

# Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*

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Artemisinin-resistant *Plasmodium falciparum* malaria has emerged in western Cambodia. Resistance is characterized by prolonged in vivo parasite clearance times (PCTs) following artesunate treatment. The biological basis is unclear. The hypothesis that delayed parasite clearance results from a stage-specific reduction in artemisinin sensitivity of the circulating young asexual parasite ring stages was examined. A mathematical model was developed, describing the intrahost parasite stage-specific pharmacokinetic–pharmacodynamic relationships. Model parameters were estimated using detailed pharmacokinetic and parasite clearance data from 39 patients with uncomplicated falciparum malaria treated with artesunate from Pailin (western Cambodia) where artemisinin resistance was evident and 40 patients from Wang Pha (northwestern Thailand) where efficacy was preserved. The mathematical model reproduced the observed parasite clearance for each patient with an accurate goodness of fit (rmsd: 0.03–0.67 in log<sub>10</sub> scale). The parameter sets that provided the best fits with the observed in vivo data consist of a highly conserved concentration–effect relationship for the trophozoite and schizont parasite stages, but a variable relationship for the ring stages. The model-derived assessment suggests that the efficacy of artesunate on ring stage parasites is reduced significantly in Pailin. This result supports the hypothesis that artemisinin resistance mainly reflects reduced ring-stage susceptibility and predicts that doubling the frequency of dosing will accelerate clearance of artemisinin-resistant parasites.

Artemisinin combination therapies are now the recommended first-line treatment for uncomplicated falciparum in all malaria endemic countries, and artesunate is the recommended treatment for severe malaria in adults (1). The emergence of artemisinin resistance on the Cambodian–Thai border is therefore alarming (2). The genetic basis for artemisinin resistance is currently unknown. Artemisinin resistance was characterized by significant reductions in in vivo parasite clearance rates (2) and has been shown to be heritable (3), but was not reflected by conventional in vitro drug susceptibility tests (2). Conventional tests do not differentiate between stages of parasite development and are therefore unsuitable for assessing stage-specific drug resistance phenotypes. Furthermore, the constant drug exposure in vitro is very different from the profile of in vivo exposure for rapidly eliminated drugs such as the artemisinin derivatives.

Key pharmacological differences between the artemisinin derivatives and other antimalarials are their rapid elimination and broad stage specificity, which includes the circulating ring-stage parasites (4, 5). These drugs have a greater effect on ring stages than any other antimalarial class (4). It is this effect that accounts for the more rapid parasite clearances with the artemisinin derivatives compared with all other antimalarial drug classes. It has been hypothesized that loss of artemisinin sensitivity in the ring-stage parasite could explain the observed slow parasite clearance times (PCTs) in Western Cambodia (2). To test this hypothesis, a mathematical model for parasite maturation,

multiplication, and antimalarial pharmacodynamics (PD) was developed to estimate the stage-specific drug effects.

Several mathematical models have been proposed to explain the dynamics of malaria parasites in an infected host with and without treatment by an antimalarial drug. Models without treatment are often based on the model of Anderson, May, and Gupta (6), who described the dynamics of parasites in terms of the rates of change of three quantities: *i*) infected erythrocytes, *ii*) uninfected erythrocytes, and *iii*) merozoites. These models have not been validated using patient data (7). Phenomena that affect parasite densities, which in *Plasmodium falciparum* infections include the degree of synchrony of the erythrocytic phase and parasitized red cell sequestration, are not examined by these models (8). Those models that have incorporated such phenomena are mainly mechanistic and have been described by White et al. (9) and Hoshen et al. (10). They consider the changing quantities of circulating and sequestered infected erythrocytes with a multiplication rate per asexual cycle. The synchrony of the erythrocyte phase in these models can be adjusted by changing the mean and the variance of the age distribution over the asexual parasite life span. There are a few published examples of in vivo pharmacokinetic–pharmacodynamic models that consider antimalarial treatment (8, 10–16). These models examine parasite growth and killing during treatment, using differential or difference equations to describe the dynamics of the parasite population, and include modeled functions of pharmacokinetic–pharmacodynamic (PK–PD) relationships.

To incorporate parasite stage distributions and stage specificity of drug action into an examination of artemisinin resistance, a unique difference equations model was developed on the basis of a combination of two previous models (9, 10) with the addition of stage specificity of drug action. The process of fitting the model to observations began with the null hypothesis of no stage specificity of drug action. Thus any stage specificity of drug action would be derived from the application of the model to the data rather than from any prior assumptions. Detailed parasite clearance and pharmacokinetic data from clinical studies on artemisinin resistance in Pailin (western Cambodia) and Wang Pha (northwestern Thailand) were compared (2). A mixed-effects model was used in the analysis of grouped data to analyze and compare model predictions for each study site.

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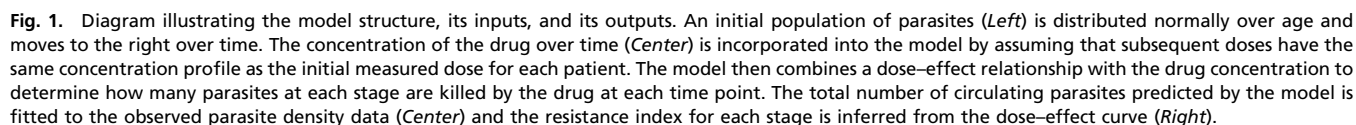
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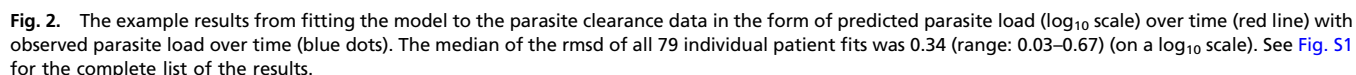
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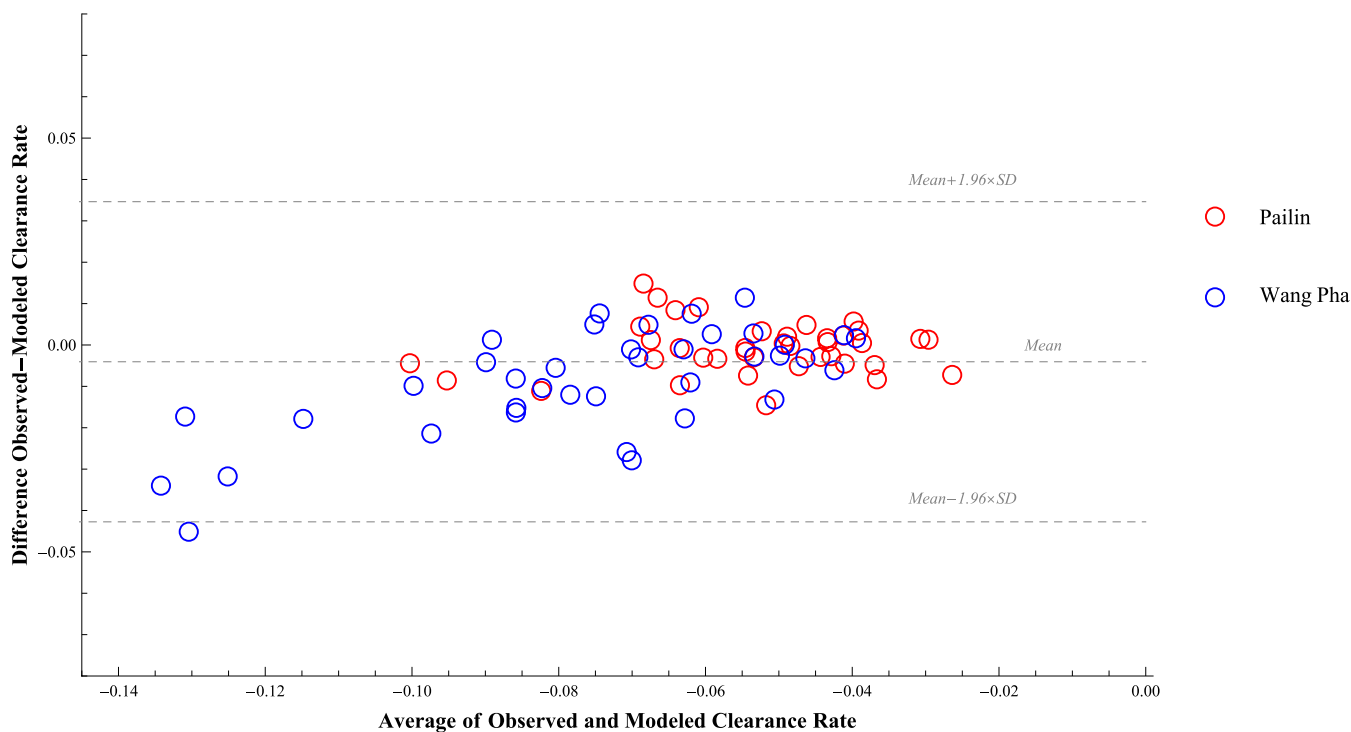
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A deterministic model for parasite maturation and multiplication during the asexual blood stages was developed. It was assumed that on presentation with acute falciparum malaria the distribution of the ages of asexual stage parasites (from 1 to 48 h) conforms to a unimodal Gaussian distribution (9). In falciparum malaria only young unsequestered parasites circulate and can be counted in peripheral blood smears. This model incorporates individual pharmacokinetic data [the profile of dihydroartemisinin (DHA) concentrations ([Fig. S1](#))] and stage-specific PK–PD relationships to predict the numbers of circulating parasites that are observed in blood samples over time for each patient. This stage-

A single quantity defining resistance level is required to determine statistically significant differences in resistance levels between stages and geographical settings to accompany the

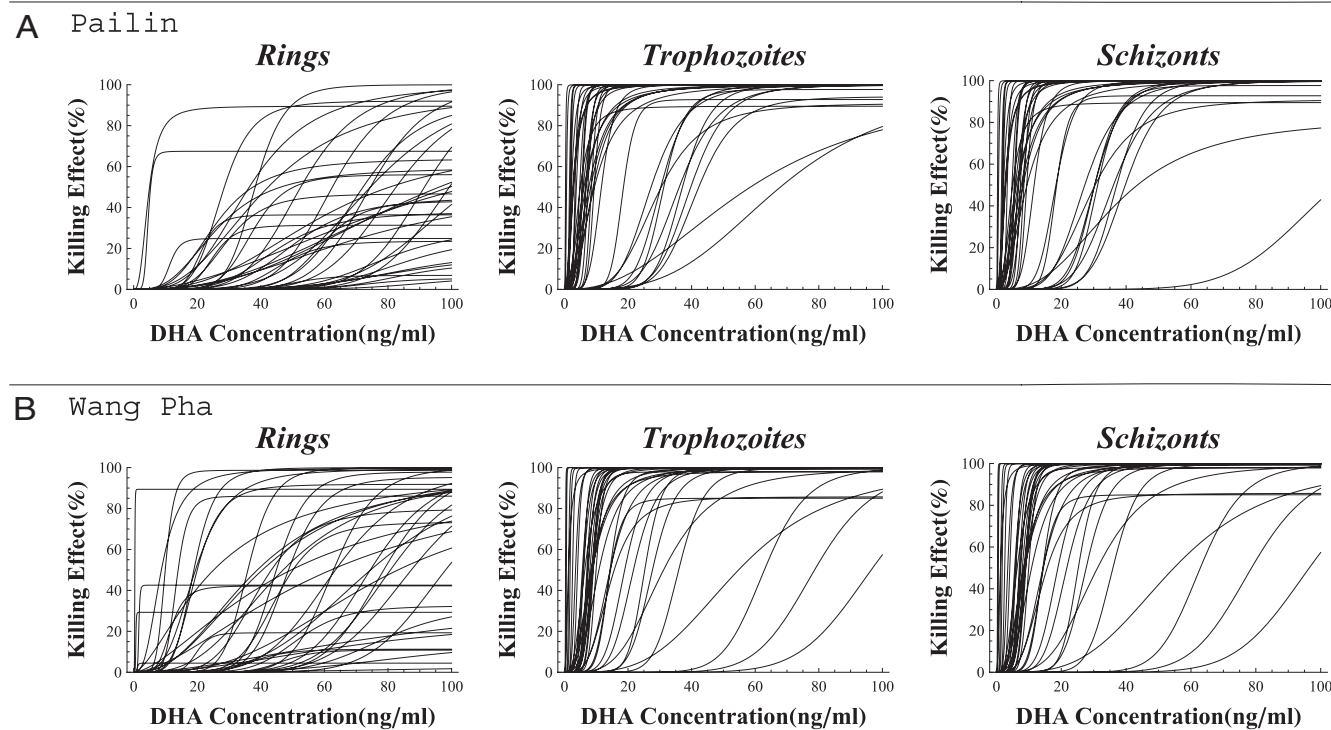




**Fig. 3.** The Bland–Altman plot shows the parasite clearance rates from the observed parasite count data were in agreement with the modeled data. The range of agreement was defined as mean  $\pm$  1.96 SD. Parasite clearance for each patient was expressed as the slope of the linear portion of the log parasitemia vs. time relationship.

graphical comparison of concentration–effect curves. For each parasite stage in each patient, the concentration–effect relationship was therefore simplified further into a single value termed the

resistance index (R.I.), which summarizes the shape of the curve such that a high value corresponds to a high level of resistance and a low value corresponds to a low level of resistance. See [SI](#)



**Fig. 4.** The inferred in vivo concentration–effect curves of each asexual stage generated from the best fit parameters for each patient from (A) Pailin (western Cambodia) and (B) Wang Pha (northwestern Thailand).

**Table 1. Results from fitting the mixed-effects models (SI Methods) with the estimates of resistance index obtained from the 10 best models in each patient using STATA 10**

log	Coefficient	SE	z	$P >  z $	95% confidence interval
R.I. Pailin	0.91	0.18	5.06	$0 < 0.001$	[0.56, 1.26]
R.I. trophozoite	−1.74	0.08	−21.81	$0 < 0.001$	[−1.90, −1.59]
R.I. schizont	−1.78	0.08	−22.35	$0 < 0.001$	[−1.95, −1.63]
R.I. Pailin trophozoite	−0.66	0.11	−5.83	$0 < 0.001$	[−0.89, −0.44]
R.I. Pailin schizont	−0.64	0.11	−5.65	$0 < 0.001$	[−0.86, −0.42]
Constant	−0.59	0.13	−4.69	$0 < 0.001$	[−0.84, −0.35]
Random effect	Parameters	Estimate	SE	95% confidence interval	
Between patient	SD (ID)	0.69	0.06	[0.58, 0.83]	
Within patient	SD ( $n$ )	0.26	0.04	[0.46, 0.61]	
	SD (residual)	1.13	0.02	[1.09, 1.17]	

The R.I. followed a lognormal distribution (Kolmogorov–Smirnov test) so the log-transformed R.I. was used for this statistical analysis.

**Methods** for more details about the R.I. Fig. 1 shows the outline model structure with its inputs and outputs. The model parameters and their sampling ranges are shown in Table S1.

The model-generated parasite clearance data closely mimicked the patients' observed parasite clearance data (see Fig. 2 and Fig. S2 for the full panels). Clearance times and clearance rates derived from the model were similar to those observed in the clinical studies. Parasitemia was output every 6 h (the usual measurement interval in these studies) with the parasite clearance time being the first time when this measure was predicted to be below the detection limit for two consecutive predictions below the detection limit. Fig. 3 shows the Bland–Altman plot of the parasite clearance rates from the observed and modeled data.

The concentration–effect curves from the best set of fitted parameters ( $EC_{50}$ ,  $E_{max}$ ,  $\gamma$ ) at each stage are shown in Fig. 4. From 100,000 sets of random parameters for each patient, the 10 best fitting parameter sets for each patient were recorded. Table S2 summarizes these sets of parameter estimates for each patient in Pailin and Wang Pha. The R.I.s were calculated using the 10 best fits for each parasite stage in each individual patient in each study site.

A two-level mixed-effects linear regression model showed that the mean R.I. for ring stage parasites in Pailin was 2.5 [95% confidence interval (CI): 1.8–3.5] times higher than the R.I. of rings in Wang Pha, indicating relative resistance of ring-stage parasites in Pailin. In contrast, the R.I. values for trophozoite-stage parasites and schizonts were not significantly different between the locations (both  $P > 0.1$ ) [Tables 1–3, SI Methods (Comparison of R.I.s), and Table S3–S7]. Examination of residuals showed heteroscedasticity in parameter sets where the ring stage susceptibilities were set as similar between the two study sites and the trophozoite and schizont susceptibilities were allowed to vary. In particular, fits to the initial changes in parasitemia, which are determined predominantly by ring stage susceptibility, were poor. As ring stages circulate, whereas trophozoites and schizonts are sequestered, this result suggests that delayed parasite clearance in Pailin is caused by reduced artesunate susceptibility of ring-stage parasites.

**Modeling Alternative Dosing Regimens.** Once-daily dosing of a rapidly eliminated drug is suitable provided the drug has broad stage specificity. The reduced ring-stage susceptibility of the artemisinin-resistant parasites opens a window of insensitivity, such that some patients' infecting parasite populations may not become susceptible until they have received the second dose, when the rings will have matured into more sensitive stages (trophozoites and schizonts). The model was used to compare two artesunate treatment regimens: 2 mg/kg every 24 h (i.e., the standard AS7 dosing regimen) with 2 mg/kg every 12 h (doubling the frequency of dosing) and 4 mg/kg every 24 h (doubling the dose). The model predicts that doubling the frequency of dosing would decrease the clearance time significantly (Fig. 5).

## Discussion

Artemisinin resistance in *P. falciparum* is a major threat for malaria control. There is an urgent need for a better description and understanding of the resistant phenotype. In this study, a mathematical model was applied to detailed PK–PD data to provide insights into the in vivo phenotype of artemisinin resistance in *P. falciparum*. After accounting for possible confounding factors, the model predicted that the most likely parameter responsible for the observed delayed parasite clearance in Pailin, western Cambodia is a reduced concentration-dependent killing rate of ring-stage malaria parasites. In the development of the model, considerable effort was made to incorporate and quantify known possible sources of variation affecting parasite clearance, such as antimalarial drug concentrations, total body parasite numbers on admission, and parasite population stage distributions. The parameter estimation method used for fitting the observed data in the model did not prespecify any parasite stage-specific dose effect (that is, DHA concentration–killing rate) relationship, but best fits were obtained for the Cambodian data when ring-stage susceptibility was reduced. The model output therefore strongly suggests that the artemisinin resistance that has developed in western Cambodia is focused at the ring stage of asexual parasite development. In acute malaria slow parasite clearance following treatment may result from reduced susceptibility of any stage of parasite development, but

**Table 2. Results from comparing the resistance indexes between stages in the same study site**

Comparison	Wang Pha			Pailin		
	Coefficient	95% CI	$P$ value	Coefficient	95% CI	$P$ value
Trophozoites vs. rings	−1.74	[−1.90, −1.59]	$0 < 0.001$	−2.40	[−2.57, −2.25]	$0 < 0.001$
Schizonts vs. rings	−1.78	[−1.94, −1.63]	$0 < 0.001$	−2.43	[−2.59, −2.27]	$0 < 0.001$
Trophozoites vs. schizonts	0.04	[−0.11, 0.20]	0.59	0.02	[−0.14, 0.18]	0.77



**Table 3. Results from comparing resistance indexes between site and stage**

		Pailin								
		Rings			Trophozoites			Schizonts		
		Coefficient	95% CI	P value	Coefficient	95% CI	P value	Coefficient	95% CI	P value
Wang Pha	Rings	0.91	[0.56, 1.26]	0 < 0.001	-1.49	[-1.85, -1.14]	0	-1.52	[-1.87, -1.16]	0 < 0.001
	Trophozoites	-2.65	[-3.00, -2.30]	0 < 0.001	0.248	[-0.10, 0.60]	0.168	-2.25	[-0.58, 0.13]	0.21
	Schizonts	-2.70	[-3.05, -2.34]	0 < 0.001	0.29	[-0.06, 0.64]	0.105	0.27	[-0.08, 0.62]	0.14

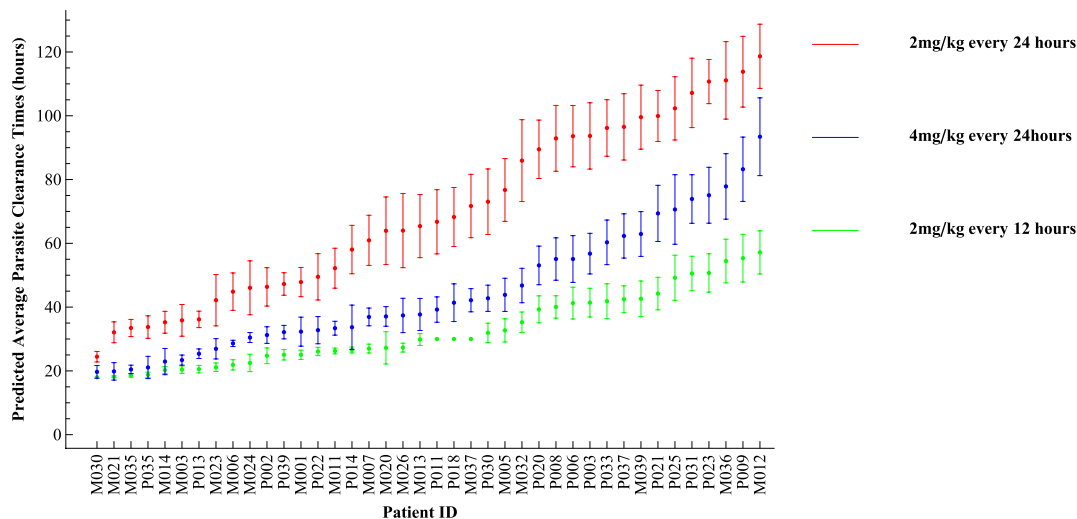
early rapid reduction of parasite densities reflects either sequestration or ring-stage killing (9, 17). The key pharmacodynamic difference between artemisinins and other antimalarial drugs is that they kill ring-stage parasites rapidly. Reduction in ring-stage killing changes the shape of the population parasite clearance curve such that the curve becomes more similar to curves following other antimalarial drug treatments such as quinine that do not affect circulating ring stages significantly. In this comparison of parasite clearance in artemisinin-resistant and artemisinin-sensitive infections forcing ring-stage susceptibility to be similar in Pailin and Wang Pha resulted in poorer fitting models with a consistent pattern of residuals in the early changes in parasitemia.

A reduced drug effect at the ring stage is sufficient to explain the current observations. This model made two main simplifying assumptions. First, it was assumed that natural infections were unimodal and normally distributed, whereas infections with two or more “broods” in different stages of development can be present in a minority of cases (9). The second assumption is that the asexual life cycle is set at 48 h. Whereas this duration is enshrined in classical malariology, and is a reasonable assumption fitting in vivo observations, it is clear from in vitro studies that there is variability in the life cycle duration (18). The parameters derived from the modeled data from Pailin and Wang Pha were not completely separated. Recent microsatellite genotyping of the parasites from Pailin suggests that ~20% of the malaria infections there could still be sensitive to artemisinin (3). This result may well explain some of the overlap between the groups. These results also explain the apparent discrepancy between the in vivo findings of a markedly delayed parasite clearance rate and the relatively unaffected conventional in vitro susceptibility assessments (2). Conventional in vitro methods expose parasites to constant concentrations over the entire life cycle. If only the ring stage but not trophozoite susceptibility is

affected, the test will have limited power to detect the resistant in vivo phenotype.

Artesunate and DHA have plasma terminal half-lives of ~1 h. This time period translates to an effective plasma concentration above the IC<sub>99</sub> of ~6 h/d in the case of the usual once-daily dosing regimen. Despite the short plasma half-life of the artemisinins, a once-daily dose is currently recommended. This dose is highly effective only because of the broad stage susceptibility of sensitive parasite strains. However, if ring-stage parasites are resistant, the timing of the drug relative to the predominant stage of development becomes more important, and a once-daily dosing might not hit the infecting parasite population at its most sensitive stages. Indeed as most uncomplicated falciparum malaria infections do present with a predominance of ring stages (19), the first dose would be expected to have a submaximal effect. The model was used to investigate alternative dosing regimens for artemisinins and predicted that doubling the frequency of dosing to twice per day is likely to reduce clearance times more effectively than doubling the dose for once-daily regimens. The shape of the concentration–effect relationships derived suggested that little would be gained by increasing individual doses. However, doubling the frequency of dosing doubles the duration for which parasitocidal drug concentrations are present in the blood whereas, because of first-order elimination, doubling the dose results in a shorter duration of drug exposure; with an increased risk this result occurs when the parasites are refractory. A clinical trial testing a multiple-dosing scheme is now underway.

Other mechanisms have been proposed to explain artemisinin resistance in vivo, such as dormancy of the ring-stage parasites (20). Under normal conditions of artemisinin exposure in natural infections a small proportion of ring-stage parasites stop maturing but are not killed by the drug (21). These parasites are thought to

**Fig. 5.** The predicted average parasite clearance time with different artesunate dose regimens.

explain the 10% recrudescence rates that follow 7-d courses of artemisinin derivatives either alone or combined with a second rapidly eliminated drug. For dormancy to explain slow log-linear parasite clearance a very high proportion of all parasites would have to become dormant; however, they would also have to be cleared relatively rapidly. Otherwise there would be a markedly biphasic parasite elimination curve. This result is not consistent with the current parasitemia–time profiles. Artemisinin resistance may well affect dormancy, but an increased dormancy fraction is unlikely to explain the observed parasite clearance profiles.

In conclusion, we developed an intrahost mathematical model describing parasitological responses after artesunate therapy for uncomplicated falciparum malaria. The model identifies selective reduction in ring-stage sensitivity as the cause of the markedly delayed parasite clearance rates observed in western Cambodia.

## Methods

**Clinical and Pharmacokinetic Data.** Data were obtained from clinical studies performed in Pailin in western Cambodia where artemisinin resistance has

emerged and in Wang Pha in northwestern Thailand in 2007 and 2008 (2). In these studies, treatment was with either 2 mg·kg<sup>−1</sup>·d<sup>−1</sup> of artesunate monotherapy for 7 d (AS7) or 4 mg·kg<sup>−1</sup>·d<sup>−1</sup> artesunate monotherapy for 3 d plus 15 mg·kg<sup>−1</sup> mefloquine on day 3 and 10 mg·kg<sup>−1</sup> on day 4 (MAS3) (2). In these studies parasite counts were performed at least six times per hour and plasma concentrations of artesunate and DHA, the active metabolite of artesunate, were measured in all patients using a frequent sampling scheme. Because the additional action of mefloquine is not modeled here, the parasite count data from patients treated with MAS3 were truncated and only data up to 72 h after admission were considered.

**Mathematical Model.** Details of the mathematical model and its application to the data can be found in [SI Methods](#).

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