INVESTIGATIONS OF INCIDENCE OF PRETREATMENT, DRUG SENSITIVITY *IN VITRO*, AND PLASMA LEVELS OF PYRIMETHAMINE IN PATIENTS WITH MULTIDRUG RESISTANT FALCIPARUM MALARIA FOLLOWING THE THREE COMBINATION REGIMENS OF ARTEMETHER/PYRIMETHAMINE

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Abstract. The study was carried out to investigate the status of *in vitro* susceptibility of *Plasmodium falciparum* to pyrimethamine (PYR) in multidrug resistant area of the Thai-Myanmar border, the incidence of unregulated use of the combination of PYR with sulfadoxine (Fansidar®) in this area and the relevance of pharmacodynamic and pharmacokinetic factors in determining the treatment outcome from the three combination regimens of ART/PYR (1-, 2- and 3-day regimens), in patients with acute uncomplicated falciparum malaria. The majority of patients had baseline PYR concentrations in the range of 1-100 (50.6%) or 100-500 (34.8%) ng/ml, while concentrations of more than 500 ng/ml were found in only 1.1%. All of the isolates exhibited high grade resistance to PYR with the minimum inhibition concentration (MIC) of as high as 10^{-5} M. No association was observed between treatment outcome and the presence of baseline plasma PYR concentrations. In addition, lack of association between plasma concentrations during the acute phase (day-1 and -2) and treatment outcome was found.

INTRODUCTION

The increasing problem of parasite resistant to available antimalarials has prompted the use of combinations of existing drugs, in addition to the development of novel antimalarials. During the years 1973-1984, a combination of sulfadoxine (S) and pyrimethamine (PYR) named Fansidar® (SP) was employed in Thailand (Karbwang and Harinasuta, 1992). However, the development of resistance to this combination (Chongsuphajaisiddhi and Sabcharoen, 1981) has limited its further use in this country. Combination therapy of artemisinin derivatives with antimalarials that act upon different loci of *P. falciparum* blood stage, and whose elimination half-lives are long, is currently one promising strategy for controlling these multidrug resistant *P. falciparum* (Bunnag *et al*, 1996; Karbwang *et al*, 1995; Na-Bangchang *et al*, 1997a). Apart from the prominent role of combination regimens of artemisinin derivatives and mefloquine, combined regimens of the first with PYR may also offer an effective alternative combination candidate, especially in areas with less intense PYR-resistance. Treatment with the combination ART/PYR has been shown to be associated with a significant improvement of clinical efficacy of ART in a multidrug resistant area along the Thai-Myanmar border (Na-Bangchang *et al*, 1996). In the light of the intriguing *in vitro* data which demonstrated the antagonistic antimalarial activity of this combination (Chawira *et al*, 1987), this finding therefore raised the question with respect to the approach whereby this combination works. The present investigation was a part of the previous comparative trial for assessing the efficacy of the three combination regimens of ART/PYR in multidrug resistant *P. falciparum* malaria (Na-Bangchang *et al*, 1996). The objectives of the study were to investigate (a) the status of *in vitro* susceptibility of *P. falciparum* to PYR in the multidrug resistant area of Thai-Myanmar border after the termination of its official use, (b) the incidence of unregulated use of PYR (as Fansidar®) in this area, and (c) the relevance of pharmacodynamic (intrinsic activity of PYR in resistant parasite) and pharmacokinetic (plasma concentrations of PYR) factors in determining the treatment outcome from the combination ART/PYR.

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PATIENTS AND METHODS

Patients and study site

The study was carried out in 1995 during the rainy season (June-August). Patients presenting to the out-patient malaria clinic in Mae Sot, Tak Province (along the Thai-Myanmar border) were screened for *P. falciparum* infection. This part of the country is well-documented as a multidrug resistant area. Uncomplicated malaria patients with parasitemia lower than 100,000 per µl, aged between 15 and 55 years, who had no previous history of antimalarial treatment within the last 1 month, were recruited to the study provided that informed consents for participation were obtained.

Treatment groups

The patients were randomized to receive three oral drug regimens ART/PYR as follows:

Regimen I: a 1-day regimen of ART/PYR, consisting of artemether (300 mg: Artenam®, Arenco nv, Belgium) plus pyrimethamine (100 mg: Daraprim®; Wellcome) on the first day, then placebo on the 2 consecutive days;

Regimen II: a 2-day regimen of ART/PYR, consisting of artemether (300 mg) plus pyrimethamine (100 mg) on the first day, then artemether (150 mg) plus pyrimethamine (50 mg) on the second day, and placebo on the third day;

Regimen III: a 3-day regimen of ART/PYR, consisting of artemether (300 mg) plus pyrimethamine (100 mg) on the first day, then artemether (150 mg) plus pyrimethamine (50 mg) on the second day, and third day.

Blood sample collection

Blood samples (5 ml each) were collected into the sodium-heparinized plastic tubes prior to the doses of each regimen (day-0) and on the day of recrudescence (day-R) Following regimen I, II and III, additional blood samplings were done on day 1, day 2, and days 1, 2, 3, 7, 14, 21, 28, respectively. Concentrations of PYR in plasma were determined using high performance liquid chromatography according to the method of Na-Bangchang *et al* (1997b). The method was shown to be free from chromatographic interference from other antimalarial drugs including mefloquine, quinine, primaquine and artemisinin derivatives.

In vitro sensitivity test

Susceptibility of *P. falciparum* to PYR was investigated in all patients prior to treatment and at the time of recrudescence, using the standard *in vitro* technique of Rieckmann *et al* (1979) with modifications. In brief, the final volume in each well of the microtiter plate (96 wells, flat bottom, 8 x 12 matrix; Nunclon®, Denmark) was 100 µl. The inoculum in each of the duplicate wells consisted of 50 µl of the prepared parasite cell suspension (0.5-1% parasitemia, 5% cell suspension, with 30% AB serum), and 50 µl of PYR solution or low *p*-amino benzoic acid (PABA), low folic acid medium with sodium bicarbonate and 30% AB serum (control well). Following 48 hours incubation (37°C, an atmosphere of 96% N_2, 1% O_2 and 3% CO_2 in a candle jar), thin blood smears were made from each well, stained with Giemsa stain and examined for the number and morphology of the ring, trophozoite and schizont stages *P. falciparum*. The assay was considered successful if at least 4 to 5-fold increase in number of parasitized cells in the control wells was achieved. The number of infected erythrocytes with normal appearance were counted per 10,000 red blood cells. Sensitivity to PYR was expressed as the minimum inhibitory concentration (MIC) of PYR required to inhibit parasite growth.

Data analysis

Comparison of plasma PYR concentrations in patients with treatment failure and those with sensitive treatment outcome was done by Mann-Whitney U test. Association of baseline PYR concentration and treatment outcome was evaluated using Chi-square test. Significance level was set at $p < 0.05$ for all tests.

RESULTS

Incidence of patients with baseline PYR concentrations

Baseline pretreatment with PYR was monitored in 89 multidrug resistant falciparum malaria patients. The majority of patients had concentrations between 1-100 (50.6%) or 100-500 (34.8%) ng/ml, while concentrations of more than 500 ng/ml were found in only 1.1% (Table 1).

In vitro sensitivity

The *in vitro* sensitivity test was successful in 31 *P. falciparum* isolates. All isolates collected before the treatment (day-0) and on the day of recrudescence (day-R) exhibited high grade resistance to PYR with the MIC of as high as 10^{-5} M.

PYR concentrations following drug administration

Median plasma PYR concentrations in all three regimens (presented as lines) and concentrations in

Table 1
Baseline concentrations of PYR in plasma of 89 acute uncomplicated falciparum malaria patients before treatment with the three regimens of ART/PYR combination; data are presented as number and (percentage).

PYR level (ng/ml)	No. of patients (%)
* No basline	12 (13.5)
* With baseline (median, range)	
1-100 (22, 3-96)	45 (50.6)
>100-500 (147, 108-494)	31 (34.8)
>500 (606, 606-606)	1 (1.1)
Total	89 (100)

patients with treatment failure from all regimens (presented as dots: 3, 2, 9 cases in Regimen-I, II and III, respectively) are shown in Fig 1. In Regimen-III group, blood sampling was extended until the elimination phase, and thus allowing accurate calculation of terminal half-life [$t_{1/2z}$; median (range) = 108 (72-282) hours]. On the day of recrudescence (from any regimen), plasma PYR concentrations varied between 0 and 330 ng/ml (median 143 ng/ml).

Association between treatment outcome and baseline PYR concentrations

No association was found between treatment outcome and the presence of baseline plasma PYR concentrations.

Association between treatment outcome and PYR concentrations during acute phase malaria infection

PYR concentrations in plasma on day-1 and day-2 in patient with sensitive response and treatment failure following treatments with any regimen are shown in Fig 2. No significant association between treatment outcome and plasma PYR concentrations on day-1 or day-2 was observed. In those with treatment failure, 3 (33.3%) and 3 (33.3%) cases had PYR concentrations lower than the lower limit of 95% CI for median on day-1 (577 ng/ml) and day-2 (751 ng/ml), whereas 6 (66.7%) and 7 (70%) cases had concentrations higher than these limits. In the group with sensitive response on the other hand, the corresponding values for day-1 *vs* day-2 were 10 (29.4%) and 24 (70%) *vs* 18 (32.1%) and 38 (67.9%), respectively.

DISCUSSION

The existence of highly PYR resistant strains of *P. falciparum* in Thailand has been confirmed by *in vitro* and *in vivo* lines of evidence obtained from the present investigations. The resistance has continued despite the restricted usage of sulfadoxine/pyrimethamine (SP; Fansidar®) under the governmental control by the Malaria Control Program

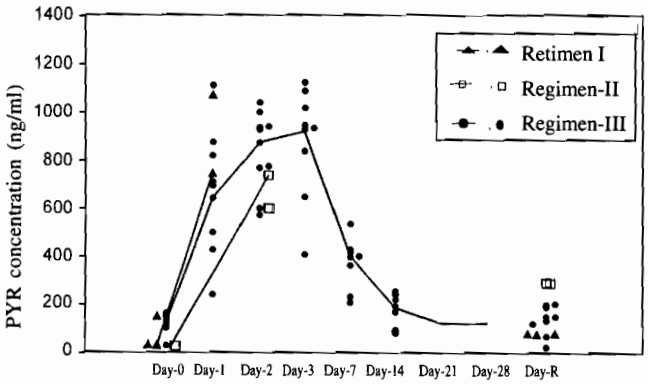

Fig 1–Plasma concentrations of pyrimethamine (PYR) following treatment with the three combination regimens of ART/PYR and on the day of recrudescence (day-R). The lines represent the median concentrations and the dot markers represent concentrations in individuals with subsequent treatment failure.

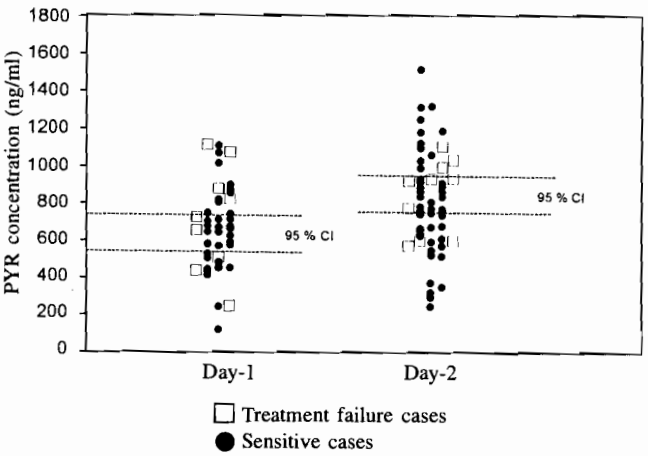

Fig 2–Pyrimenthamine (PYR) concentrations on day-1 and day-2 of treatment failure and sensitive cases treated wiht the three combination regimens of ART/PYR [median (95% CI): day-1 = 673 (577-745) ng/ml; day-2 = 786 (751-922) ng/ml].

(Thimasarn *et al*, 1994). The drug has had a limited role as the first-line treatment for uncomplicated falciparum malaria since the report of resistance in 1981 and subsequent spreading thereafter (Chongsuphajaisiddhi and Sabcharoen, 1981). Its role has been preserved for presumptive or first-line treatment only in some certain PYR-sensitive areas, but not in areas with prominent multidrug resistance such as that of the Thai-Myanmar border (Karbwang and Harinasuta, 1992). Unregulated use of antimalarial drugs particularly SP (as package drug) in the community is however, widespread and information obtained from patients with respect to their previous history of antimalarial treatment are often difficult to be ascertained. This assertion is mirrored by the finding of remarkably high incidence of patients who had baseline concentrations of PYR in their plasma prior to treatment of the present episode of falciparum infection. The observed pretreatment concentrations of PYR in the high concentration range, notably 100-500 ng/ml implicates a relatively short lapse between the previous and present episode of the infection. Based on the median plasma concentration-time profile seen in patients (Fig 1), it was speculated that most treatment failure cases occurred approximately one week after the primary treatment with Fansidar®.

The concentration of PYR of at least 10^{-5} M (MIC; 2,480 ng/ml) was found to be required to inhibit the growth of PYR-resistant strains of *P. falciparum in vitro*. This level of susceptibility of the parasites to the drug has been more or less stable since 1994 (Subdivision of Treatment Policy, Malaria Division, 1997). Such this high MIC is definitely, not achieved in plasma following the administration of the conventional dose of PYR. When PYR is given as the combined therapeutic regimen –SP at a single oral dose of 75-100 mg PYR, plasma maximum concentrations of 400-1,000 ng/ml are reached at 1-8 hours (Cavallito *et al*, 1978; Na-Bangchang *et al*, 1997b; Tan-ariya *et al*, 1998; Weidekamm *et al*, 1982; 1987). In the present study, median concentration of 921 ng/ml was attained during the first day (day-1) of a single dose of 100 mg. This eventually declined to lower levels on the later phase. The influence of disease state on the disposition kinetics of PYR appears to occur in patients with acute malaria in such a way to improve systemic exposure of PYR (increased concentration and prolonged half-life) compared with that in healthy subjects (Na-Bangchang *et al*, 1997b; Tan-ariya *et al*, 1998); yet the levels attained were still far lower than the minimum inhibitory concentration. Either patients who had successful treatment outcome or those with subsequent treatment failure were inevitably subjected to subtherapeutic plasma PYR level. In addition, lack of association between plasma PYR concentrations during the acute phase (day-1 or -2) and treatment outcome was observed in this study. This points to the fact that, as the degree of PYR-resistance rises, therapeutic plasma level of PYR could not be achieved from the present dose of PYR to cope with the resistant parasites.

It is worthy of note that, irrespective of the plasma PYR levels achieved, radical cures were obtained in a number of patients when additional doses of ART were coadministered. The modest benefit of this combination has been evident from the results of the comparative clinical trial for assessing the efficacy of these three combination regimens of ART/PYR in multidrug resistant uncomplicated falciparum malaria (Na-Bangchang *et al*, 1996). When given as monotherapeutic regimen at a short duration of 1-3 days ART, cure rate of virtually 0% was obtained (Bunnag *et al*, 1991). With the addition of 2-3 doses of PYR at an extended duration of 2 or 3 days (Regimen II and III), the cure rates of ART were improved to 27.8-75% (Na-Bangchang *et al*, 1996). Furthermore, unlike the combination – SP, high grade resistance (RII and RIII) was not encountered with any of these combination regimens. In support of the beneficial combination therapy from ART/PYR, a recent *ex vivo* study where the conditions do mimic the normal parasite-host relation, demonstrated synergistic or at least additive blood schizontocidal activity of sera obtained from healthy subjects following the coadministration of ART and PYR (Tan-ariya *et al*, 1998). The higher maximum concentrations of PYR achieved as a consequence of pharmacokinetic interaction with ART may only partly explain the improved cure rate from ART/PYR combination. The major contributing factor is likely to be the pharmacodynamic synergistic blood schizontocidal activity of the combination (Tan-ariya *et al*, 1998). Rapid initial clinical response seen in patients (PCT, FCT) may have been solely accounted for by the rapid and potent action of ART (Na-Bangchang *et al*, 1996) while radical cure been accounted for by PYR in successfully treated patients. Obviously, eradication of the highly PYR-resistant strains which constituted the majority of parasite pool could not be accomplished by subtherapeutic plasma concentrations of PYR. However these parasites were ART-sensitive and thus were almost completely eliminated by ART (Na-Bangchang *et al*, 1996) and left over for PYR to remove, parasite residuals which required relatively lower concentration of PYR (less PYR-resistant).

In spite of the fact that PYR has a relatively narrow time window of activity in the later half of parasite cycle (24-48 hours) than ART, its longer half-life would allow the levels to be maintained until all these less PYR-resistant parasites have been eliminated. The role of PYR including other slowly eliminated antimalarials such as mefloquine, in improving clinical efficacy of artemisinin derivatives when using as a short course treatment (1-3 days), as well as in protecting them from resistance developing remain to be proved.

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