

## *Activity correlation between artemisinin and dihydro-artemisinin in fresh isolates of Plasmodium falciparum from Thailand*

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### **Introduction**

Artemisinin is a sesquiterpene lactone that occurs naturally in the wormweed (*Artemisia annua* L.), an annual plant of wide distribution in the temperate zone between the Balcans and China. Artemisinin was first extracted from the plant, structurally identified and described by Chinese scientists in the early 1970s (1). The compound has strong antimalarial activity directed against asexual and sexual blood forms of all *Plasmodium* species so far investigated (9). Its blood schizontocidal activity is very rapid, accounting for short fever and parasite clearance times, albeit not exhaustive at the recommended dose regimens and thus fraught with the problem of recrudescence (6). The main indication for the use of artemisinin and its derivatives is the treatment of falciparum malaria, especially in areas with resistance to the common blood schizontocidal compounds.

Following oral administration, the relative bioavailability of artemisinin appears to be low. Earlier, this was ascribed to rapid first-pass metabolism. However, more recent evidence points to poor absorption from the gastro-intestinal tract (8). Since artemisinin shows very low solubility with injectable vehicles, precluding its formulation for parenteral administration, derivatives with better solubility characteristics were obtained by semi-synthesis, i. e. by chemical modification starting from the natural product. Artemether, the oil-soluble methyl ether of artemisinin, and Na-artesunate, the water soluble sodium hemisuccinate ester of artemisinin, were the first derivatives developed for therapeutic use.

Common to artemisinin, artemether and Na-artesunate is their metabolic conversion to dihydro-artemisinin, a compound with an extremely short half-life (4). The structural modification is limited to the terminal oxygen of the artemisinin molecule. Dihydro-artemisinin is the most active of the artemisinin analogues. The metabolic conversion to dihydro-artemisinin is slowest with the parent compound since it involves reduction. It is faster with artemether, involving a common demethylation reaction. With Na-artesunate it is almost instantaneous as it involves only straightforward hydrolysis.

Following the discovery that artemether and Na-artesunate are highly effective also after oral administration (3), the use of artemisinin has declined, the more so as the required dose levels with the derivatives are considerably lower than those of artemisinin. Nevertheless, there may still be an important place for artemisinin as it has a longer half-life as compared to artemether and Na-artesunate, and thus the capability of a protracted release of the more active dihydro-artemisinin.

Due to the substantial rate of recrudescences after monotherapy it is quite difficult to assess the sensitivity of *Plasmodium falciparum* to artemisinin and its derivatives by an in vivo test. In vitro tests are therefore an important tool for monitoring the sensitivity to this group of drugs (14). However, the development of such tests is difficult due to the notorious instability of Na-artesunate and dihydro-artemisinin, and the ability of artemether to bond with the microplate material, leading to inactivation. As the clinical-parasitological activity of Na-artesunate and artemether relies almost entirely on that of dihydro-artemisinin, it was therefore of interest to compare the activity of dihydro-artemisinin with that of the parent compound and to correlate the specific activities of both. These aspects were addressed in the study described below. It was done with the primary objective of determining the feasibility of using the activity of artemisinin as a reliable quantitative indicator of the activity of dihydro-artemisinin. This has important practical implications for field work since predosed artemisinin plates have a shelf-life (under refrigeration at 4-6°C) of 6 months as compared to only 5 weeks with dihydro-artemisinin.

## Material and Methods

The studies were carried out in May/June 1995 and 1997 at the malaria clinic of Mae Hong Son, in northwestern Thailand, near the border to Myanmar. The malaria clinic receives patients from Mae Hong Son Province and, when the border is open, also from neighbouring Myanmar. Malaria incidence is low within Mae Hong Son Province. It is much higher in the neighbouring Shan State of Myanmar. Artemisinin and its derivatives are not available in Thailand, while they can be easily obtained in Myanmar, where their sale is not limited to pharmacies.

The studies were part of the Thai programme for the monitoring of the drug sensitivity of *P. falciparum*. They were conducted in cooperation between the Ministry of Public Health of Thailand and the Institute of Specific Prophylaxis and Tropical Medicine, University of Vienna, Austria.

**Patients** The parasite isolates were obtained from symptomatic patients with *P. falciparum* mono-infections who had come to the malaria clinic for diagnosis and treatment. Treatment within the preceding 9 weeks for mefloquine or 4 weeks for other antimalarials and an asexual parasite density outside the range of 1000-80000/μl blood were exclusion criteria. Blood samples for the in vitro test and the precise determination of parasite density were taken after having confirmed mono-infection and absence of exclusion criteria.

**In vitro Test** The sensitivity of the *P. falciparum* isolates to artemisinin and dihydro-artemisinin has been tested in accordance with the WHO standard method for the determination of the inhibition of schizont maturation (10). The pre-dosed test plates were prepared at the Institute of Specific Prophylaxis and Tropical Medicine, University of Vienna. Medium RPMI-1640 LPLF, heparinized micropipettes and most of the other disposable material have been obtained through the WHO Regional Office for the Western Pacific, Manila, Philippines. In view of the short shelf-life of the dihydro-artemisinin plates, the parallel tests were completed within 4 weeks from the production of the plates.

The artemisinin and dihydro-artemisinin doses on the test plates (FALCON 3070, Becton Dickinson) and the corresponding concentrations in blood-medium mixture (BMM) are listed in Table 1.

After finger puncture 100 μl blood were obtained by sterile heparinized micropipette and immediately diluted with 900 μl RPMI-1640 LPLF medium. Aliquots of 50 μl of the homogeneous blood-medium-mixture (BMM) were then added to all scheduled wells of the test plates. The plates were then closed with sterile covers (FALCON 3071). After gentle agitation, the plates were placed in a pre-warmed candle jar and incubated for 24 hours at 37.5°C. After incubation the plates were remo-

Table 1:

Doses of artemisinin (ART) and dihydro-artemisinin (DHA) on the micro-test plates, and corresponding concentrations in blood-medium mixture (BMM).

Well	ART dose pmol	ART conc. nM	DHA dose pmol	DHA conc. nM
A	Control	Control	Control	Control
B	0.15	3	0.05	1
C	0.50	10	0.15	3
D	1.50	30	0.50	10
E	5.00	100	1.50	30
F	15.00	300	5.00	100
G	50.00	1000	15.00	300
H	150.00	3000	50.00	1000

Table 2:

Main activity parameters of artemisinin (ART) and dihydro-artemisinin (DHA) derived from paired tests in fresh isolates of *Plasmodium falciparum* from Mae Hong Son, Thailand, 1995 and 1997.

PARAMETER	DHA 1995	DHA 1997	ART 1995	ART 1997
% SMI* at				
1 nM	—	40.04	—	—
3 nM	45.42	83.78	13.92	6.37
10 nM	88.45	99.59	22.79	41.45
30 nM	96.36	100.00	43.77	81.85
100 nM	99.66	100.00	80.80	95.83
300 nM	100.00	100.00	94.45	99.82
1000 nM	100.00	-	96.30	99.97
3000 nM	100.00	-	98.48	100.00
No. of isolates	22	23	22	23
r (for regression)	0.9855	0.9896	0.9816	0.9929
$\chi^2$ for heterogeneity	0.5773	0.6646	2.3610	0.5804
S (slope)	3.2735	2.6818	6.3319	2.9839
EC-50 nM	3.1000	1.1100	29.0700	12.9200
EC-90 nM	14.3000	3.9600	313.5800	52.8500
GMCOP** nM	60.8200	8.4700	304.3400	125.2400

\* SMI = Inhibition of schizont maturation  
\*\* GMCOP = Geometric mean cut-off point of schizont maturation

ved from the candle jar, and the supernatants of the wells decanted. Thick films were made of the well sediments, placing all 8 samples of the test line on one and the same slide. After thorough drying, overnight, the slides were stained according to GIEMSA, at pH 6.8 - 6.9.

Schizont counts were made with the sediments of all wells, counting the number of schizonts per 200 asexual forms (all stages). According to convention, tests with a minimum of 10 % schizonts in well A, the drug-free control well, were considered successful (12).

The pre-incubation (asexual) parasite density has been assessed by parallel counts of asexual parasites and white blood cells, according to the WHO standard method (13).

#### Evaluation of the Sensitivity Tests

The drug-dependent inhibition of schizont maturation by artemisinin and dihydro-artemisinin shows a log-normal pattern. The test results were therefore evaluated according to the method of LITCHFIELD and WILCOXON (5). In this procedure the drug concentrations are transformed to logarithms, and the inhibitions (%) into probits. This results in linear regressions which can be mathematically determined according to the rule of the least squares. The calculation of regression parameters and effective concentrations (EC) for individual and grouped isolates was facilitated by the use of computer adaptations of the basic method (2, 11). Correlation analysis of paired data followed the conventional mathematical methods (7).

#### Results

A total of 22 isolates were successfully tested in parallel, with artemisinin and dihydro-artemisinin in 1995. In 1997 the number of isolates was 23. The main response parameters for the two drugs are summarized in Table 2. Figures 1 and 2 show the comparisons between the response of *P. falciparum* to artemisinin and dihydro-artemisinin for 1995 and 1997, respectively. The regressions indicate a good fit of the data points to the regression lines, and relatively narrow confidence intervals. It is evident that the sensitivity to artemisinin and dihydro-artemisinin was higher in 1997 as compared to 1995, a point to be discussed later.

In 1995 the EC-50 for artemisinin was 29.07 nM as against 12.92 nM in 1997. The difference was more marked at the EC-90, with 313.58 vs 52.85 nM, largely due to the larger slope (S) in 1995. Similarly, the EC-50 for dihydro-artemisinin showed significant differences with 3.10 nM for 1995 and 1.11 nM for 1997 (with  $PR = 2.7946 > f_{PR} = 2.1274$ ). At the EC-90 the difference was slightly more marked, with 14.30 nM for 1995 and 3.96 nM for 1997 (with  $PR = 3.6132 > f_{PR} = 2.8561$ ). The geometric mean cut-off points of schizont maturation showed significant differences between 1995 and 1997, with  $t = 2.3653$  ( $p < 0.05$ ) for artemisinin and  $t = 4.7150$  ( $p < 0.001$ ) for dihydro-artemisinin.

The correlation between the response of the individual parasite isolates to artemisinin and dihydro-artemisinin, at the EC-50 and EC-90 levels, shows high significance for 1995 ( $r = 0.8787$ ;  $p < 0.0001$ ) (Fig. 3). The same holds for 1997 ( $r = 0.8381$ ;  $p < 0.0001$ ) (Fig. 4).

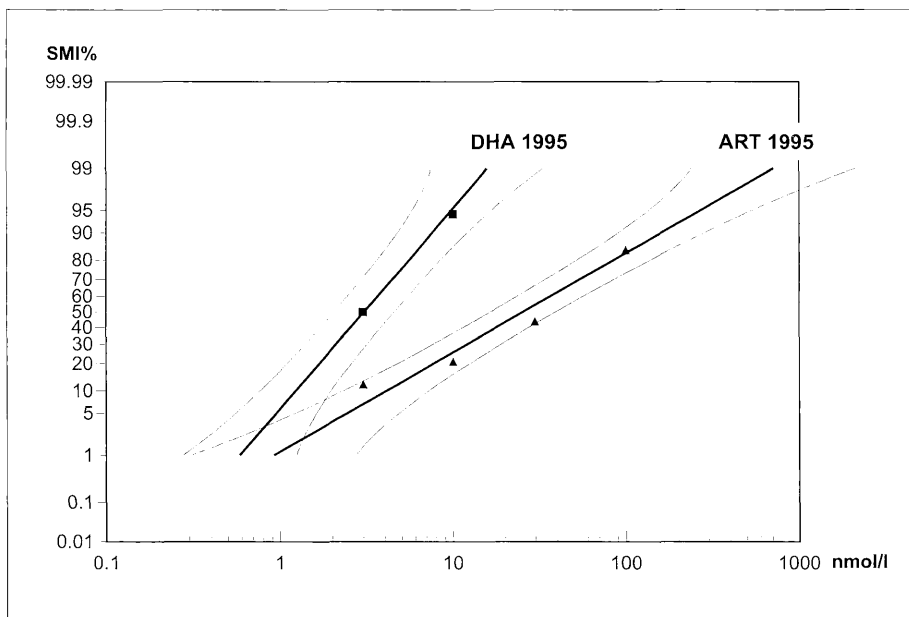


Figure 1:

Sensitivity of fresh isolates of *Plasmodium falciparum* from Mae Hong Son, Thailand, to artemisinin and dihydro-artemisinin in 1995, with data points and 95 % confidence intervals. Results of paired tests only (n = 22 each).

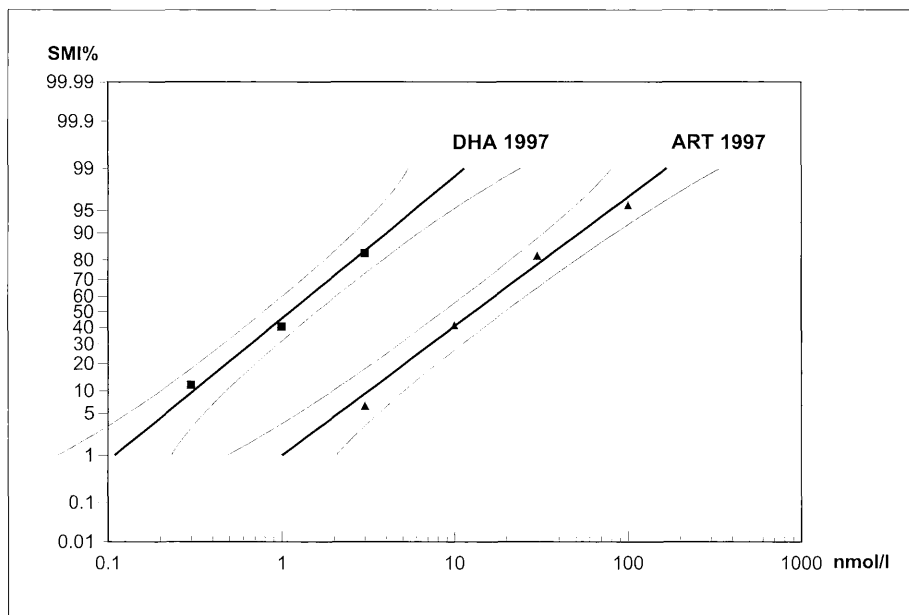


Figure 2:

Sensitivity of fresh isolates of *Plasmodium falciparum* from Mae Hong Son, Thailand, to artemisinin and dihydro-artemisinin in 1997, with data points and 95 % confidence intervals. Results of paired tests only (n = 23 each).

Although not immediately evident from the response patterns observed with artemisinin and dihydro-artemisinin (Fig. 1 and Fig. 2), the amalgamation of the grouped data shows a highly regular quantitative activity correlation between the concentrations of artemisinin and dihydro-artemisinin that is best expressed in the form of a HOERL regression ( $y = a \cdot b^x \cdot x^c$ ). Based on 14 data points (7 each derived from the regressions for 1995 and 1997) in the range of artemisinin concentrations between 0.39 and 2179.38 nM and the corresponding dihydro-artemisinin concentrations, a highly significant correlation is obtained ( $r = 0.9988$ ;  $p < 0.000005$ ;  $SE = 0.6912$ ;  $a = 0.2108$ ;  $b = 0.9999$ ;  $c = 0.7431$ ).

## Discussion

The comparison of the response data for artemisinin and dihydro-artemisinin in *P. falciparum* at Mae Hong Son shows an increase of sensitivity between 1995 and 1997. This surprising phenomenon may be explained by the different origin of the infections in both years. In 1995 the majority of patients came from Myanmar or were Thais who had contracted the infection in Myanmar. Crossing of the border was easy at that time. Subsequently, the border was closed in the aftermath of disturbances. This has largely limited the clientele of the malaria clinic to patients who had contracted the infections on Thai territory. In Myanmar, artemisinin and its derivatives are available since the early 1980s and it has to be assumed that the sensitivity of local *P. falciparum* has decreased as a result of the liberal use of these drugs. In Thailand, on the other hand, the use of artemisinin and its derivatives is strictly regulated and limited to the health services of two scheduled areas. Mae Hong Son does not belong to the scheduled areas.

In this study it was possible to cover a relatively wide drug sensitivity range. The sensitivity tests yielded precise and conclusive results for artemisinin and dihydro-artemisinin, both in 1995 and 1997. They also provided highly significant evidence for the strong correlation between the antimalarial activities of artemisinin and dihydro-artemisinin in the individual *P. falciparum* isolates and even more

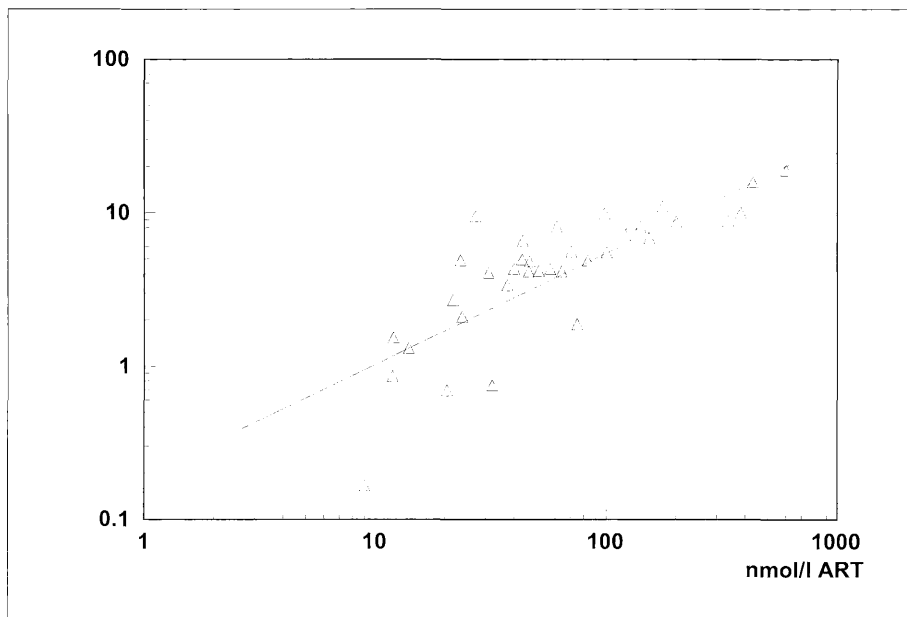


Figure 3:  
Scatter diagram of the correlation between artemisinin (ART) and dihydro-artemisinin (DHA) activity in *Plasmodium falciparum* isolates from Mae Hong Son, Thailand, 1995. (EC-50 and EC-90 data).

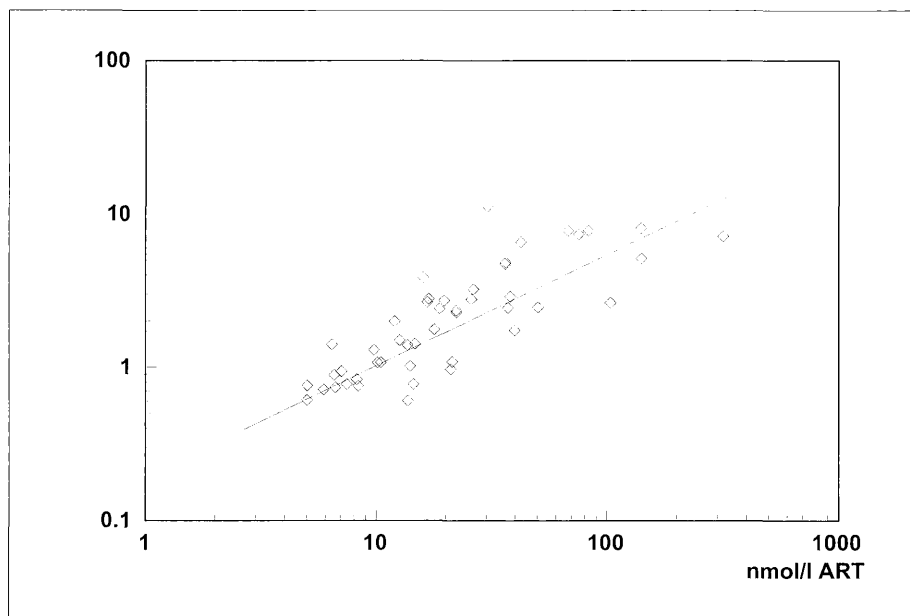


Figure 4:  
Scatter diagram of the correlation between artemisinin (ART) and dihydro-artemisinin (DHA) Activity in *Plasmodium falciparum* isolates from Mae Hong Son, Thailand, 1997. (EC-50 and EC-90 data).

so for the grouped data. On the basis of these results it is possible to use sensitivity tests with artemisinin as substitutes for tests with dihydro-artemisinin. This facilitates sensitivity testing in the field where the short shelf-life of the dihydro-artemisinin plates is a major practical constraint.

Data conversion is simple. To obtain the estimated sensitivity to dihydro-artemisinin (y) the results of the tests with artemisinin (x), namely the EC-1, EC-16, EC-50, EC-84, EC-90, EC-95 and EC-99 values, are converted according to the formula

$$y = a \cdot b^x \cdot x^c$$

where  $a = 0.2108$ ;  $b = 0.9999$ ;  $c = 0.7431$ . This will provide the same EC-range for dihydro-artemisinin. These data can be entered in the appropriate computer programme (2, 11) in order to obtain an adjusted regression. In the use with grouped data the method has a very narrow margin of error.

## Summary

The sensitivity of *Plasmodium falciparum* to artemisinin and its main active metabolite, dihydro-artemisinin, was investigated in 1995 and 1997 at Mae Hong Son, northwestern Thailand, near the border to Myanmar. Paired in vitro tests with both compounds were successfully conducted with 45 parasite isolates. The sensitivity to artemisinin and dihydro-artemisinin was higher in 1997 as compared to 1995, a phenomenon apparently related to the different origin of infections during both observation periods. Dihydro-artemisinin showed consistently higher activity as compared to artemisinin. The activities of both compounds are strongly correlated. The highly significant activity correlation ( $p < 0.000005$ ) can be used to estimate the response parameters for dihydro-artemisinin from the sensitivity data of artemisinin. This has important implications for in vitro testing as the short shelf-life of dihydro-artemisinin plates practically precludes their use in field investigations.

## Key words

*Plasmodium falciparum*, drug sensitivity, artemisinin, dihydro-artemisinin.

**Zusammenfassung** *Wirkungskorrelation zwischen Artemisinin und Dihydro-Artemisinin in frischen Isolaten von Plasmodium falciparum in Thailand*

In vitro Untersuchungen über die Empfindlichkeit von *Plasmodium falciparum* gegenüber Artemisinin und Dihydro-Artemisinin wurden 1995 und 1997 in Mae Hong Son, Nordwest-Thailand, nahe der Grenze zu Myanmar durchgeführt. Insgesamt 45 Parasitenisolate wurden erfolgreich mit beiden Verbindungen getestet. Die Sensibilität gegenüber Artemisinin und Dihydro-Artemisinin war 1997 höher als im Jahre 1995, was auf die unterschiedliche Herkunft der Infektionen zurückzuführen sein dürfte. Die relative Hemmwirkung von Dihydro-Artemisinin war durchgehend höher als jene von Artemisinin. Beide Substanzen zeigen ausgeprägte Wirkungskorrelation ( $p < 0,000005$ ) über den gesamten pharmakokinetisch möglichen Bereich. Dies erlaubt die Errechnung von Hemmparametern für Dihydro-Artemisinin aus den Artemisininindaten, eine wichtige Erleichterung, da die kurze Lagerungsdauer der mit Dihydro-Artemisinin vordosierten Platten deren Verwendung in Feldstudien praktisch ausschließt.

**Schlüsselwörter** *Plasmodium falciparum, Arzneimittelempfindlichkeit, Artemisinin, Dihydro-Artemisinin.*

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