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IN VITRO ACTIVITY OF TAFENOQUINE ALONE AND IN COMBINATION WITH ARTEMISININ AGAINST *PLASMODIUM FALCIPARUM*

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Abstract. Emergence and spread of drug-resistant falciparum malaria has created an urgent demand for alternative therapeutic agents. This study was conducted to assess the *in vitro* blood schizontocidal activity of tafenoquine, the most advanced candidate drug of the 8-aminoquinolines, and of its 1:1 combination with artemisinin in fresh isolates of *Plasmodium falciparum* in an area with multi-drug resistance, measuring the inhibition of schizont maturation. In 43 successfully tested parasite isolates, the mean effective concentrations (ECs) of tafenoquine were 209 nmol/L for the EC_{50}, and 1,414 nmol/L for the EC_{90}. Tafenoquine showed no significant activity relationships with mefloquine, artemisinin, and chloroquine. With quinine, a highly significant activity relationship was observed at the EC_{50}, but not at the EC_{90}. The EC_{50} and EC_{90} of the tafenoquine-artemisinin combination were 15.9 nmol/L and 84.3 nmol/L. The combination was synergistic. Tafenoquine appears to be a promising candidate for treating multidrug-resistant falciparum malaria, especially in combination with artemisinin derivatives.

INTRODUCTION

Over the past decades, the emergence and spread of drug-resistant strains of *Plasmodium falciparum* caused increasing difficulties in the therapy and prophylaxis of malaria. The ability of *P. falciparum* to quickly adapt to recently introduced drugs hastened the pace of the development of new antimalarial agents. In addition, there is also a growing demand for alternative therapeutic agents for *Plasmodium vivax* infections due to chloroquine resistance of asexual blood forms and low sensitivity of hypnozoites to primaquine in some areas.[1]

The 8-aminoquinoline primaquine is the only available drug for antirelapse treatment of vivax malaria. Apart from the hypnozoitocidal activity against *P. vivax*, primaquine shows good tissue schizontocidal and gametocytocidal efficacy, but insufficient blood schizontocidal activity against *P. falciparum*. The need for an increased dosage in the treatment of infection with *P. vivax* strains with primarily low primaquine sensitivity, e.g., the Chesson strain, highlights the limitations of this drug. Primaquine may cause methemoglobinemia and can lead to life-threatening hemolysis in glucose-6-phosphate dehydrogenase (G6PD)–deficient patients.[2] This problem led to a systematic exploration of the 8-aminoquinolines to identify compounds with higher blood schizontocidal and hypnozoitocidal activity, an improved safety profile, and more convenient drug regimens.

Among the 8-aminoquinolines, compound WR 238 605 (tafenoquine), a 5-phenoxy primaquine derivative, is the most advanced candidate compound. It originates from the antimalarial drug research program of the Walter Reed Army Institute for Research. The first report on the drug was in 1985. In preclinical studies, it was found to fulfill expectations since it showed high activity against hypnozoites and tissue schizonts, as well as asexual blood forms.[3,4] Its ability to interrupt the sexual phase of the parasites' life cycle is due to the inhibition of oozyst maturation and the interruption of sporozoite invasion into the salivary glands of the mosquito; however, the drug shows little direct gametocytocidal activity.[5] Like primaquine, tafenoquine is also not devoid of the risk of side effects. It seems to have the potential of forming methemoglobin and it may cause hemolysis in G6PD-deficient individuals.[4] One major difference between primaquine and the 5-phenoxy primaquine derivate tafenoquine (WR 238 605), is the considerably longer mean plasma half-life of the new drug. While primaquine has a mean half-life of approximately six hours, tafenoquine was found to have a mean ± SD half-life of 361 ± 40 hours. This fact allows for more convenient dosing, but at the same time increases the risk of promoting the development of drug resistance if used on a large-scale in areas with intensive malaria transmission.[4,6]

In several clinical studies, tafenoquine was also tested as a prophylactic and anti-relapse treatment with encouraging results.[4,7,8] So far, the potential of tafenoquine as a blood schizontocide for the treatment of multidrug-resistant forms of falciparum malaria has not been methodically explored. The results of studies in animal models and of an *in vitro* screening investigation of 13 different 8-aminoquinolines showed that tafenoquine has a blood schizontocidal activity that is within a therapeutically achievable concentration range. Furthermore, it showed no cross-relationships with classical antimalarial drugs, but a resistance-reversing effect with chloroquine.[3,9]

The aim of this study was the exploration of the potential of tafenoquine as an alternative candidate for the treatment of multidrug-resistant falciparum malaria and the assessment of baseline sensitivity data, based on the inhibition of schizont maturation.[10] Tafenoquine was also tested in a 1:1 (mol/mol) combination with artemisinin. Apart from speeding up clinical response, drug combinations may delay the emergence and spread of drug resistance.[11–13] Since tafenoquine is a slow-acting antimalarial, artemisinins may be ideal partners due to their rapid onset of action, affordability, and the absence of significant *in vivo* resistance.[14]

MATERIALS AND METHODS

The study took place in the northwestern border regions of Thailand at the Malaria Clinic of Mae Sot in close proximity to the Thailand-Myanmar border from July to August 2000. In this area, which is thought to harbor the most resistant forms of falciparum malaria in the world, *P. falciparum* shows

a high degree of resistance to chloroquine and antifolates, and a considerably reduced sensitivity to mefloquine and quinine.[11]

The study was conducted in the course of the regular monitoring of the sensitivity of *P. falciparum* in Thailand under the auspices of the Malaria Division of the Ministry of Public Health of Thailand. These activities are covered by a clearance from the Ethical Committee of the Ministry of Public Health of Thailand. Outpatients with clinically manifested falciparum malaria were invited to participate in this study and informed consent was obtained from all individuals included in the study. All participants had microscopically confirmed *P. falciparum* monoinfections. The geometric mean asexual parasite density was 29,595/μL (95% confidence interval [CI] = 22,473–38,972/μL). The patients had a (reported) minimum of four weeks without antimalarial treatment prior to inclusion. The intensive migration between the neighboring border regions is reflected by the fact that the majority (59%) of the patients had acquired their infections in Myanmar. Only seven of the 58 patients included in the study were females and the mean ± SD age was 24.1 ± 9.6 years.

The test procedure was based on the standard World Health Organization (WHO) *in vitro* micro-test technique for the assessment of the response of *P. falciparum* to antimalarial drugs.[15] This test system measures the drug-dependent inhibition of schizont maturation. Tests were conducted with tafenoquine (batch no. TFN-A-02C2, 14.04.99; SmithKlineBeecham; now GlaxoSmithKline, Worthing, West-Sussex, UK), artemisinin (Laboratory Standard; Academy of Military Science, Beijing, People's Republic of China), and a 1:1 tafenoquine-artemisinin mixture. To establish a comparable standard for further *in vitro* testing, the WHO *in vitro* microtechnique was adapted to the use of tafenoquine. In addition, parallel routine tests (WHO test kit; Regional Office for the Western Pacific, Manila, The Philippines) were conducted with chloroquine, mefloquine, and quinine.

Blood (0.3 ml) was collected from each patient in sterile, heparinized capillary tubes and added to 7.4 ml of RPMI 1640 culture medium with reduced content of p-aminobenzoic acid and folic acid (3.9% blood-medium-mixture). Fifty microliters of the blood-medium-mixture (BMM) was added to the wells of Falcon® 3070 plates Becton-Dickinson and Company, Franklin Lakes, NJ. These test plates were predosed with ascending increasing quantities of tafenoquine (0.5–500 pmol/well corresponding 10–10,000 nmol/L of BMM), artemisinin (0.15–150 pmol/well), and a 1:1 combination of both drugs (0.1–100 pmol/well). After 24 hours of incubation at 37.5°C, the cultures were harvested and a thick film prepared from each well. After thorough drying, the thick films were stained with Giemsa solution at pH 6.85. The number of schizonts (≥ 3 nuclei) per 200 asexual parasites was microscopically counted. Isolates with a schizont maturation rate of less than 10% in the control well were excluded.

Regression parameters and effective concentrations were estimated according to the classical method of Litchfield and Wilcoxon.[16,17] Activity correlations between different drugs were calculated by Pearson's parametric test or Spearman's rank test. All tests were performed at a two-sided significance level of $\alpha = 5\%$ ($P < 0.05$). Assuming a fully additive activity of tafenoquine and artemisinin the expected effective concentration (EC) values and the expected regression parameters were calculated by the following formula:

$$\text{Exp.inhib.\%} = [\text{obs.abs.Inhib.A} + (1 - \text{obs.abs.Inhib.A}) \times \text{obs.abs.Inhib.B}] \times 100$$

Exp = expected obs = observed abs = absolute
A = drug A B = drug B

The quotient of observed and expected effective concentrations at different EC levels indicates antagonism (> 2), partially additive (1 < x < 2), fully additive activity (= 1), or synergism (< 1).

RESULTS

Tafenoquine. Of a total of 58 isolates, 43 (74%) were successfully tested for their susceptibility to tafenoquine. Fifteen isolates had to be excluded due mostly to pre-incubation asexual parasite densities > 80,000/μL and/or schizont maturation < 10%. The geometric mean of the cut-off points (i.e., the lowest concentrations at which no schizont maturation was observed) was 4,360 nmol/L. All isolates except for one were fully inhibited within the test range. When the log-concentration/probit-response regression for tafenoquine was calculated, the chi-square value for heterogeneity was 8.99, which was below the limit of 11.07. This indicated an acceptable fit of the observed data points to the log-normal regression model. The mean 50% effective concentration (EC_{50}) for tafenoquine was 208.7 nmol/L (95% CI = 134.0–325.1 nmol/L). The corresponding EC_{90} and EC_{99} values were 1,413.6 nmol/L (95% CI = 766.6–2,606.5 nmol/L) and 6,722.9 nmol/L (95% CI = 2,783.6–16,237.3 nmol/L), respectively, (Table 1 and Figure 1).

Correlation analysis. Parallel *in vitro* tests were conducted with tafenoquine, quinine, mefloquine, chloroquine, and artemisinin. Individual EC_{50} and EC_{90} values were calculated for all drugs and isolates. The results of the correlation analysis (Pearson) are listed in Table 2. While a highly significant activity correlation was observed between tafenoquine and quinine at the EC_{50} level ($P = 0.0016$), this was not the case at the EC_{90}. For all other drugs (mefloquine, chloroquine, and artemisinin) the activity correlation remained well below the threshold of statistical significance. Correlation analysis was repeated using the Spearman rank correlation test, and this yielded practically the same results as those obtained with the Pearson method.

Interaction. Forty *P. falciparum* isolates were successfully tested for their response to artemisinin, tafenoquine, and their combination. The chi-square values were 1.32 for artemisinin and 0.34 for the drug combination 0.34; therefore, these values were well within permissible limits.

The geometric mean cut-off concentration for the 1:1 taf-

TABLE 1

Effective concentrations (ECs) and 95% confidence intervals (CIs) (nmol/L) of tafenoquine (n = 43) and artemisinin (n = 40) for *Plasmodium falciparum*

	Tafenoquine		Artemisinin	
EC	Mean	95% CI	Mean	95% CI
EC_1	6.5	2.7–15.6	0.3	0.1–0.7
EC_{50}	208.7	134.0–325.1	12.1	8.2–17.9
EC_{90}	1,413.6	766.6–2,606.5	88.5	51.1–153.1
EC_{95}	2,431.3	1,213.4–4871.5	155.4	83.1–290.6
EC_{99}	6,722.9	2,783.6–16,237.3	447.2	201.3–993.7

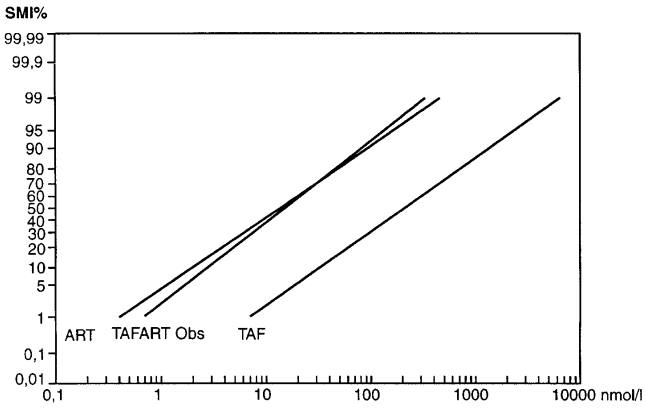

FIGURE 1. Log concentration-probit regression lines for the inhibition of *Plasmodium falciparum* by tafenoquine (TAF), artemisinin (ART), and a 1:1 (mol/mol) combination of tafenoquine and artemisinin (TAFART). SMI = inhibition of schizont maturation; Obs. = observed.

enoquine-artemisinin drug mixture was 119.3 nmol/L. Schizont maturation was fully inhibited at a concentration of 600 nmol/L in all isolates. An EC_{50} of 15.3 nmol/L (95% CI = 10.2–22.9 nmol/L), an EC_{90} of 81.6 nmol/l, and an EC_{99} of 319.4 nmol/l were observed (additional ECs and the corresponding CIs are shown in Table 3).

For artemisinin, an EC_{50} of 12.1 nmol/L (95% CI = 8.2–17.9 nmol/L) was found and the corresponding EC_{90} and EC_{99} values were 88.5 nmol/L and 447.2 nmol/L, respectively (Table 1 and Figure 1). The geometric mean cut-off concentration was 166.0 nmol/L.

The EC values for tafenoquine with the 40 samples tested in parallel with the drug combination (EC_{50} = 209.7 nmol/L, EC_{90} = 1,416.0 nmol/L, EC_{99} = 6,717.2 nmol/l) and the geometric mean cut-off concentration (4,392.4 nmol/L) were very similar to those observed in the larger sample of 43 isolates. Also, the regression lines showed close resemblance as evident from a slope ratio of 1.0024.

Assuming fully additive activity, the expected effective concentrations of the combination were calculated on the basis of the response to the single compounds. The expected regression parameters are shown in Table 2. Throughout the range of EC_1 and EC_{99}, the observed EC values were lower than those expected. The interaction-quotient (observed/expected) was therefore less than 1, indicating synergism between tafenoquine and artemisinin (Table 2 and Figure 2). Synergism became stronger with increasing ECs. The observed regression line of the combination (slope [S] = 3.66) was steeper when compared with that of artemisinin (S = 4.68) or tafenoquine (S = 4.40).

To identify the dominating compound in the 1:1 tafenoquine-artemisinin drug combination, the EC_{50} and EC_{90} values for tafenoquine, artemisinin, and their mixture were calculated for the individual parasite isolates. On this basis, the correlations of artemisinin and tafenoquine with the drug mixture were calculated. Artemisinin and the drug mixture showed correlation coefficients at the EC_{50} and EC_{90} levels of 0.69 and 0.75, respectively, indicating highly significant correlations ($P < 0.001$). The correlation coefficients of tafenoquine and the drug mixture were 0.40 ($P = 0.01$) at the EC_{50}, and 0.16 ($P > 0.05$) at the EC_{90}.

DISCUSSION

The *in vitro* microtest system permits a quantitative assessment of the sensitivity of asexual blood forms of *P. falciparum* to tafenoquine. The overall success rate of 74% (43 of 58 isolates) is within an acceptable range. Most (15) of the excluded isolates had pre-incubation parasite densities greater than 80 000/μL, thus disqualifying them from inclusion since parasitemia above this threshold leads to an early exhaustion of the buffering capacity of the culture medium.[15] This usually results in poor schizont maturation, i.e., schizont counts < 10%. It is difficult to avoid this constraint since the degree of parasitemia often changes during the interval between primary blood examination and sampling for the test, and precise pre-incubation counts become available only after having set up the test.

In this study, tafenoquine was found to possess marked blood schizontocidal activity in *P. falciparum* in an area with a high percentage of multidrug-resistant parasite populations. The observed EC values were in the same range as those found with quinine, while artemisinin showed 6–10 times lower values for the EC_{50} and EC_{90}. Related to blood-medium-mixture, tafenoquine EC values were 2.4 (EC_{50}) to 5 (EC_{90}) times higher than those of chloroquine and 10 (EC_{50}) to 20 (EC_{90}) times higher than the ECs for mefloquine.

There are no comparable data available from *in vitro* tests measuring the inhibition of schizont maturation. Therefore, the comparison is essentially limited to a study with the hypoxanthine uptake method in seven culture-adapted *P. falciparum* clones and isolates.[9] In the cited study, the EC_{50} values varied between 59.4 and 1470 nmol/L, with a mean of 436 nmol/L (i.e., a range similar to that observed in our study). Tafenoquine showed a two times higher EC_{50} value compared with chloroquine and an approximately 20 times higher value than mefloquine. In contrast, in a *P. cynomolgi/Macaca mulatta* model, tafenoquine showed a three-fold higher blood schizontocidal activity than chloroquine, halofantrine, and mefloquine (S.K. Puri et al., unpublished data). If one takes the different methods into consideration, the EC values of tafenoquine seem to lie in about the same range in these studies, but the activity relationships to chloroquine and mefloquine differ considerably, essentially reflecting the intrinsic sensitivity pattern of the parasite species and strains. As far as the studies with *P. falciparum* are concerned, the differences may be mainly attributed to the selection of *P. falciparum* strains/clones and their relatively small number, and to a lesser extent to methodologic features.

The comparison of tafenoquine with the structurally related drug primaquine may be of particular interest. Published *in vitro* data for the blood schizontocidal activity of primaquine in *P. falciparum* show a range of EC_{50} values between 0.6 and 14 μmol/L.[18–20] Apart from methodologic differences, our results for tafenoquine therefore showed an activity 3–67 times higher than that of primaquine. Other studies, which describe a 4–100 times higher blood schizontocidal activity of tafenoquine compared with primaquine in the *P. berghei* and *P. yoelii* mouse model and a 10 times higher activity in the *P. vivax/Aotus trivirgatus* model, tend to support these findings.[3,21] In this context, it is important to note that primaquine as well as tafenoquine showed lower EC values in chloroquine-resistant strains than in chloroquine-sensitive strains.[3,18] A regimen consisting of simultaneous

TABLE 2

Analysis for activity correlation (Pearson) between tafenoquine and various antimalarial compounds at a 50% effective concentration (EC_{50}) and EC_{90} in fresh isolates of *Plasmodium falciparum*

Drug compared	No. of isolates	at EC_{50}		at EC_{90}	
		r	*P*	r	*P*
Quinine	41	0.4773	0.0016*	0.2146	0.1684
Mefloquine	32	–0.1001	0.3309	0.0519	0.3291
Chloroquine	40	0.1726	0.2481	–0.2596	0.1048
Artemisinin	41	0.1904	0.2118	–0.0379	0.4114

* Significant activity correlation ($P < 0.05$).

chloroquine-primaquine administration might therefore improve the treatment of chloroquine-resistant *P. vivax* infections.

Analogous to previous studies, no activity associations were found between tafenoquine on the one hand and chloroquine and mefloquine on the other.[9] Although in our study artemisinin showed no correlation with tafenoquine, a significant activity relation with quinine was observed at the EC_{50} level. Since correlation analysis provides an insight into the mode of action and cross-sensitivities between different drugs, these results may be seen as an indication for the relative independence of tafenoquine from the sensitivity of *P. falciparum* to currently used antimalarials except for quinine.

So far little is known about the blood schizontocidal mode of action of 8-aminoquinolines. The antiplasmodial activity of primaquine has been ascribed to the competition with ubiquinone, a substance involved in the mitochondrial electron transport chain.[4] In *Pneumocystis carinii,* tafenoquine caused ultrastructural membrane changes and damage, suggesting that oxidative impact may be a major mechanism of action.[22] Further *in vitro* studies in *P. falciparum* showed that tafenoquine inhibits dihydroorotate dehydrogenase to a lesser extent than hypoxanthine uptake, indicating that parasite viability is depressed by a mode of action other than the inhibition of the mitochondrial electron transport system.[23] From this point of view, the observed correlation to quinine may give an interesting perspective. Quinine is a class 2 blood schizontocide belonging to the 4-quinolinemethanols, whose major target is known to be the iron metabolism of the parasite. The significant correlation between the activities of quinine and tafenoquine suggests the possibility that both compounds may share this mechanism at least in part, a hypothesis requiring further research.

TABLE 3

Expected (Exp) and observed (Obs) effective concentration (EC) values and 95% confidence intervals (CIs) of the tafenoquine-artemisinin combination (TAFART) and the mathematically derived interaction coefficient (Obs/Exp) based on parallel observations in 40 *Plasmodium falciparum* isolates

	TAFART observed	95% CI	TAFART expected	Obs/Exp
EC_1	0.73	0.32–1.66	0.80	0.91
EC_{16}	4.18	2.80–6.25	5.33	0.78
EC_{50}	15.31	10.24–22.90	21.85	0.70
EC_{85}	56.12	37.53–83.90	89.62	0.63
EC_{90}	81.64	46.49–143.35	134.70	0.61
EC_{95}	131.20	69.06–249.23	225.58	0.58
EC_{99}	319.42	140.99–723.67	593.35	0.54

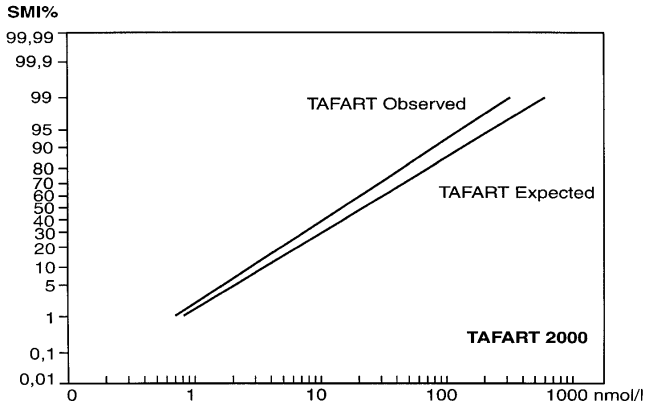

FIGURE 2. Observed and expected log-probit regressions for the tafenoquine-artemisinin combination in *Plasmodium falciparum*. SMI = inhibition of schizont maturation; TAFART = combination of tafenoquine and artemisinin.

Since treatment with drug combinations is discussed as a means of slowing down the emergence and spread of resistance in falciparum malaria and of accelerating the onset of therapeutic response, artemisinin was tested as a potential partner drug for tafenoquine. The rapid onset of action, the absence of *in vivo* resistance, the good tolerability, and the affordable cost may make artemisinin derivates very interesting candidates for such combinations. In this context, Peters cited the potentiating effect of an artemisinin-primaquine combination in artemisinin-resistant strains,[12] while in another study no interaction between tafenoquin and artemisinin was observed.[3] In the course of this study the 1:1 combination was found to have more than additive activity. Therefore, lower drug concentrations were required to inhibit schizont maturation. Artemisinin was apparently the principal factor in this combination, as suggested by the highly significant correlation of the combination with artemisinin, while the correlation with tafenoquine was less strong (EC_{50}) or below the threshold of statistical significance (EC_{90}). The EC_{99} (usually considered the minimum inhibitory concentration) of the combination represents only approximately 1/40 of the tafenoquine concentration required when tafenoquine is used alone. This is fully within a pharmacokinetically feasible range. Although artemisinin appears to be the lead factor in the combination, the EC_{99} of the combination represents only approximately one-third of the artemisinin concentration as compared to artemisinin alone. Thus, the synergism is obviously not due to a mere enhancement of the effect of tafenoquine by artemisinin.

In conclusion, the results of this study suggest an interesting potential of the 8-aminoquinoline tafenoquine as a blood schizontocidal agent in the treatment of multidrug-resistant falciparum malaria. A combination with artemisinin derivatives seems to be especially promising. In addition to the enhanced blood schizontocidal activity, such combination treatment may have an impact on malaria transmission and the occurrence and spread of resistance due to the ability of the single compounds to inhibit the sporogony of *P. falciparum*.[5,11,24] However, the potential side effects of tafenoquine, such as the production of methemoglobin and the risk of hemolysis in G6PD-deficient patients, have to be taken into consideration. This problem should be resolved by the

development of a simple, cheap, fast and reliable screening test for G6PD deficiency.

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