

## *In vitro* ANTIMALARIAL DRUG SENSITIVITY TESTING

Principal Investigators : H. Kyle Webster, MAJ, MSC  
Ellen F. Boudreau, MAJ, MC  
Katchrinnee Pavanand, M.D.  
Kosol Yongvanichit, BS  
Lorrin W. Pang, CPT, MC  
Phung Phintuyothin, GEN, MC, RTA (RET)

Associate Investigators : Prasit Sookto  
Niphon Chuanak  
Jose Solivan-Perez, SP5, USA

**OBJECTIVE :** To determine the antimalarial activity of chloroquine, quinine, mefloquine and halofantrine against strains of *P. falciparum* isolated from naturally acquired malaria infections using a semiautomated radioisotope microdilution method.

**BACKGROUND :** Antimalarial drug resistance is a serious and persistent problem in Thailand and other malarious regions of the world (1). In response to the drug resistance problem the US Army Drug Development Program at the Walter Reed Army Institute of Research has produced several new antimalarial drugs. One of these drugs, mefloquine, had reached the stage of controlled mass use for treatment of multi-drug resistant *P. falciparum* malaria. Another new drug halofantrine - a phenanthrenemethanol, had reached the stage of limited field trials.

There are both *in vivo* and *in vitro* methods by which to assess the susceptibility (sensitivity) of malaria parasites to blood schizontocidal drugs. The major value of *in vitro* tests is that they help eliminate the difficulties associated with *in vivo* methods and thus provide an objective means for measurement of drug susceptibility. Basically, this involves quantifying dose-response characteristics for individual drugs thereby permitting the identification of drug resistance patterns in specific geographical areas.

The purpose of this study was to implement a multi-drug *in vitro* test using a semiautomated radioisotope microdilution technique. These studies were done in cooperation with the Royal Thai Navy and the Malaria Division, Thai Ministry of Public Health.

**METHODS :** *Sample preparation.* Heparinized venous whole blood (5 ml) was collected from adult Thai males after informed consent. The RBC were washed in RPMI media. Direct testing was done in the field using the RBC suspension. Indirect testing involved cryopreservation of the parasitized erythrocytes.

*In vitro* testing by the radioisotope microdilution technique (RMT). This procedure is basically that developed by Desjardins and Colleagues (1979) (2) with some modifications. The technique is based on incorporation of (<sup>3</sup>H) hypoxanthine, a nucleic acid precursor, by parasitized RBC in microculture. Inhibition of uptake of (<sup>3</sup>H) hypoxanthine by the parasites serves as an

indicator of antimalarial activity. The basic steps in the procedure are outlined in Figure 1. The stages of the technique are as follows:

a. Malaria culture (24 hours)(microculture in the presence of serial dilutions of 4 antimalarial drugs:

1. Halofantrine
2. Mefloquine
3. Quinine
4. Chloroquine)

b. Addition of radioisotope ( $^3\text{H}$ -hypoxanthine) to microculture plates:

1. Add isotope to microcultures.
2. Incubate for 24 hour.

c. Harvesting microcultures for counting of radioisotope:

1. Use of MASH to collect parasites (DNA/RNA) on filter paper.
2. Storage and transport of filter paper discs.

d. Counting of radioisotope by liquid scintillation spectrometry (Bangkok).

e. Calculation of the inhibitory dose (ID) 50 from radioisotope incorporation data.

*Computation of the inhibitory dose (ID)-50.* The inhibitory dose - 50 (ID50) is defined as that dose (concentration in ng/ml) of drug producing a 50% inhibition of the uptake of ( $^3\text{H}$ ) hypoxanthine by the parasite as compared to incorporation in the absence of drug. Each microtiter plate was set-up with 4 drugs. A two-fold serial dilution for a total of 11 concentrations over a 1024-fold range was made for each drug. The drugs and their respective concentration ranges were:

Chloroquine	(0.5 - 512 ng/ml),
Quinine	(0.9 - 921 ng/ml),
Mefloquine	(0.2 - 205 ng/ml) and
Halofantrine	(0.2 - 205 ng/ml).

The DPM data over the range of tested drug concentration allows construction of a concentration - response curve. The concentration - response curves over these ranges were characteristically sigmoidal after logarithmic transformation of the concentration. Nonlinear regression analysis was used to interpret the concentration-response curves based on a computer program obtained from the Division of Experimental Therapeutics, WRAIR. Figure 2 is an example of concentration-response curves for two *P. falciparum* strains tested against chloroquine. The Gambian strain (ID 50 = 8 ng/ml) was sensitive as compared to the resistant Chantaburi (ID 50 = 127) strain.

**RESULTS :** *Reproducibility of the test system.* Table 1, gives representative data on the reproducibility of the radioisotope test. This technique is more sensitive and precise than traditional microscopic methods.

*In vitro* testing of *P. falciparum* from natural malaria infections. Table 2 shows the *in vitro* antimalarial activity of the four antimalarial drugs against strains of *P. falciparum* from naturally acquired malaria infections. Testing was done at three study sites: Chantaburi, Phrabuddabat and Trat. There were no significant difference for the ID 50's of the four drugs when the three collection sites were compared. This probably reflects a similar geographic focus for the strains of *P. falciparum* involved in these infections. Most of the malaria cases were determined to have originated near the Thai-Kampuchean border. As would be predicted the ID 50's for chloroquine and quinine were high indicating decreased susceptibility to these drugs whereas the ID 50's for mefloquine and halofantrine were low and indicate sensitivity for these drugs.

When individual data are examined, however, some alarming drug susceptibility responses emerge. Table 3, shows the results of *in vitro* testing on 15 patients at Chantaburi. There were 4 individuals with high ID 50's for mefloquine (> 15 ng/ml). One of these CH 12, was confirmed as the first RII mefloquine resistant case in Thailand (3). The other 3 cases were treated with halofantrine. However, had they been treated with mefloquine treatment failure would have been predicted based on the high ID 50 values. The data in Table 3 also show a correlation for the high mefloquine ID 50's and corresponding high ID 50 values for quinine (> 148 ng/ml).

There were 7 treatment failures with halofantrine in the first 36 patients to receive this drug at Chantaburi (indicated by an asterisk (\*) in Table 3). This was an alarmingly high number of failures. It was, therefore, important to quickly determine whether parasite resistance to halofantrine was involved - a sufficient reason to stop the study - or whether there was a host factor involved (e.g. absorption of the drug). *In vitro* testing of the 7 *P. falciparum* strains revealed that the mean ID 50 for this group was not significantly different than that of the mean for all cases (15) studied at Chantaburi (1.00 vs 0.67 ng/ml Table 4). This suggested that parasite resistance was not involved. Therefore a decision was made to change the treatment schedule for administration of the drug. There were no additional treatment failures in the next 20 patients indicating a problem in bio-availability of halofantrine under the original treatment schedule.

**CONCLUSIONS :** The radioisotope microdilution technique for antimalarial drug susceptibility testing appears to be a dependable, sensitive and precise *in vitro* method for determination of antimalarial activity of schizontocidal drugs.

This method has been used to establish base-line data for chloroquine, quinine, mefloquine and halofantrine in selected geographic areas of Thailand.

The method was valuable for *in vitro/in vivo* correlation in which a RII mefloquine case was confirmed and in which parasite resistance was ruled out in 7 cases of treatment failure with the new drug halofantrine.

Table 1. Reproducibility of the radioisotope test.

<u>Controls</u> (Parasitized (0.55%) RBC, no drug)	
DPM	19740 ± 517* (n = 220)
<u>Background</u>	
DPM	521 ± 27 (n = 20)

ID 50 for 3 replicate tests of the same *P. falciparum* strain

	<u>ID 50 (ng/ml)</u>
Quinine	595.98** (548-647)
Mefloquine	13.75 (11-17)

(\*) Mean ± SEM

(\*\*) Geometric mean (95% confidence interval)

Table 2. *In vitro* antimalarial activity of antimalarial drugs against strains of *P. falciparum* from naturally acquired malaria infections in Thailand.

Source	<u>ID 50 (ng/ml)</u>			
	Chloroquine	Quinine	Mefloquine	Halofantrine
Chantaburi (n = 15)	95.26* (76-119)	148.32 (112-197)	5.45 (3-9)	0.67 (0.4-1.2)
Phrabuddabat (n = 8)	94.73 (63-142)	197.24 (126-308)	5.32 (4-8)	0.63 (0.4-1.1)
Trat (n = 10)	101.86 (80-130)	169.90 (123-235)	5.17 (4-6)	0.73 (0.5-1.0)
All cases (n = 33)	97.08 (84-112)	165.61 (138-197)	5.33 (4-7)	0.68 (0.5-0.9)

\* Geometric mean (95% confidence interval).

Table 3. *In vitro* antimalarial activity of antimalarial drugs against strains of *P. falciparum* from selected cases of naturally acquired malaria infections in patients at Ft. Taksin Marine Barracks, Chantaburi, Thailand.

Case	Treatment Drug	Treatment Outcome	Test Parasitemia	ID 50 (ng/ml)			
				Chloroquine	Quinine	Mefloquine	Halofantrine
CH 12	M	R II	0.6	53.44	149.55	15.65	2.44
CH 14	H	R I	0.7	62.96	182.33	3.75	0.68*
CH 15	H	R I	0.9	112.74	121.09	2.93	0.61*
CH 18	H	R II	0.4	141.42	278.00	15.05	1.11*
CH 20	H	R I	0.9	90.84	210.49	9.32	0.95*
CH 21	H	S	0.7	109.48	125.08	4.54	0.79
CH 22	H	S	0.7	75.57	88.03	2.72	0.28
CH 24	H	R I	0.8	105.79	203.35	32.74	4.25**
CH 27	H	S	0.7	67.75	85.23	2.28	0.25
CH 30	H	S	0.6	44.04	47.90	2.58	0.28
CH 31	H	S	0.3	88.31	168.91	1.53	0.29
CH 33	M	S	0.9	107.39	137.76	3.55	0.11
CH 34	H	R I	0.5	155.41	360.58	19.17	1.71*
CH 35	H	S	0.5	163.12	217.66	4.57	2.11
CH 36	H	R I	0.5	157.96	114.52	3.95	0.32*

Table 4. *In vitro* antimalarial activity of halofantrine against strains of *P. falciparum* isolated from treatment failures at Chantaburi (Ft. Taksin)

	Halofantrine ID 50 (ng/ml)
Treatment failure cases (n = 7)	1.00* (0.3-1.7)
Chantaburi all cases (n = 15)	0.67 (0.4-1.2)
Thailand - all cases studied (n = 33)	0.68 (0.5-0.9)

\* Geometric mean (95% confidence interval).

FIGURE 1

ANTIMALARIAL SUSCEPTIBILITY TESTING USING THE RADIOISOTOPE MICRODILUTION TECHNIQUE.

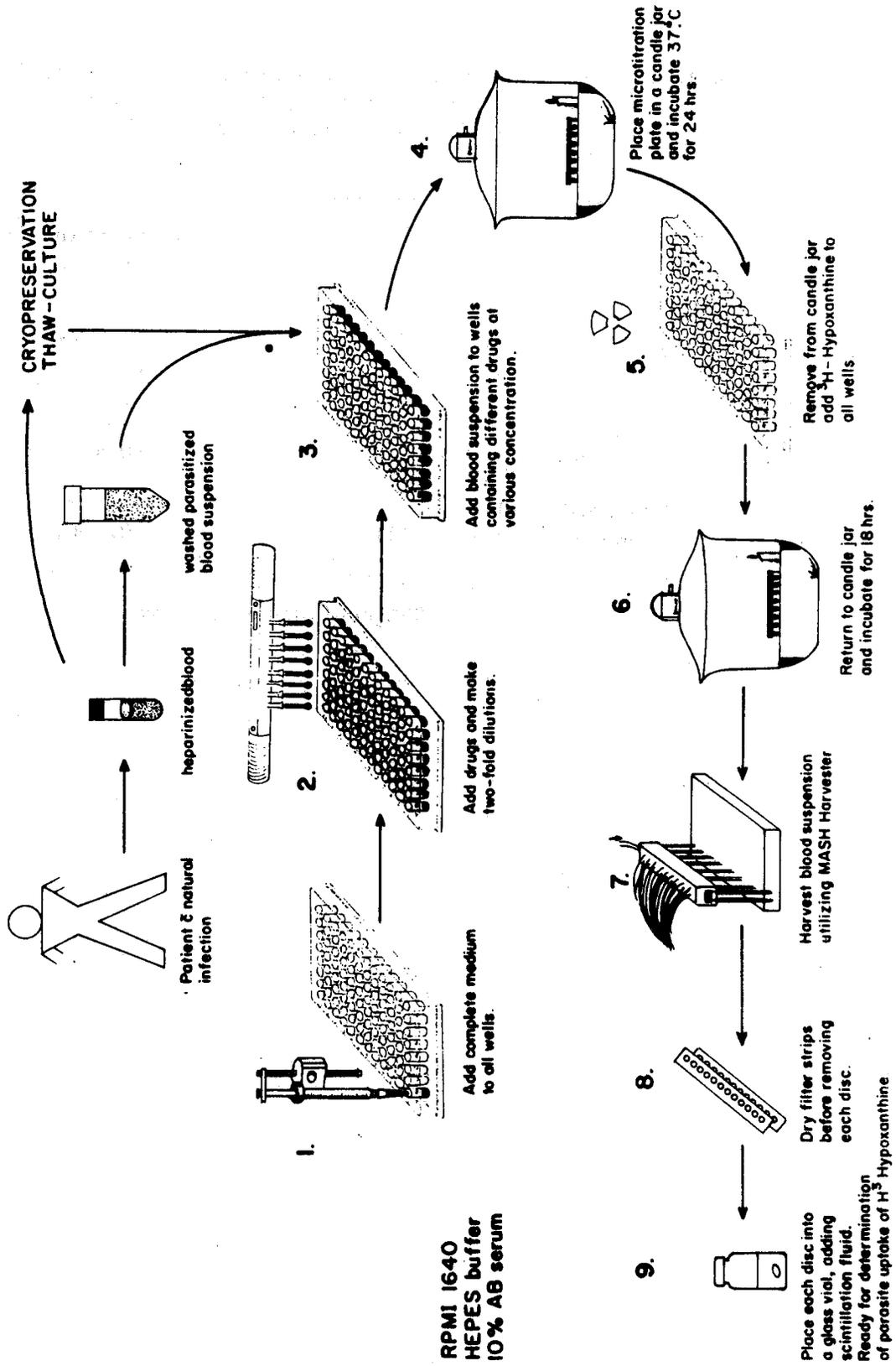
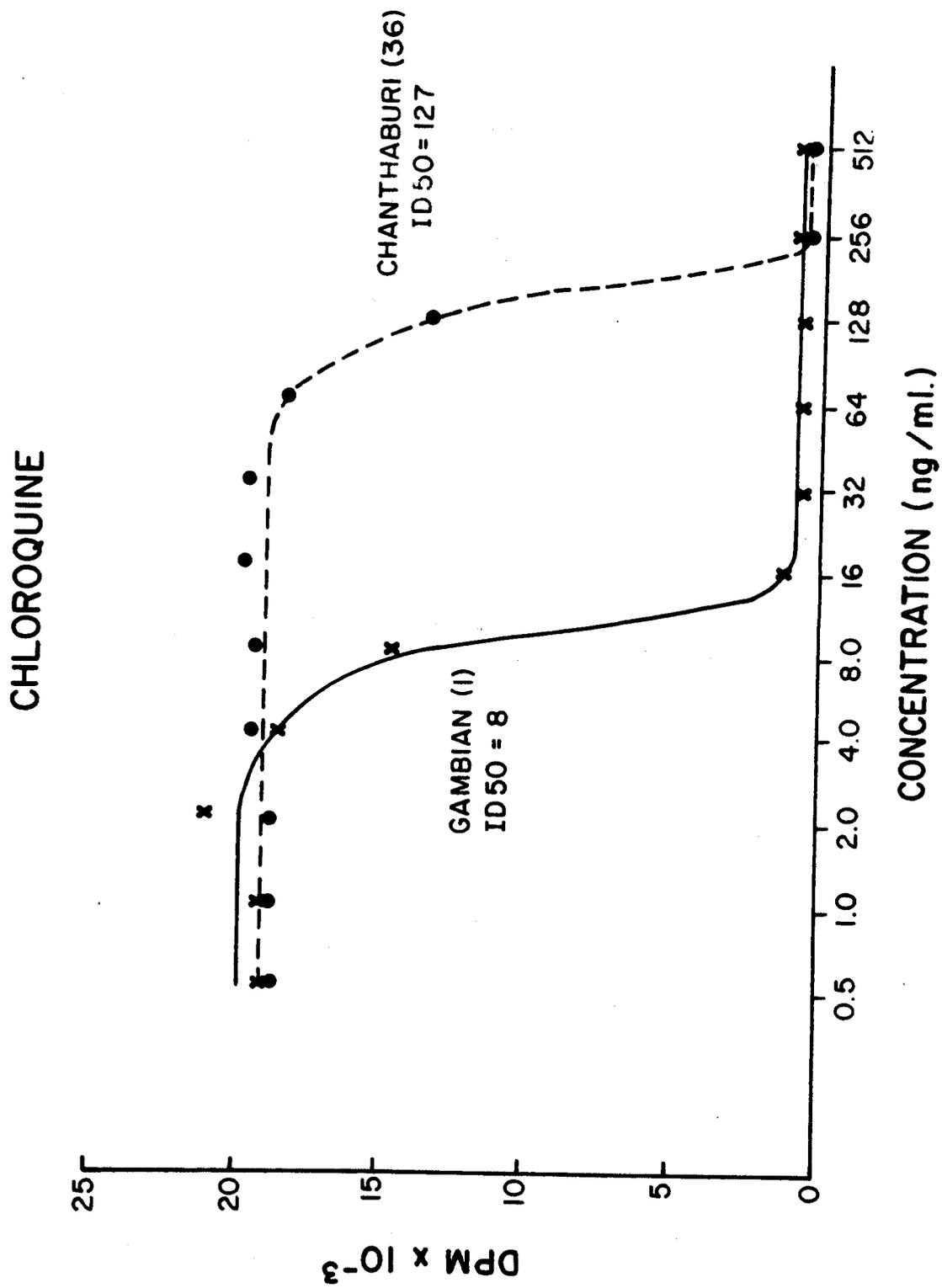


FIGURE 2



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