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In vitro susceptibility of *Plasmodium falciparum* collected from pyrimethamine-sulfadoxine sensitive and resistant areas in Thailand

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Seventy Plasmodium falciparum *isolates, collected from two geographically separate areas of Thailand, were tested for their* in vitro *responses to pyrimethamine, sulfadoxine, and a combination of these two drugs. The effects of pyrimethamine and pyrimethamine-sulfadoxine combinations against* P. falciparum *isolates were found to be significantly greater in a northern area where the combined drug was an effective therapeutic agent than in a south-eastern area, near the Thai-Kampuchean border, where the combined drug was no longer effective. However, the actions of sulfadoxine against parasites obtained from the two areas were not significantly different. There was no significant difference between the mean values of plasma 4-aminobenzoic acid (PABA) in falciparum malaria patients and in healthy controls. The test for PABA determinations used in this study gave positive readings with both PABA and sulfadoxine.*

A countrywide *in vitro* study between 1977 and 1980 in Thailand showed that 96.8% of 557 fresh *Plasmodium falciparum* isolates were resistant to chloroquine (*1*), and the clinical responses of malaria patients in the Hospital for Tropical Diseases, Bangkok, were most often RII and RIII responses. After the emergence of chloroquine resistance in 1962 (*2*), a combination of pyrimethamine and sulfadoxine (Fansidar)[a] became one of the main antimalarial drugs for the treatment of uncomplicated falciparum malaria in this country. Between 1967 and 1977 a single dose of Fansidar gave radical cure rates of 76.7-89.5% (*3-6*). However, studies in 1978-79 of various groups of falciparum malaria patients showed high failure rates of 50-90% after treatment with this drug combination (*7-9*). Between November 1980 and May 1981, an investigation in five separate areas of the country showed that, after a single full dose of 1.5 g sulfadoxine and 75 mg pyrimethamine (3 tablets of Fansidar), the radical cure rates in the north (Petchabun malaria clinic) (90%) and south (Sadoa malaria clinic) (82%) were significantly higher than those in the south-central (Chantaburi malaria clinic) (32%), north-eastern (Kuchinarai clinic) (39%), and north-central (Mae Sot clinic) (42%) parts of the country (*10*).

In the *in vitro* test, a culture medium containing 25-30 μg/l of 4-aminobenzoic acid (PABA), which provides sufficient PABA for the growth and propagation of *P. falciparum* in continuous culture, has been used to evaluate the sensitivity to sulfadoxine, while Waymouth medium has been suggested for use in pyrimethamine and sulfadoxine testing (*11*). The *in-vitro* microtest of Rieckmann et al. (*12*) has been widely used in field evaluations of drug resistance in malaria, but the PABA concentrations in the test system were not measured.

The object of the present study was to investigate the *in-vitro* sensitivity of fresh *P. falciparum* isolates (collected from patients living in Fansidar-sensitive and resistant areas) to pyrimethamine and sulfadoxine, and to determine the plasma 4-aminobenzoic acid concentrations in malaria patients.

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[a] Fansidar is a combination of pyrimethamine and sulfadoxine prepared by Hoffmann-La Roche, Basle, Switzerland.

MATERIALS AND METHODS

The study was begun on 12 July 1982 in the Petchabun malaria clinic (a Fansidar-sensitive area) and later in the Chantaburi malaria clinic (a Fansidar-resistant area) (*10*). The two clinics are approximately 500 kilometres apart. Patients attending the Petchabun clinic live in forested villages from which migration is minimal, whereas patients attending the Chantaburi clinic are generally migratory gem-mining workers. The study was completed on 26 October 1982.

Only patients with a negative result in urine tests for evidence of prior ingestion of 4-aminoquinolines and sulfonamides, using the Dill-Glazko test (*13*) and the lignin test (*14*, respectively, were included in this study.

After explanation of the trial, venous blood samples from each of the consenting uncomplicated falciparum malaria patients presenting at the Petchabum clinic and the Chantaburi clinic were collected into a heparinized syringe. Parasitized blood was thus taken from 83 patients; *in vitro* maturation to schizonts occurred in 70 isolates (84.3%) (40 from Petchabun and 30 from Chantaburi). All 70 isolates were tested for sensitivity to sulfadoxine and pyrimethamine-sulfadoxine, and 68 for pyrimethamine sensitivity.

Initial parasitaemia in the Petchabun and Chantaburi patients was in the range 1510–44 107 and 907–53 520 ring forms per μl blood, respectively. Owing to a technical error, the erythrocyte volume fraction (ECF) of the Petchabun patients was not measured; the ECF of 36 Chantaburi patients was in the range 0.23–0.48 (mean, 0.36).

The in vitro *test system*

The blood samples were assayed immediately or within 1 hour after collection. They were tested for parasite sensitivity to pyrimethamine, sulfadoxine, and combined pyrimethamine and sulfadoxine (in a ratio of 1 to 200), using an adaptation of the microtest described by Rieckmann et al. (*21*) and Yisunsri & Rieckmann (*15*). The fixed drug ratio (1:200) of pyrimethamine and sulfadoxine was derived from plasma concentrations of the two drugs 24 hours after ingestion of Fansidar (*16*).

The culture medium (Waymouth MB/752/1)[b] consisted of Waymouth formula powder 14.16 g/l, sodium bicarbonate 2.24 g/l, Hepes buffer 5.94 g/l, and gentamycin sulfate 8 mg/l, and the final pH was 7.4. The pure powder forms of pyrimethamine and sulfadoxine were kindly supplied by F. Hoffmann-La Roche & Co. Stock solutions of pyrimethamine and sulfadoxine were prepared separately. Pyrimethamine powder was dissolved with a small amount of 0.5% lactic acid, and the sulfadoxine powder was dissolved with a small amount of 50% sodium hydroxide. Double-distilled water was added until the concentrations of the stock solutions of pyrimethamine and sulfadoxine were 10×10^{-3} mol/l and 2.5×10^{-1} mol/l, respectively. Fifteen dilutions of each drug and of the drug combination were made in double-distilled water. The ranges of drug dilutions were: pyrimethamine 4×10^{-8} to 3.6×10^{-5} mol/l, sulfadoxine 1×10^{-5} to 7.2×10^{-3} mol/l, and pyrimethamine-sulfadoxine 4×10^{-8} to 8×10^{-6} mol/l and 3.6×10^{-5} to 7.2×10^{-3} mol/l. The drug dilutions, the pre-dosed plates (flat-bottomed, 8×12 wells) with three control wells, and the medium were prepared just before entering each field. The plates and medium were kept in a refrigerator throughout the study period.

Each well was inoculated with a mixture of 45 μl of Waymouth medium and 5 μl of the patient's blood. The plates were gently rocked to dissolve the drug and then placed in a candle jar. After incubation in a covered water bath[c] at 38.5–40 °C for 48 hours, thick films were prepared from each well and stained with Giemsa solution. The number of apparently normal schizonts with three or more nuclei per 100 leukocytes was determined and expressed as a percentage of the mean of three control wells. Morphologically abnormal schizonts that did not have distinctly pink-stained chromatin and blue cytoplasm were also recorded. The minimum inhibitory concentration (MIC) of the drug was defined as the lowest concentration at which normal schizont formation was completely inhibited.

The pH values of the blood-medium-drug mixtures ranged from 7.18 to 7.41, whereas the pH of the stock solutions of pyrimethamine and sulfadoxine were 2.1 and 9.9, respectively.

Determination of sulfadoxine and 4-aminobenzoic acid (PABA)

Plasma collected from 79 patients in the Petchabun and Chantaburi clinics and plasma from 176 apparently healthy adults were tested for sulfadoxine and PABA concentrations. Determination of sulfadoxine followed the methods previously described (*17*) and determination of PABA concentrations followed the method described by Horwitz (*18*).

Evaluation of the specificity of the methods used for PABA determination

Plasma samples from four volunteers in the Department of Tropical Paediatrics, Faculty of

[b] Grand Island Biological Company, New York, USA.

[c] From Lab-Line Instruments, Inc., Illinois, USA.

Tropical Medicine, Mahidol University, Bangkok, were collected daily for 6 days before and for 5 days after the administration of 3 tablets of Fansidar, and their sulfadoxine and PABA concentrations were determined.

Data analysis

The PABA concentration in each well was calculated using the formula: PABA in the well = plasma PABA (1 − ECF)/10. An assumed mean ECF of 0.36 was used for the Petchabun patients and individual ECF values were used for the Chantaburi patients in the calculation.

Student's "t" test was used to compare the log-transformed values of the MICs of the drugs acting on the Petchabun and Chantaburi parasites. Owing to a non-normal distribution of the data, the geometric means and their S.E. were tabulated.

RESULTS

The 48-hour duration of the test meant that the parasites were exposed to the drugs for a full cycle of asexual multiplication. In the presence of effective concentrations of antifolate drug combinations, ring forms do not mature into schizonts with a normal appearance; normal merozoite formation and subsequent reinvasion of erythrocytes are mostly prevented. This was in marked contrast to the large numbers of small ring forms that were frequently observed in wells containing no drug.

The susceptibilities of *P. falciparum* to the tested drugs are summarized in Table 1. There was no statistically significant difference between the MICs of sulfadoxine acting on the Petchabun and Chantaburi parasites. However, the MICs of pyrimethamine and the pyrimethamine-sulfadoxine combination acting on the Petchabun parasites were significantly lower than those on the Chantaburi parasites ($P < 0.001$ and < 0.001, respectively). The geometric mean values of the MICs of pyrimethamine and pyrimethamine-sulfadoxine on the Petchabun parasites were 3-fold and 2.6-fold lower than on the Chantaburi parasites, respectively.

Synergism of the two drugs was consistently noted in all isolates studied. Thus, evaluation of the geometric means of the MICs of the drugs acting on the parasites from the two areas shows that the drug combination resulted in a 2.6-fold increase in the activity of pyrimethamine and a 8.5-fold increase in the activity of sulfadoxine.

Plasma from 45 out of 79 patients (60%) showed detectable levels of sulfadoxine ranging from 11 to 121 mg/l. However, plasma containing sulfadoxine from the four volunteers gave a false positive reading for PABA concentrations, as shown in Table 2. The unusually high plasma PABA concentrations in these 45 patients, ranging from 4320 to 47 440 μg/l, were considered to be false positives and excluded.

Plasma PABA concentrations in 176 controls and 34 patients whose plasma had no detectable sulfadoxine and the calculated PABA concentration in

Table 1. Minimal inhibitory concentrations of pyrimethamine, sulfadoxine, and combined pyrimethamine/sulfadoxine in *in vitro* tests with fresh *P. falciparum* isolates collected from the Petchabun and Chantaburi malaria clinics in Thailand, 1982

	Minimal inhibitory concentrations (mol/l)		
	Pyrimethamine	Sulfadoxine	Pyrimethamine/sulfadoxine
Petchabun ($n = 40$)[a]			
Range	1.2×10^{-7} to 5.0×10^{-6}	1.6×10^{-4} to 4.8×10^{-3}	$8.0 \times 10^{-8}/1.6 \times 10^{-6}$ to $2.0 \times 10^{-6}/4.0 \times 10^{-4}$
Geometric mean ± SE	$1.6 \times 10^{-6} \pm 0.02 \times 10^{-6}$	$1.6 \times 10^{-3} \pm 0.02 \times 10^{-3}$	$6.6 \times 10^{-7}/1.5 \times 10^{-4} \pm 0.2 \times 10^{-7}/0.2 \times 10^{-4}$
Median	2.0×10^{-6}	2.4×10^{-3}	$8.0 \times 10^{-7}/1.6 \times 10^{-4}$
Chantaburi ($n = 30$)[a]			
Range	1×10^{-6} to 2.0×10^{-5}	4.0×10^{-4} to 6.0×10^{-3}	$1 \times 10^{-6}/2.0 \times 10^{-4}$ to $4.0 \times 10^{-6}/8.0 \times 10^{-4}$
Geometric mean ± SE	$5.0 \times 10^{-6} \pm 0.03 \times 10^{-6}$	$2.1 \times 10^{-3} \pm 0.03 \times 10^{-3}$	$1.7 \times 10^{-6}/3.5 \times 10^{-4} \pm 0.02 \times 10^{-6}/0.2 \times 10^{-4}$
Median	5.0×10^{-6}	2.4×10^{-3}	$2.0 \times 10^{-6}/4.0 \times 10^{-4}$

[a] n = number of isolates studied; only 28 isolates from the Chantaburi clinic were available for the pyrimethamine assay.

Table 2. Plasma PABA and total sulfadoxine concentrations in four volunteer subjects collected 6 days before and 5 days after the administration of 3 tablets of Fansidar (pyrimethamine-sulfadoxine)

	Before administration of pyrimethamine-sulfadoxine		After administration of pyrimethamine-sulfadoxine	
	PABA (μg/l)	Sulfadoxine (mg/l)	PABA (μg/l)	Sulfadoxine (mg/l)
1.[a] Range	30-111	–	50 530-61 613	146-177
Mean	68	0	55 789	161
2. Range	37-74	–	65 798-75 563	205-271
Mean	61	0	72 621	232
3. Range	30-111	–	50 375-73 780	145-242
Mean	56	0	62 744	194
4. Range	74-185	–	52 080-62 620	148-215
Mean	111	0	57 433	171

[a] The numbers 1-4 refer to the four volunteer subjects tested.

Table 3. Range, mean and median values of PABA concentrations in plasma (from 176 control subjects and 34 patients), in which sulfadoxine was not detectable, and the calculated PABA concentrations in wells containing a blood-medium-drug mixture from the 34 patients

	Plasma PABA concentration (μg/l)		PABA concentration in wells containing blood-medium-drug mixture (μg/l)
	Controls	Patients	
Range	20-271.4	80-160	4.6-36.9
Mean	98.7	273.8	17.4
Median	75.8	240.0	16.2

wells containing blood-medium-drug mixtures are summarized in Table 3. There was no significant difference between the plasma PABA concentrations in the patients and the controls.

DISCUSSION

The results of this study show that the degree of sulfadoxine susceptibilities of the parasites collected from the two areas were similar and that the higher susceptibility of the Petchabun parasites to pyrimethamine and the pyrimethamine-sulfadoxine combination was therefore due to the pyrimethamine susceptibility of the parasites.

The *in vitro* pyrimethamine and sulfadoxine assays indicate a wide range of sensitivity of the *P. falciparum* isolates in Thailand to these drugs. However, there is a positive correlation between the *in vitro* sensitivity to pyrimethamine-sulfadoxine in the present study and the *in vivo* response to a single dose of 3 tablets of Fansidar recently reported by Pinichpongse et al. (*10*), the time interval between these two studies being approximately one year. Our results fully support the view that the *in vitro* test could be used to detect the presence and prevalence of *P. falciparum* resistance to the drug combination.

However, after the completion of this study, Desjardins (personal communication, 1983) found that the growth rates of *P. falciparum* were affected by both the PABA and folic acid concentrations in the culture medium, and that the 1:80 ratio of pyrimethamine to sulfadoxine in the drug combination test approaches the optimum ratio of the independently determined activities of each drug *in vitro*. Chulay and coworkers determined the sensitivities of two isolates of parasites to the drugs by ^{3}H-hypoxanthine incorporation techniques and reported that the growing parasites in modified RPMI medium (with no PABA and no folic acid) were inhibited by 10^{-6} mol/l of sulfadoxine. A 1000-fold reduction of

sulfadoxine activity was observed in the medium containing 0.01 mg folic acid/l. They also noted that in normal RPMI medium 1640 (containing 1 mg folic acid and 1 mg PABA per litre) there was about 10-fold reduction in pyrimethamine activity and that sulfadoxine did not cause 50% parasite inhibition, even at concentrations exceeding the achievable blood concentrations. They found that medium containing PABA at concentrations above 0.5 mg/l was a growth inhibitor (*19*). Waymouth MB/752/1 medium used in our study contains 0.4 mg folic acid/l and no PABA.

It is therefore apparent that there is a need for a standardized field test for these drugs, and this subject was discussed at a recent meeting.[d] The report of the meeting proposed that the WHO standardized test for detecting the susceptibility of *P. falciparum* to pyrimethamine and sulfadoxine in the field should utilize a modified RPMI 1640 medium with low concentrations of PABA (0.5 μg/l) and folic acid (10 μg/l), because this has been found to be the most suitable medium considering the control growth rates of the parasites and the effects on the test of both PABA and folic acid. In addition, alkalization with NaOH for dissolving sulfadoxine in the culture medium is generally not recommended because of its variable effect on the parasite growth rate. Currently, investigators who collaborated in the studies on the WHO standard technique are evaluating the drugs' effect by comparing the number of parasites present at the end of the incubation period with the number at the beginning, i.e., the quantitative effect on parasite invasion of new red cells.

It may be noted that the methodology used in our study may not necessarily meet all the required criteria. However, the synergistic effects of pyrimethamine and sulfadoxine in combination confirm previous reports (*20*, *21*). The samples for PABA determination must be checked to see that they are sulfonamide-free, because any cross-reaction is most likely to be due to the structural similarity of PABA and the sulfonamides. Sulfadoxine was detected in 60% of lignin-negative plasma from patients, which shows that the urine lignin test for sulfonamides is not a sensitive test.

[d] WERNSDORFER, W. H. & DESJARDINS, R. E. *Report on informal discussions of investigators engaged in research on antifolate antimalarials, Bangkok, 27-29 April 1983.*

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RÉSUMÉ

SENSIBILITÉ *IN VITRO* DE *PLASMODIUM FALCIPARUM* RECUEILLIS DANS DES ZONES SENSIBLES ET DES ZONES RÉSISTANTES À LA PYRIMÉTHAMINE-SULFADOXINE

Une étude a été effectuée pour observer et comparer la sensibilité *in vitro* d'isolements frais de *P. falciparum* recueillis dans des zones récemment signalées comme sensibles à la pyriméthamine-sulfadoxine (Fansidar) (dispensaire antipaludique de Petchabun) et des zones résistantes à ce médicament (dispensaire antipaludique de Chantaburi), en Thaïlande. Les malades fréquentant le dispensaire de Petchabun, situé dans le nord, habitaient dans des villages forestiers d'où les migrations étaient insignifiantes. Ceux qui fréquentaient le dispensaire de Chantaburi, situé dans le sud-est, près de la frontière avec le Kampuchéa, étaient des travailleurs de mines de pierres précieuses.

A partir de 83 malades atteints de paludisme à *P. falciparum* qui ont fréquenté les deux dispensaires de juillet à octobre 1982, on a prélevé des échantillons de sang et on les a examinés dans l'heure suivante pour déterminer la sensibilité à la pyriméthamine, la sulfadoxine et la pyriméthamine-sulfadoxine, au moyen d'une adaptation du microtest décrit par Rieckmann et al. en 1978 et Yisunsri & Rieckmann en 1980. La plus faible concentration de médicament inhibant complètement la formation normale de schizontes a été prise comme concentration minimale inhibitrice.

On a observé une maturation *in vitro* jusqu'au stade de schizontes dans 70 isolements (84,3%) à savoir 40 provenant du dispensaire antipaludique de Petchabun et 30 de celui de Chantaburi. L'action de la pyriméthamine et des asso-

ciations pyriméthamine-sulfadoxine s'est révélée nettement plus forte à l'égard des isolements de *P. falciparum* provenant de Petchabun qu'à l'égard de ceux de Chantaburi ($P < 0{,}001$), mais l'effet de la sulfadoxine sur les parasites provenant des deux zones n'était pas significativement différent. Les effets synergiques des deux médicaments ont été constamment observés dans tous les isolements examinés. Le pH du mélange sang-milieu-médicament dans les cupules (allant de 7,18 à 7,41) ainsi que les concentrations de PABA dans les cupules (de 4,6 à 36,9 µg/l) ne doivent pas avoir perturbé la croissance des parasites ni influé sur les valeurs de la concentration minimale inhibitrice observée dans la présente étude. Les différences entre les concentrations plasmatiques de PABA chez 34 malades et 176 témoins n'étaient pas significatives, ces concentrations étant comprises entre 20 et 271 µg/l. Des plasmas contenant de la sulfadoxine et provenant de quatre volontaires ont donné des résultats faussement positifs en ce qui concerne le PABA. Il convient donc que les échantillons destinés à la détermination de cette dernière substance soient exempts de sulfamides.

Dans la présente étude, il y a une corrélation positive entre la sensibilité *in vitro* à la pyriméthamine et à la sulfadoxine et la réponse *in vivo* à l'association pyriméthamine-sulfadoxine récemment signalée. Nos résultats appuient pleinement l'opinion selon laquelle l'épreuve *in vitro* pourrait être utilisée pour déceler sur le terrain la fréquence des isolements de *P. falciparum* résistants à la pyriméthamine-sulfadoxine.

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