

In vitro susceptibility of *Plasmodium falciparum* isolates to halofantrine in the Central Province of Papua New Guinea

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SUMMARY

Halofantrine is a newer antimalarial drug which has not been approved for clinical use in Papua New Guinea. We assessed 21 Central Province isolates of *Plasmodium falciparum* for their in vitro susceptibility to halofantrine. The concentration required to inhibit 50% of parasite growth (IC₅₀) ranged from 0.05 to 7.0 nM with a mean of 1.90 nM and a median of 1.50 nM. The minimum inhibitory concentration (MIC) values ranged from 2.5 to 50 nM with a median of 5.0 nM. All but one isolate had an MIC of 10 nM or less. These results indicate that halofantrine would be a suitable alternative for the treatment of *P. falciparum* malaria in the region in the future, if and when the need arises, provided that its use was carefully monitored.

Introduction

Malaria remains one of the major diseases in Papua New Guinea (PNG) and chemotherapy is being increasingly employed to control the morbidity and mortality caused by it. However, with a rapid spread of drug-resistant falciparum malaria throughout the world, including PNG (1,2), the number of drugs available to treat malaria effectively has become scarce. The recently developed 9-phenanthrenemethanol derivative, halofantrine (Hf), has been shown to be active in vitro (3,4) as well as in vivo (5-7) against chloroquine-resistant and multidrug-resistant strains of *Plasmodium falciparum*. The drug has been available in some parts of the world since 1988 (8). Though available, halofantrine has not been approved for malaria treatment in PNG and its use has been restricted to clinical trials (9).

There are some major setbacks with halofantrine, however. These include treatment failures despite adequate therapeutic plasma concentration (10), reports of reduced susceptibility of *P. falciparum* to halofantrine

(11), cardiac toxicity (12), and an apparent cross-resistance between halofantrine and mefloquine in vitro (13). The cross-resistance between halofantrine and mefloquine would have been predicted anyway, based on their structural similarity. The reports of treatment failures and of reduced susceptibility of *P. falciparum* strains and isolates are of serious concern because these events occurred in areas where halofantrine had never been used clinically. These observations may well suggest innate resistance or increased tolerance to halofantrine of certain wild strains of *P. falciparum*. Further studies are needed before a role can be established for halofantrine in the treatment of drug-resistant malaria in PNG.

In vitro susceptibility studies of halofantrine have been conducted in many parts of the world, but nothing is known about its in vitro antimalarial activity against local isolates of *P. falciparum* in PNG. As part of an ongoing assessment of halofantrine in PNG, we investigated the in vitro susceptibility to halofantrine of local *P. falciparum* isolates collected from the southern region of Central

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Province, PNG. We report here our findings in the context of other similar studies elsewhere.

Materials and Methods

Plasmodium falciparum isolates

Isolates were obtained from malaria patients with *Plasmodium falciparum* mono-infection attending the Outpatient Clinic, Port Moresby General Hospital (PMGH); the hospital is located in the National Capital District within the region of Central Province, Papua New Guinea. Venous blood (5ml) was obtained from all eligible patients at presentation who had a parasitaemia greater than 500 parasites per μ l of blood. Blood was taken before antimalarial therapy was given. Subjects who had taken antimalarial drugs within 3 weeks of presentation were excluded.

In vitro microtest technique

The drug susceptibility of *P. falciparum* was determined using the World Health Organization (WHO) standardized in vitro microtest system (14) developed by Rieckmann et al. in 1978 (15) adapting the methodology used for the cultivation of *P. falciparum* (16). The technique involves quantitating schizont maturation following cultivation of infected erythrocytes in plates charged with defined quantities of drug.

Preparation of isolates for the in vitro test

Infected erythrocytes collected from the malaria patients were washed three times with culture medium (RPMI 1640, 25 nM NaHCO₃, 25 nM HEPES plus nonimmune human AB serum) before assaying. Samples with high parasitaemia were diluted with fresh washed human type O-positive red blood cells to reach a parasitaemia of 1%. All chemicals for parasite culture, including human nonimmune sera, were commercially obtained from Sigma (USA) except for RPMI 1640 (Gibco).

Preparation of halofantrine drug plates

Halofantrine hydrochloride (racemate) was obtained from Smith Kline & Beecham (Welwyn, UK). A stock solution (10⁻² M) of halofantrine was prepared by dissolving 53.7 mg (molecular weight = 537) in 10 ml of 70% alcohol in water as described previously (17).

The stock solution was diluted 1000 times with culture medium to give a working concentration of 10⁻⁵ M from which various test concentrations (0.1, 1.0, 2.5, 5, 10, 50 and 100 nM) were made by serial dilution with culture medium. These test concentrations were made to determine the lowest and highest concentrations required to assess baseline responses of the local parasite isolates. The results of the study will help to determine the optimal range for in vitro studies in the future.

An aliquot (100 μ l) of different drug concentrations was dispensed into the wells of a 96-well sterile microtitre plate, one concentration per row of 12 wells. The first row was the positive control, containing culture medium only, 100 μ l per well.

In vitro microtest

An aliquot (10 μ l) of washed parasitized erythrocytes was added to the drug solutions in the wells. Each isolate was tested in two wells at each drug concentration, ie, the study was done in duplicate. The plates were placed in a candle jar (desiccator) and when the candle was almost extinguished the jar was sealed and placed in an incubator at 38°C for 24-36 hours. The schizont maturation was assessed microscopically. A test was considered assessable if there were 20% or more schizonts in control wells. Depending on the maturation stages, the supernatant in each well was removed and thin film smears were prepared for each well. The smears were left to dry, fixed in methanol and stained with 20% buffered (pH 7.2) Giemsa stain for 10 to 15 minutes. The schizonts with two or more nuclei were counted against 100 asexual parasites.

Calculation of MIC and IC₅₀

The IC₅₀ was calculated from the simple graph of the percentage schizont maturation against the logarithm of the drug concentration. It was defined as the concentration required to inhibit 50% of parasite growth to schizont development. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which growth of at least 95% of the parasites to schizont development was inhibited.

Results

21 isolates were tested for susceptibility to halofantrine. The results of the in vitro test are presented in Table 1. The mean halofantrine IC₅₀ value of the isolates was 1.90 nM with a standard deviation of 1.93, a standard error of 0.42 and a 95% confidence interval of 1.02-2.78 nM. The median IC₅₀ value was 1.50 nM. The MIC values ranged from 2.5 to 50 nM with a median of 5.0 nM. With the exception of one isolate, all isolates had a halofantrine MIC of 10 nM (6/21) or less (14/21).

The lowest and highest IC₅₀ were 0.05 nM and 7.0 nM, respectively. Although these two extreme values revealed a wide difference in the range of IC₅₀ values for halofantrine, this drug was active at IC₅₀ values < 10 nM against all the local isolates. There is no consensus on the threshold concentration of in vitro resistance for halofantrine so we were unable to define any reference point for clinical resistance in this study.

Discussion

Although multidrug-resistant falciparum malaria is not a serious problem in PNG, chloroquine-resistant strains of *P. falciparum* are widespread. Quinine remains the drug of choice for severe and complicated malaria, including chloroquine-resistant malaria, and its clinical efficacy remains unquestionable. However, recent in vivo reports of relative quinine resistance in the northern part of the country indicate that quinine can no longer be relied on to give a full parasitocidal effect (18). Mefloquine, though available, is seldom prescribed and halofantrine, a newer antimalarial drug, is awaiting approval for clinical use. We took this opportunity to assess and document the in vitro susceptibility pattern of *P. falciparum* isolates to halofantrine before it is approved for wider clinical use for malaria treatment. In 21 isolates tested the in vitro drug susceptibility profile for halofantrine showed fairly low IC₅₀ values, ranging from 0.05 to 7.0 nM (Table 1) with a mean IC₅₀ value of 1.90 nM. There were two isolates with IC₅₀ of 7.0 nM lying outside the 95% confidence interval of 1.02-2.78 nM and those in the range 0.05 to 3.5 had an arithmetic mean of 1.41±0.97 nM

(median = 1.50), which better reflects the mean IC₅₀ of isolates judged to be halofantrine susceptible. These low IC₅₀ values were expected, since halofantrine has never been used in PNG for malaria treatment but the number of isolates tested in our study was small, and we may not have detected any inherently less susceptible isolates existing in the study area. Therefore further assessment with a larger sample size is required for a more informative interpretation.

The reported in vitro susceptibility of *P. falciparum* isolates to halofantrine varies widely between regions. The Central Province *P. falciparum* isolates are comparable to those from Thailand in their susceptibility to halofantrine but are less susceptible to the drug than the Philippine isolates. The mean IC₅₀ value obtained in the present study was 1.90 nM compared to the value of 1.23 nM from six southern Thailand isolates (19) while that of isolates from four regions in northeastern Thailand ranged from 1.26 to 1.46 nM (0.63-0.73 ng/ml) (20). In contrast, the Philippine *P. falciparum* isolates, as determined by Bustos et al. (21), were more susceptible to halofantrine with a median IC₅₀ value of 0.50 nM, compared to ours of 1.50 nM. However, several investigators have reported higher mean IC₅₀ values than ours from other malaria-endemic areas. Lambros et al. reported a mean IC₅₀ value of 3.06 nM (1.53 ng/ml) in 70 Malaysian falciparum isolates (22), while Oduola et al. (23) and Freese et al. (3) reported mean IC₅₀ values of 5.93 nM from West African and 4.62 nM from southern African isolates respectively. Ringwald et al. investigated *P. falciparum* isolates obtained from thirteen different countries in the African continent (11). The mean IC₅₀ values in these countries ranged from 4.0 to 10.0 nM. Recently, Brasseur et al. reported mean IC₅₀ values of 12.2 nM from 11 Congo isolates and 4.3 nM from 10 Cameroon isolates (24). A high mean IC₅₀ value for Congo was not surprising because halofantrine represented 25% of all antimalarial drugs sold there in 1991-1992 while Cameroon had not received government approval for halofantrine use. Three years earlier, the reported IC₅₀ value for 8 Congo isolates had been 4.0 nM (11), so that within a span of a few years the IC₅₀ value had increased by 3-fold. Thus there is a high frequency and degree of resistance to halofantrine in the Congo as a consequence of halofantrine drug

TABLE 1IN VITRO SUSCEPTIBILITY OF CENTRAL PROVINCE *P. FALCIPARUM* ISOLATES TO HALOFANTRINE

Isolates	Halofantrine	
	IC ₅₀ * (nM)	MIC** (nM)
CP1	7.00	50.0
CP2	2.00	5.0
CP3	2.50	5.0
CP4	1.50	2.5
CP5	2.00	5.0
CP6	1.50	2.5
CP7	3.50	5.0
CP8	0.25	5.0
CP9	0.26	2.5
CP10	0.36	5.0
CP11	2.00	10.0
CP12	0.50	10.0
CP13	0.05	2.5
CP14	7.00	10.0
CP15	2.00	10.0
CP16	1.50	5.0
CP17	2.40	5.0
CP18	1.80	10.0
CP19	0.65	2.5
CP20	0.28	5.0
CP21	0.80	10.0

*IC₅₀=drug concentration at which 50% of the growing asexual parasites (trophozoites) were inhibited from maturation (development into schizonts)

**MIC=the minimum (lowest) drug concentration at which growth of 95% of the asexual parasites (development from trophozoites to schizonts) was inhibited

pressure over three years. Where similar drug pressures were absent there appears to be a low frequency of drug resistance, indicated by high in vitro susceptibility to halofantrine. It seems that halofantrine resistance can be easily induced with uncontrolled and widespread use of the drug.

Although it is not possible to make direct comparisons between in vitro tests carried out by different laboratories, in vitro results alone may be sensitive enough to detect small

differences in drug susceptibility patterns among different parasite isolates, as is shown in Table 2 for some *P. falciparum* isolates from the Asia-Pacific region. Overall, the results of the various studies have given an indication of relative susceptibility to halofantrine of *P. falciparum* isolates from different geographical areas within the Asia-Pacific region.

The MIC profile of halofantrine in our study also showed fairly low values. 95% (20/21) of the isolates in our study had an MIC

of 10.0 nM or less and only one isolate had an MIC of 50.0 nM, being the second highest concentration tested. The MIC results of this study compare very well with those of an investigation carried out in six Thai isolates (19) which ranged from 1.94 to 7.79 nM. Some African studies (3,5) revealed highly variable and wide-ranging MIC values, some of which are much higher than the MIC values obtained in this study. The MIC may be an accurate parameter for assessing the susceptibility of malaria parasites to drugs. Nevertheless, though the in vitro microtest technique is suitable for measuring parasite susceptibility to halofantrine, more data are required to establish an MIC predictive of in vivo resistance.

The relationship between the in vitro IC₅₀ and the in vivo response to halofantrine has yet to be determined and the threshold of halofantrine resistance is still not defined. Few cases of R1 and R2/R3 resistance to halofantrine treatment have been reported (7,9,24). The in vitro tests on some of these isolates revealed a wide range of IC₅₀ values from 7.0 nM with R1 to 26 nM or higher with R2/R3 resistance. 2 of the 21 Central Province isolates tested had an IC₅₀ of 7.0 nM and the practical implication of such an observation to the proposed use of halofantrine in PNG is not yet clear. One of these two isolates had an MIC of 50 nM, and it appears that this isolate is inherently less susceptible to halofantrine than the others. It may mean that halofantrine

TABLE 2

MEAN IC₅₀ VALUES OF SOME *P. FALCIPARUM* ISOLATES FROM THE ASIA-PACIFIC REGION

Country	Halofantrine IC ₅₀ *	No of isolates	Source
Thailand			Ref. 20
Chantaburi	1.34 nM (0.67 ng/ml)	15	
Phrabuddabat	1.26 nM (0.63 ng/ml)	8	
Borai	1.46 nM (0.73 ng/ml)	10	
Yala	1.23 nM	6	Ref. 19
Malaysia			Ref. 22
Pahange	4.05 nM (2.03 ng/ml)	27	
Perak	2.74 nM (1.37ng/ml)	37	
Selangor	4.03 nM (2.02 ng/ml)	6	
Philippines	0.50 nM (median IC ₅₀)	37	Ref. 21
Papua New Guinea			
Central Province	1.90 nM (median 1.50 nM)	19	Present study

*Mean of the 50% inhibitory concentrations expressed as nanomolar (nM) or, in parentheses, ng/ml equivalent; for the Philippines the value given is the median, and both mean and median are stated for the present study

should be introduced cautiously into the region. However, further *in vitro* and *in vivo* testing of halofantrine in PNG is required to confirm this observation. The majority of the isolates, however, exhibited high *in vitro* susceptibility to halofantrine, and if corroborated *in vivo* this means that halofantrine may be of value in controlling *P. falciparum* malaria in PNG, particularly against chloroquine-resistant malaria in the future.

The *in vitro* assessment provides baseline data on the susceptibility of Central Province *P. falciparum* isolates to halofantrine. The results of our study and those of others, taken collectively, indicate some variability in susceptibility of *P. falciparum* parasites to halofantrine. Some of these variations may be due to local variation in susceptibility of different strains existing in different regions, reflecting biological diversity of malarial parasites as a result of evolutionary selection. The proportion of naturally less susceptible parasites in an inoculum may be an important determinant of the parasites' response to drugs and the size of the proportion may depend on certain attributes such as drug pressure and the intensity of malaria transmission in the locality. Drug pressure imposed by the test drug (or by a structurally similar analogue) on the *P. falciparum* parasites will lead to the selection of resistant strains and may increase the likelihood of significant drug resistance (24). Hypothetically, this phenomenon would be aided by a high level of malaria transmission. In such a locality high-level resistance would be the result of continuous and intensive serial passage of *P. falciparum* in nonimmune or immune subjects, with large numbers of parasites being exposed to a high level of drug pressure at each passage in the local community.

The *in vitro* techniques used for assessing and reporting susceptibility data may also contribute to the difference in susceptibility patterns of *P. falciparum* to halofantrine between regions. Currently, two techniques are employed, the *in vitro* microtest (15) and a radioisotope technique (20). The fact that we employed the former technique does not prejudice our results but the precision and sensitivity of the two methods may differ, and

comparisons have to be made with caution. In addition, the method of calculating the mean IC_{50} value differs from one study to another: some studies express the mean IC_{50} value as an arithmetic mean while others use a geometric mean. This is another attribute that requires attention in the interpretation and comparison of different IC_{50} values from various studies around the world. Therefore, variations between *in vitro* drug susceptibility studies are seemingly the result of both technical and biological factors, and these should be taken into account in comparative analysis of drug susceptibility data from different geographical regions.

We hope these data will be useful in decision-making if the standard treatments have to be modified in the event of high chloroquine treatment failure. The observations in this study should also be regarded as the beginning of a data base for epidemiological comparison in succeeding years, as halofantrine may be expected to receive widespread use in PNG in the future.

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