research, Chulalongkorn University, Thailand.

Parasite cultivation

K1 and G112 *P. falciparum* stains were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% human B serum and 25 mM HEPES. Cultures were kept at 37°C under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂ (Trager and Jensen, 1976). The levels of parasitemia in the cultures were kept between 2 and 10%, with a 5% hematocrit.

Preparation of drug solutions

Standard powder of dihydroartemisinin and fosmidomycin (99.9% purity) were kindly provided by Dafra Pharma, Belgium and Chiracon, German, respectively. Stock solutions of the two drugs were prepared at a concentration of 10 mM in 50% ethanol. On the day of experiment, stock solutions of the two drugs were diluted with serum-free medium in order to obtain the required concentrations. Concentration-response assays were first carried out to obtain the 50% inhibitory concentration (IC₅₀) values for both drugs. This was then followed by a combination assay based on a method described by Fivelman *et al* (2004). The drug dilutions were made to allow the IC₅₀ of the individual drug to fall to about a fourth of the two-fold serial dilution. The dilutions of fosmidomycin:dihydroartemisinin were then prepared in fixed ratios of 5000:0, 4500:2, 3500:6, 2500:10, 1500:14, 500:18, and 0:20 nM. Each drug combination was 2-fold serially diluted and prepared in sterile flat-bottom 96-well microtiter plates (Nunc, Denmark).

Assessment of antimalarial interactions between dihydroartemisinin and fosmidomycin *in vitro*

Assessment of the antimalarial interaction between dihydroartemisinin and fosmidomycin was performed *in vitro* based on a schizont maturation inhibition test according to the

method of Rieckmann *et al* (1978) with modifications. In brief, an aliquot of parasite (40 μ l) with 1% parasitemia and 20% hematocrit, was added into each well of the microtiter plate. The test wells consisted of varying concentrations of fosmidomycin and dihydroartemisinin at different ratios as described above, from rows A to G. The control wells (row H) consisted of drug-free parasitized erythrocytes (pRBC). All experiments were performed in triplicate. The microtiter plates were incubated in a candle jar for 24 to 30 hours. Following incubation, a thick smear was prepared from each well of the parasite suspension and the number of schizonts was counted per 200 pRBC. The percent growth was compared among the number of schizonts in each drug-containing well and the control well. Concentration-response analysis was performed using Grafit™ (Erithacus Software, UK) in order to obtain fifty percent inhibitory concentration (IC₅₀) values. Two IC₅₀ values for each of the five combination curves were calculated separately by using the known concentration ratios of both fosmidomycin and dihydroartemisinin, the fractional inhibitory concentration of fosmidomycin (FIC fosmidomycin) and dihydroartemisinin (FIC dihydroartemisinin) were calculated for each point, and isobolograms were plotted. To obtain numeric values for the type of interaction, results were expressed as a sum of the fractional inhibitory concentrations (sum FIC), calculated as (IC₅₀ of dihydroartemisinin in a mixture divided by IC₅₀ for dihydroartemisinin alone) + (IC₅₀ for fosmidomycin in the mixture divided by the IC₅₀ of the fosmidomycin alone). Sum FIC values indicated the type of antimalarial interactions as follows: "synergism" if sum FIC < 1; "addition" if sum FIC = 1; and "antagonism" if sum FIC > 1.

Statistical analysis

The median (range) values of the FICs for the two *P. falciparum* strains were compared using the Mann-Whitney *U*-test for data not conforming to normal distribution at a statisti-

cal significance level of $\alpha = 0.05$ (SPSS version 12).

RESULTS

Determination of *in vitro* concentration-response of *P. falciparum* strains to dihydroartemisinin and fosmidomycin

The concentration-response curves of the two *P. falciparum* strains K1 and G112, to dihydroartemisinin and fosmidomycin were investigated *in vitro* based on a schizont maturation inhibition test and the results are presented in Table 1. The median (range) IC₅₀ values for dihydroartemisinin against K1 and G112 strains were 1.6 (1.2-2.0) and 2.5 (2.4-2.6) nM, respectively. The IC₅₀ values for fosmidomycin against K1 and G112 strains were 1,347 (1,068-1,625) and 786 (737-834) nM, respectively.

Antimalarial interaction between dihydroartemisinin and fosmidomycin

The median (range) sums of the FICs for the antimalarial interaction between fosmidomycin with dihydroartemisinin for the K1 and G112 *P. falciparum* strains were 1.24 (0.89-1.59) and 1.21 (0.83-1.59), respectively. The isobolograms of the interaction between the two drugs in both parasite strains are shown in Fig 1. The results indicate marked antagonism of the combination for both chloroquine-sensitive (G112) and chloroquine-resistant (K1) strains. There were no significant differences in the median sum FICs between the strains.

Table 1

In vitro sensitivity of K1 and G112 *P. falciparum* strains to fosmidomycin and dihydroartemisinin, expressed as median IC₅₀ values.

Parasite strains	Median IC ₅₀ (range) nM	
	Fosmidomycin	Dihydroartemisinin
K1	1,347 (1,068-1,625)	1.6 (1.2-2.0)
G112	786 (737-834)	2.5 (2.4-2.6)

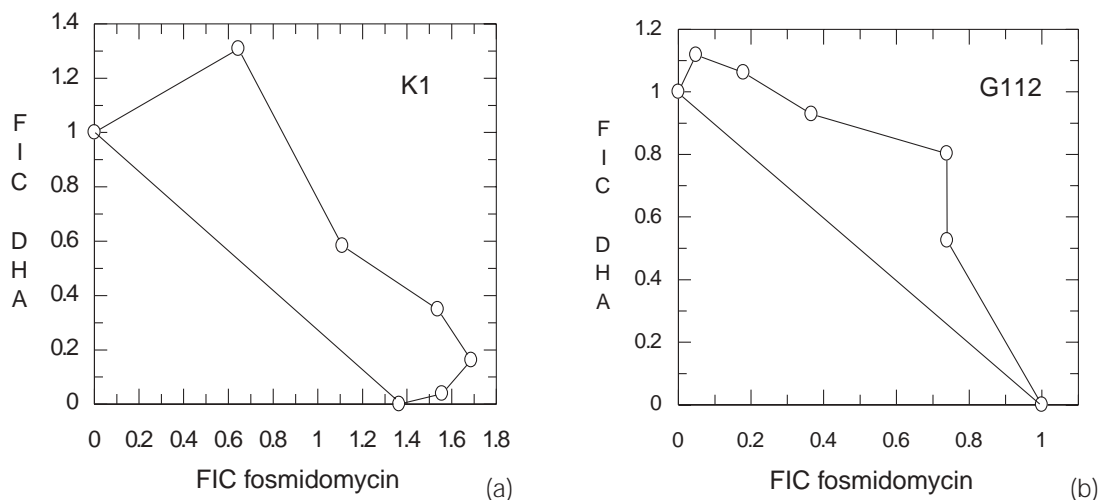


Fig 1—Representative isobolograms of the interaction between fosmidomycin with dihydroartemisinin in (a) K1 and (b) G112 *P. falciparum* strains.

DISCUSSION

The IC_{50} values for dihydroartemisinin and fosmidomycin against K1 chloroquine-resistant and G112 chloroquine-sensitive strains were similar, although a trend toward a higher IC_{50} value for fosmidomycin was observed for the K1 strain compared with the G112 strain (1,347 vs 786 nM, respectively). It has been reported previously that the IC_{50} values of fosmidomycin vary between 300 and 1,200 nM depending on the strains and the growth conditions (Jomaa *et al*, 1999; Wiesner *et al*, 2002). Although there was no statistically significant difference in the IC_{50} values for both drugs between chloroquine-resistant and chloroquine-sensitive strains, the markedly higher IC_{50} in the chloroquine-resistant parasite suggests the clinical efficacy of fosmidomycin should be carefully monitored when the drug is applied for clinical usage as resistance to this drug may develop easily, particularly in an area with intensive multidrug resistance, such as Thailand.

Due to a high rate of recrudescence when fosmidomycin is used as monotherapy, investigation of a suitable combination of partner drugs is required to develop effective fosmi-

domycin combination therapy. The combination of fosmidomycin with chloroquine, mefloquine, halofantrine, lumefantrine, artemisinin, atovaquone, proguanil, rifampicin, and ciprofloxacin have been investigated for their antimalarial effects *in vitro*, but the results showed absence of a specific interaction. An additive antimalarial effect was found between fosmidomycin and quinine, doxycycline and azithromycin. Interestingly, synergism in antimalarial activity was only observed between fosmidomycin and clindamycin, and its natural precursor, lincomycin (Wiesner *et al*, 2002).

The antagonistic interaction between fosmidomycin and artemisinin has been reported previously (Wiesner *et al*, 2002) with a mean sum $FIC \pm SEM$ of 1.33 ± 0.08 . This is in agreement with results observed in our study where median (range) sum FIC values for K1 and G112 *P. falciparum* strains were 1.24 (0.89-1.59) and 1.21 (0.83-1.59), respectively. Reports of antagonistic interactions between artemisinin derivatives and other antimalarials have been widely published (Bell, 2005) in animal models including *P. berghei* and *P. yoelii* mouse model (Chawira *et al*, 1987). However, a recent phase II clinical trial in Gabon children with uncomplicated

falciparum malaria demonstrated an improved efficacy with artesunate (an artemisinin derivative) when used in combination with fosmidomycin as a short course regimen (Borrmann *et al*, 2005). The cure rate of consecutively shortened regimens of artesunate-fosmidomycin (1-2 mg/kg of body weight and 30 mg/kg of body weight, respectively, given every 12 hours for 3 days) was virtually 100%.

The discrepancy between *in vitro* and *in vivo* observations is interesting. The rationale for using antimalarial combinations of drugs with short and relatively longer half-lives is to exploit pharmacodynamic synergistic antimalarial activity of the two partner drugs, and to delay the emergence of drug resistance. Artemisinin and its derivatives are short half-life drugs (1-3 hours) (Balint, 2001) that are usually used in combination with the long half-life drug mefloquine (14-20 Days) (Karbawang *et al*, 1988). This combination has proved effective against both sensitive and resistant strains of *P. falciparum* malaria. Fosmidomycin, on the other hand, has a much shorter half-life than mefloquine (1-2 hours) (Murakawa *et al*, 1982). Nevertheless, improved therapeutic efficacy was observed despite the short half-life of the combination partner, fosmidomycin, and the fact that the drugs were not given concurrently. This may suggest that pharmacokinetic interactions override the pharmacodynamic antagonistic effect between artemisinin derivatives and fosmidomycin. The discrepancy between *in vitro* and *in vivo* results requires further clinical study to investigate the pharmacokinetic drug interactions between the two drugs.

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