PLASMA CONTAINING ARTEMETHER-PYRIMETHAMINE HAS *EX VIVO* BLOOD SCHIZONTICIDAL ACTIVITY AGAINST *PLASMODIUM FALCIPARUM*

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Abstract. Plasma samples collected at intervals from healthy volunteers, after administration of 3 drug regimens [artemether (ART) 300 mg, pyrimethamine (PYR) 100 mg, and ART 300 mg plus PYR 100 mg] were examined for blood schizonticidal activity against K_1 strain and $T_{9/94}$ clone of *Plasmodium falciparum ex vivo*. A synergistic effect against $T_{9/94}$, a pyrimethamine sensitive clone, was observed in plasma collected after ART+PYR administration, when the test was carried out in low *p*-aminobenzoic acid, low folic acid medium. The maximum activity (A_{max}), expressed as equivalent dihydroartemisinin concentration, for plasma samples collected after the combined ART+PYR regimen [6,935 (1,330-13,400) nmol/l] was significantly higher than those for the single ART or PYR regimens [935 (397-2,000) and 9.9 (5.6-15.6) nmol/l, respectively]. In addition, the area under the activity curve (AUA) for the combined regimen [12,8397 (39,274-19,7901) nmol.h/l] was significantly higher than those for the single ART or PYR regimens [(3618 (1406-5597) or 334 (82.3-733.3) nmol.h/l, respectively]. Microscopic observation revealed that ART in the combined regimen exerted its inhibitory effect against all erythrocytic stages and that this occurred before effects of PYR activity. Prolongation of inhibitory effects for the combined ART+PYR regimen was shown to be due to PYR activity by comparison to the activity from the single ART regimen. Results clearly demonstrated no PYR activity against K_1, a pyrimethamine resistant strain, in plasma samples collected after the single PYR regimen and the ART+PYR regimen. Microscopic examination confirmed that growth inhibition of K_1 was caused by ART activity only.

INTRODUCTION

The increasing prevalence of multidrug-resistant strains of *Plasmodium falciparum* has necessitated the development of new antimalarial compounds. Artemisinin and its derivatives represent the most promising new agents. Artemether (ART), one artemisinin derivative, has been shown to be rapidly and highly effective against multidrug-resistant *P. falciparum* (Jiang *et al*, 1982; Arnold *et al*, 1990). However, due to its short half-life (Karbwang *et al*, 1997a), a long treatment course of more than 5 days is required in order to achieve a 100% cure rate. One strategic aim at improving the clinical effectiveness (efficacy, patient's compliance) of artemether is to use it in combination with other long half-life drugs such as mefloquine (Karbwang *et al*, 1995; Bunnag *et al*, 1996; Na-Bangchang *et al*, 1997a). Pyrimethamine (PYR), a dihydrofolate reductase inhibitor, is another antimalarial with a long half-life and it has been used for therapy or prophylaxis of falciparum malaria for decades. The drug is active against several stages of human malaria, but due to its slow blood schizonticidal action, the use of PYR alone is limited to acute falciparum malaria (Karbwang and Harinasuta, 1992). From the pharmacokinetic and pharmacodynamic points of view, *P. falciparum* in Thailand is resistant to PYR (Karbwang and Harinasuta, 1992). However, a combination of ART and PYR might result in therapeutic synergistic effects. Up to now, there have been no reports on interactions between ART and PYR in man and such interactions are important considerations for combination therapy. Thus, in this study of healthy Thai male volunteers, we examined the pharmacodynamic interaction(s) of combined ART and PYR *ex vivo*.

MATERIALS AND METHODS

Malaria parasites

K_1 strain and $T_{9.94}$ clone of *P. falciparum* were used in this study. K_1 strain was originally isolated

from a patient who was a resident of Kanchanaburi Province in western Thailand (Thaithong and Beale, 1981). $T_{9/94}$ clone was cloned from T_9 isolate which was isolated from a patient resident of Tak Province in northern Thailand (Rosario, 1981). K_1 strain is highly resistant to PYR, whereas $T_{9/94}$ is sensitive. Both isolates are resistant to chloroquine but sensitive to ART. They were routinely maintained in continuous culture (Trager and Jensen, 1976) in 60 × 15 mm disposable plastic petri dishes (Nuncon®, Denmark) using RPMI 1640 medium containing L-glutamine (formula 430-1800; Gibco, New York), with 25 mM HEPES buffer (N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid, Sigma), 32 mM $NaHCO_3$ and gentamicin (80 mg/l). The culture medium contained *p*-aminobenzoic acid (PABA) and folic acid at a concentration of 1,000 µg/l each. Before use, the medium was supplemented with 10% (v/v) type-AB serum. The initial parasitemia was 0.5% with 6-8% red blood cell suspension in a total volume of 4 ml. Parasites were synchronized with 5% D-sorbitol (Lambros and Vanderberg, 1979) for 24 or 48 hours before use.

Test media

Two types of culture media were used for *ex vivo* assessment of antimalarial activities in plasma samples. They were also used for determination of effective concentrations (EC_{50s}) of ART, dihydroartimisinin (DHA) and PYR against K_1 strain and $T_{9/94}$ clone. The first medium was RPMI 1640 formula 438-1800, which was also used for cultivation, as mentioned above. The second was RPMI 1640 special formula AS074-1800 (GIBCO, New York), which contained PABA and folic acid at concentrations of 0.5 µg/l and 10 µg/l, respectively, and was proven to be suitable for *in vitro* sensitivity tests of *P. falciparum* against PYR and sulfadoxine (Tan-ariya *et al*, 1987). It was then referred to as RPS in this study. In the *in vitro* sensitivity experiments, neither of these media were supplemented with normal human serum.

Preparation of parasite inoculum

Four different kinds *P. falciparum* inoculum were used in this study. They were (1) K_1 strain in RPMI, (2) $T_{9/94}$ clone in RPMI, (3) K_1 strain in RPS and (4) $T_{9/94}$ clone in RPS. Each inoculum was prepared as 5% red blood cell suspension which consisted of 0.5% - 1% parasitemia of mixed stages (rings: immature schizonts at 1:1). The procedures were as follows. *P. falciparum* cultures of K_1 strain and $T_{9/94}$ clone were synchronized with 5% D-sorbitol (Lambros and Vanderberg, 1979) every other day in order to obtain pure parasite stages, *ie*, rings or immature schizonts. For both $T_{9/94}$ or K_1, two different culture lines, one containing 5-6% ring stages and the other containing 5-6% immature schizont stages, were pooled, washed and collected by centrifugation (750g, 7 minutes 4°C). The packed cells were resuspended in either RPMI or RPS media (without added serum) to make 50% cell suspensions. The percentage of parasitemia was then reduced to 0.5-1% by diluting with 50% (v/v) normal 0 cell suspension. Finally, these infected cell suspensions were adjusted to 5% total cells by using medium with no serum. This preparation was referred to as parasite inoculum.

Plasma samples from healthy volunteers after antimalarial drug administration

A series of plasma samples (duplicate aliquots of 500 µl) were collected at intervals from 8 healthy Thai males after administration of antimalarial drug regimens on 3 separate occasions (randomization) as follows:

Regimen-I: a single oral dose of 300 mg ART (Arenco nv, Belgium: 50 mg per tablet)

Regimen-II: a single oral dose of 100 mg PYR (Daraprim®, Welcome: 25mg per tablet)

Regimen-III: a single oral dose of 300 mg ART, given concurrently with a single oral dose of 100 mg PYR

The wash-out period after the ART regimen (Regimen I) was at least 1 week, while that after the PYR regimens (Regimens II and III) was at least 3 weeks. The scheduled times for collecting plasma samples after Regimen I was 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 18 and 24 hours. For regimens containing PYR (Regimens II and III), collection of plasma samples followed the same routine but was also continued daily after the first 24 hours until day 7 and then once again on days 10 and 14. The collected samples were stored at-20°C until use. Plasma concentrations of artemether, dihydroartemisinin were measured by high performance liquid

chromatography with electrochemical detection according to the method of Karbwang *et al*, (1997b). Concentration of pyrimethamine in plasma were measured by high performance liquid chromatography with ultraviolet detection using the method of Na-Bangchang *et al* (1997b). Coefficients of variation for all the compounds were below 10% at the concentration range 10-2,000 ng/ml. The quantification limits of artemether, dihydroartemisinin and pyrimethamine assays were 3, 2 and 5 ng/ml, respectively.

Ex vivo assessment of blood schizonticidal activities of sampled plasma

The collected plasma samples were examined for blood schizonticidal activity against K_1 strain and $T_{9/94}$ clone in microtiter plates using the standard *in vitro* microtechnic of Rieckmann *et al* (1978) with modifications (Tan-ariya *et al*, 1997). Plasma samples collected at each interval were two-fold serially diluted with normal AB serum to dilutions of 1:2, 1:4, 1:8, 1:32, 1:64, 1:128 and 1:256. A subsample (20 µl) of each diluted plasma sample (or AB-serum control) was dispensed into duplicate wells in a microtiter plate (96 wells, flat bottom, 8 × 12 matrix; Nuncon, Denmark). Then 80 µl of the parasite inoculum described above was added to each well and the results were read after a 48 hours incubation period (37°C, in an atmosphere of 80% N_2, 17% O_2 and 3% CO_2 in a candle jar).

Evaluation of results

After 48 hours incubation, a thin blood smear was made from each well and stained with Giemsa so that the number and morphology of rings, trophozoites, and schizonts of *P. falciparum* could be recorded. To ensure that the test system was functioning properly, the control wells were required to show at least a 4 to 5-fold increase in the number of parasitized cells. This was done by counting the number of infected red blood cells per 10,000 red blood cells.

To evaluate the effect of drug-containing plasma on the parasites, samples were examined microscopically for any changes in parasite density and for viability of the remaining parasites. Viable ring stage parasites were characterized by the presence of a round red or purple nucleus and by a large vacuole occupying most of the cytoplasm (delicate blue circular ring). Viable trophozoites were characterized by the presence of a larger amount of nuclear material and cytoplasm and by an absent or smaller central vacuole. Pigment granules were seen in some viable trophozoites. Viable immature schizonts were characterized by more than two nuclei, a bright blue cytoplasm expanded to two-thirds of the erythrocyte, and prominent pigment granules. Viable mature schizonts were characterized by the presence of 12-24 merozoites, each with a small round nucleus surrounded by a minute mass of ovoid cytoplasm, and a big mass of pigment.

Blood schizonticidal activities of the plasma samples were evaluated using the maximum inhibitory dilution (MID) as an end-point. The MID was determined by noting the maximum dilution of drug-containing plasma which completely killed the parasites (at least 99% kill or inhibition; EC_{99}). For ease of comparison of inhibitory effect of drug-containing plasma from various regimens, activity was finally transformed to, and expressed as molar equivalent activity of dihydroartemisinin (DHA_{eq}; nmol/l), using plasma drug concentrations, MID, and relative antimalarial potency of each drug as factors in calculation. The relative potency was determined from the ratio of EC_{50} of dihydroartemisinin and artemether or pyrimethamine against K_1 strain or $T_{9.94}$ *clone P. falciparum*. Overall activity (DHA_{eq}) of sampled plasma after dosing with artemether alone (regimen I) was the combined activity of artemether and its *in vivo* activity meta-bolite, dihydroartemisinin, whereas, activity of sampled plasma after dosing with artemether plus pyrimethamine (regimen III) was the sum activity of artemether and pyrimethamine.

The time couses of *ex vivo* blood schizonticidal activity of sera were analyzed by model-independent method. A_{max} (the maximum blood schizonticidal activity) and t_{Amax} (the time to maximum activity) were directly observed data, and AUA (area under the activity-time curve) was calculated using linear trapezoidal rule.

Statistical analysis

Differences in blood schizonticidal activities (A_{max}, t_{Amax}, AUA) for plasma samples obtained after artemether or pyrimethamine alone and drug combination (ART+PYR) were compared using Wilcoxon Signed Rank test at a statistical significance level of $p = 0.05$.

RESULTS

Morphological changes of *P. falciparum* after being exposed to drug containing plasma

The effects of drug-containing plasma on the viability of *P. falciparum* K_1 strain (pyrimethamine resistant) and $T_{9.94}$ clone (pyrimethamine sensitive) were determined by examination of Giemsa stained slides under the light microscope. In wells with complete inhibition, abnormal and/or dead parasites were observed. Artemether (ART) exerted an effect on erythrocytic parasite stages. Some of the dead ring stages had purple-stained pyknotic nuclei with no cytoplasm (Fig 1a); others had residual cytoplasm which stained blue and had no vacuoles (Fig 1b). Infected red blood cells harboring dead rings showed no change in size, shape and color. Dead immature and mature schizonts had purple nuclei, little cytoplasm and were vacuoled and atrophic (Fig 1c). Some infected red blood cells had lysed, leaving free immature and mature schizonts in the medium; others remained intact but had distorted membranes (Fig 1d).

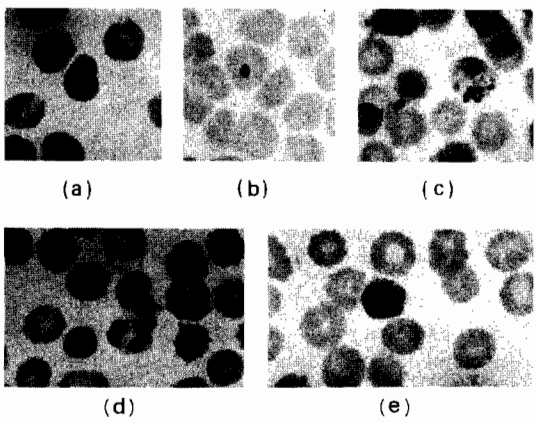


Fig 1–Abnormal or dead parasites after 48 hours exposure to ART (a-d) or PYR (e) containing plasma. (a) and (b), ring stage; (c) and (e), immature and mature schizonts.

Pyrimethamine (PYR) exhibited an effect on dividing parasite stages, *ie* schizonts. All sensitive parasites died at the schizont stage. Infected red blood cells showed no changes in shape, size or color. Dead schizonts were characterized by fragmented nuclei scattered over the cell. The cytoplasm was expanded and stained pale pink or pale blue (depending on the pH of the Giemsa stain) (Fig 1e) and it fully occupied the infected red cell.

Ex vivo blood schizonticidal activities of plasma against *P. falciparum*

Maximum inhibitory dilutions (MIDs). The MID patterns of the drug containing plasma samples against K_1 strain and $T_{9.94}$ clone are shown in Figs 2 and 3. The undiluted plasma from Regimen I (PYR alone) showed no inhibitory effect on the growth of strain K_1 in either RPMI or RPS medium. The MID patterns for plasma from Regimen I (ART alone) and Regimen III (combined ART+PYR) were very similar in both media used (Fig 2). A complete inhibitory effect was detected from hour 0.25 until hour 18. The MID patterns varied markedly over the period of investigation for the range of dilutions between 1 (undiluted plasma) and 1:64. High MIDs (1:8 - 1:64) were noted from 1 hour - 3 hours. Decreasing MIDs were detected from 4 hours through to 18 hours.

The results with $T_{9.94}$ clone contrasted with those from K_1 strain. The plasma samples from Regimen II (PYR alone) showed an inhibitory effect on parasite growth in both RPMI and RPS media. A complete inhibitory effect was seen in RPMI medium at 0.25 hour through to 14 days with various MIDs (1-1:8). In most cases, the highest MIDs were found from 1 hour-18 hours with dilution 1:8 (Fig 3b). Decreasing MIDs were apparent from 2 days - 14 days. The MID pattern for Regimen I (ART alone) was generally similar to that for K_1 strain, except that the highest MID (1:64) was achieved at 0.5 hour-1 hour. The MIDs for ART alone (1:32-1:64) during peak activity (0.25 hour-4 hours) (Fig 3a) were higher than those for PYR alone (1:8). Examining the MID pattern for Regimen III (combined ART+PYR), some additive effect was recognized, although there was no change in the highest MID observed. As shown in Fig 3c, MIDs for most of the combined regimen samples for 0.5 hour-6 hours were > 1:16, which was higher than those for PYR alone (1:8). Also during this period, only half of the total Regimen I samples (ART alone) showed MIDs at this dilution. Prolonged activity and the MID pattern during 18 hours-14 hours was more or less the same as that for Regimen II (PYR alone). With RPS medium, there

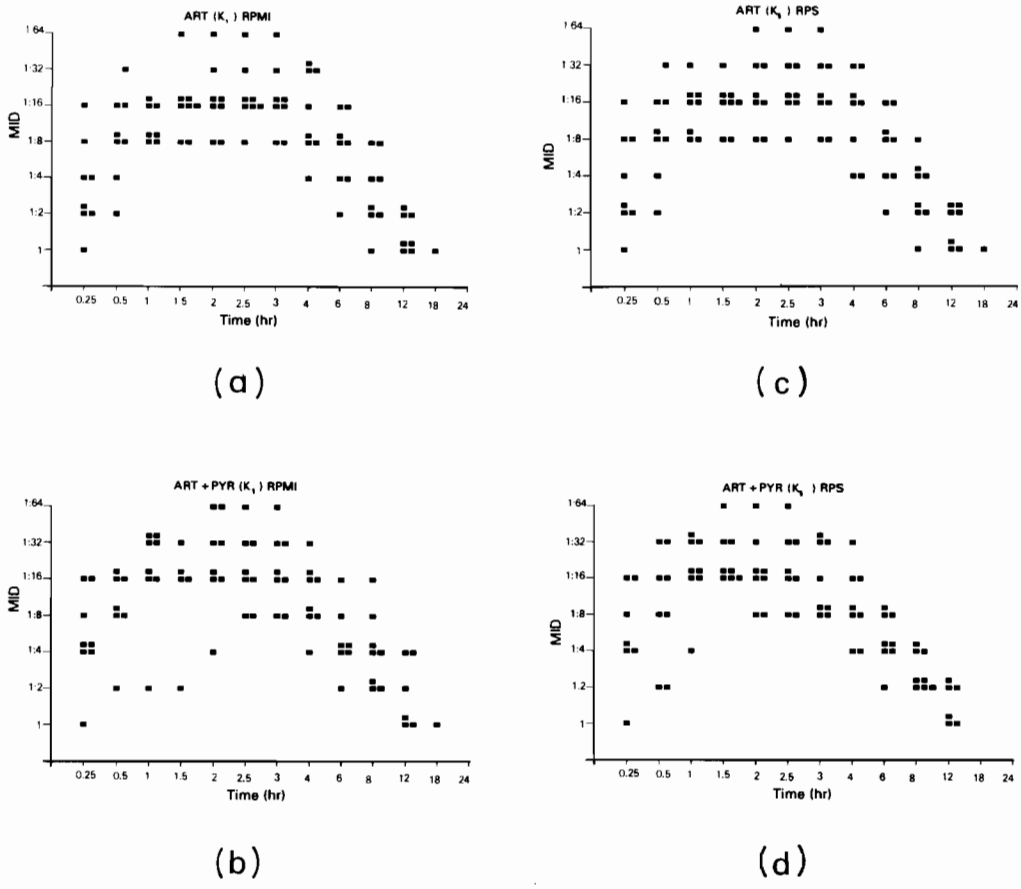


Fig 2–MID patterns for plasma collected from healthy volunteers after drug administration against K_1 strain of *P. falciparum* in RPMI medium (a and b) by (a) Regimen I (ART alone) and (b) Regimen III (ART+PYR); and in RPS medium (c and d) by (c) Regimen I (ART alone) and (d) Regimen III (ART+PYR).

was improved PYR activity (indicated by higher MIDs) and a more pronounced additive effect when compared to RPMI medium. In most cases, the highest MIDs occurred from 1 hour-18 hours at 1 : 64 (Fig 3e), which was higher than that for Regimen I (ART alone) at 1:32 (Fig 3d). The MID patterns for Regimen II (PYR alone) and Regimen III (combined ART+PYR) were similar (Figs 3e, 3f). Interestingly, the highest MIDs for one or two samples from 1 hour-4 hours increased to 1:128. The prolonged activity for both regimens was 10 days, which was shorter than that observed in RPMI medium (*ie* 14 days).

Blood schizonticidal activity of ART and PYR expressed as equivalent concentration of dihydroartemisinin (DHA). EC_{50} values for ART or DHA for the test parasites were found to be the same and independent of the types of media used. EC_{50} values of ART and DHA for K_1 strain were 0.914 nmol/l and 0.181 nmol/l and those for $T_{9.94}$ clone were 0.10 nmol/l and 0.067 nmol/l respectively. For PYR, EC_{50} value of both parasites observed in RPS medium were found to be lower than those in RPMI medium. EC_{50} value of PYR for K_1 strain in RPS and RPMI media were 12,119 and 26,394 nmol/l and those for clone $T_{9.94}$ were 26.2

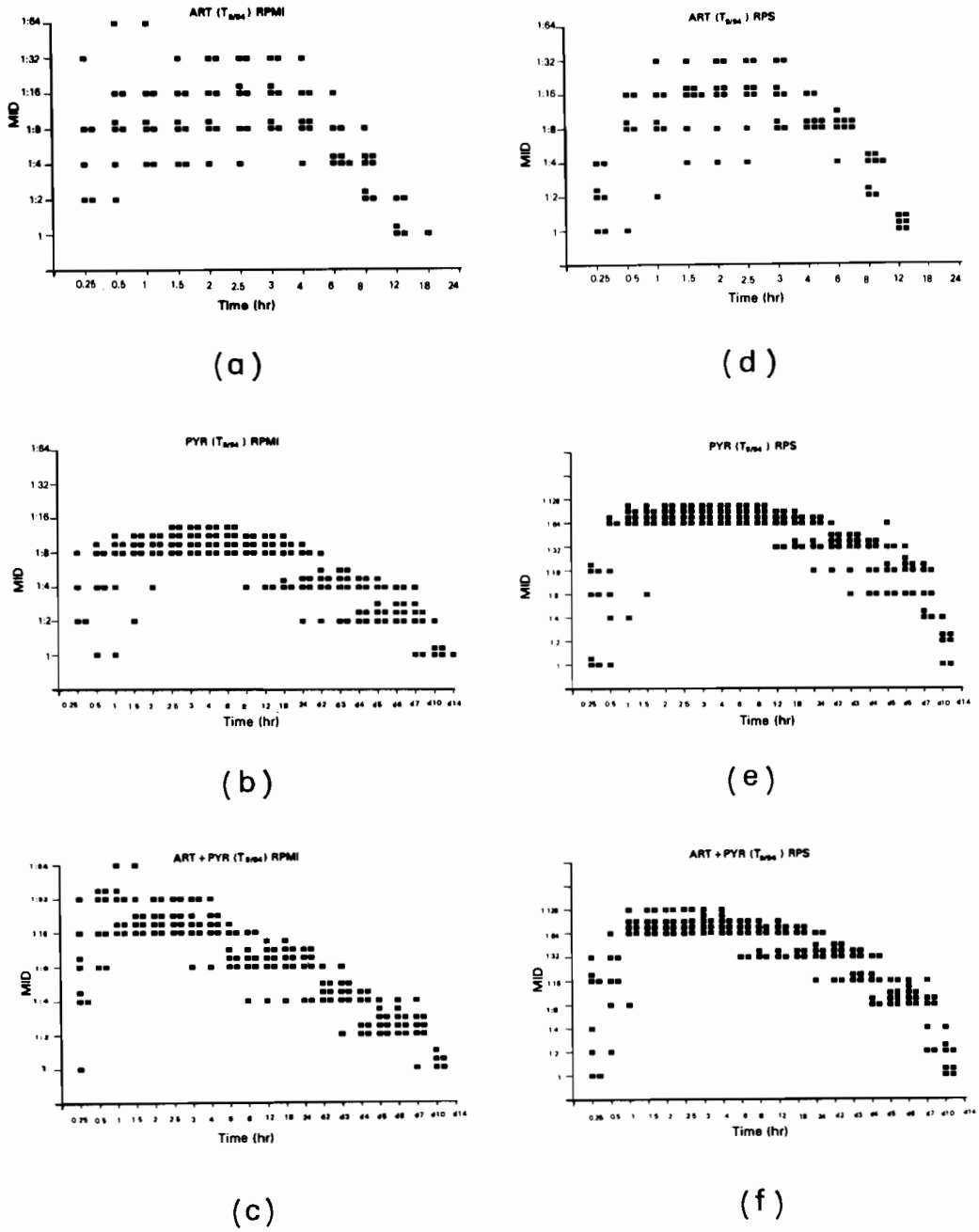
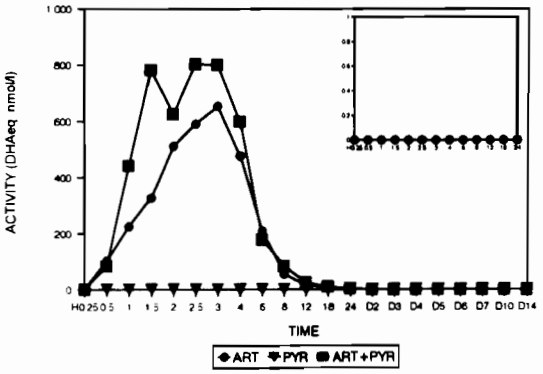
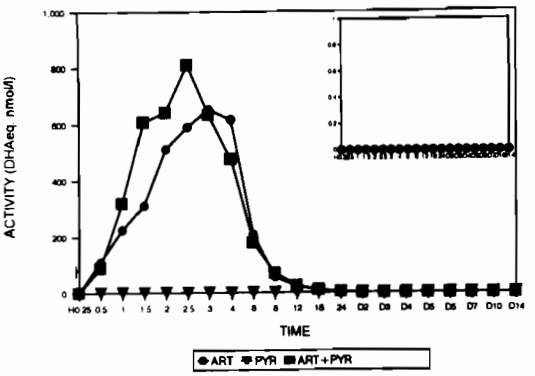


Fig 3–MID patterns for plasma collected from healthy volunteers after drug administration against $T_{9.94}$ clone of *P. falciparum* in RPMI medium (a-c) by (a) Regimen I (ART alone), (b) Regimen II (PYR alone) and (c) Regimen III (ART+PYR); and in RPS medium (d-f) by (d) Regimen I (ART alone), (e) Regimen II (PYR alone) and (f) Regimen III (ART+PYR).

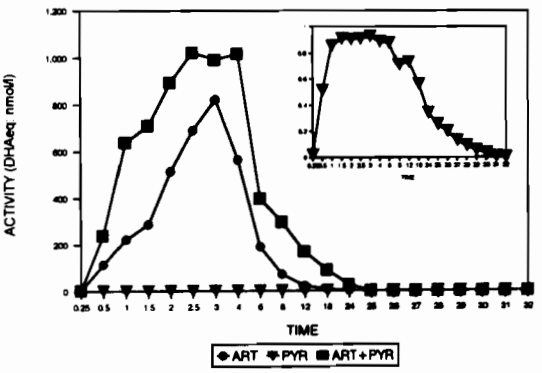


(a)

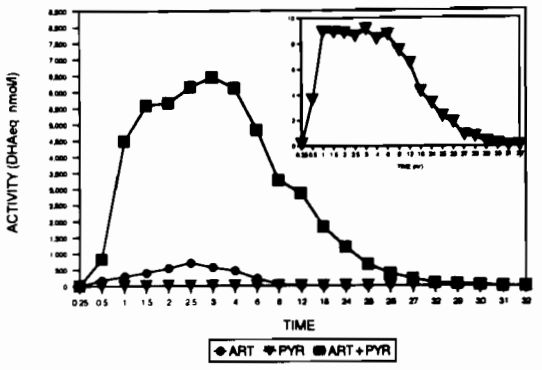


(b)

Fig 4–Blood schizonticidal activity of ART and PYR against *P. falciparum* K_1 strain (RPMI medium) when given singly (ART or PYR) or in combination (ART+PYR) in (a) RPMI or (b) RPS.



(a)



(b)

Fig 5–Blood schizonticidal activity of ART and PYR against *P. falciparum* $T_{9.94}$ clone in (a) RPMI or (b) RPS.

and 34.5 nmol/l respectively. Blood schizonticidal activity-time profiles of plasma from the 3 regimens expressed as DHA_{eq} in both RPMI and RPS media are shown in Figs 4 and 5 and the observed parameters (A_{max}, t_{Amax}, t_{lag}, AUA) are summarized in Tables 1 and 2. The plasma activity patterns for Regimen I (ART alone) and Regimen III (combined ART+PYR) were similar against K_1 strain regardless of the type of medium used (Figs 4a, 4b). Statistical analysis revealed no differences in the maximum activity (A_{max}), the time to reach maximum activity (t_{Amax}), the lag time for activity following drug administration (t_{lag}) and the area under the activity curve (AUA) (Table 1). However, pattern differences were found with $T_{9.94}$ clone in both RPMI and RPS media (Figs 5a, 5b and Table 2). In both media, plasma activity with Regimen III (combined ART+PYR) was higher than with either of the single drug regimens. However, the activity observed in RPS medium was much more pronounced. A_{max} (6,935 nmol/l) and AUA (128,397 nmol.h/l) for Regimen III in RPS medium were significantly higher than those for Regimen I (ART alone) (935 nmol/l; 3,618 nmol.h/l) and Regimen II (PYR alone) (9.9 nmol/l; 334 nmol.h/l) (Table 2). In addition, t_{max} (2.7 hours) for the combined regimen was significantly shorter than that for Regimen I (ART alone) (3 hours) but longer than that for Regimen II (PYR alone) (1.25 hours) (Table 2). A_{max} for Regimen III in RPMI (1,130 nmol/l) was significantly higher than that for Regimen II (PYR alone) (1 nmol/l) but not for Regimen I (ART alone)

(825 nmol/l). Moreover, AUA (8,413 nmol.h/l) for Regimen III was significantly higher than for Regimens I and II (3,003 and 39.9 nmol.h/l, respectively) (Table 2).

DISCUSSION

This study examined *ex vivo* blood schizonticidal activity of plasma collected after administration of ART or PYR alone, compared with that after the combined regimen ART+PYR. Overall activity was expressed as molar equivalent activity of DHA (accounted for by relative potency of ART and PYR compared with DHA) that inhibited > 95% growth inhibition.

For K_1 strain, it was found that PYR alone exerted no inhibitory effect on growth. Plasma samples from subjects on Regimen I (ART alone) and Regimen III (combined ART+PYR) showed similar MID patterns (Fig 2). There were no differences in either the highest MID or the duration of observed activity in both types of media tested. The lack of direct and/or synergistic or additive inhibitory effect of PYR against K_1 strain could be due to its marked resistance to the drug (EC_{50} in RPMI and RPS were 26,394 and 12,119 nM, respectively). The MID patterns of both Regimen I (ART alone) and III (combined ART+PYR) correlated well with the plasma concentration-time profiles of ART and DHA (Tan-ariya *et al* 1998). The highest MID range observed at 1.5 hours-4 hours was within the medium range for times taken to reach peak drug concentration in the plasma (1.5 hours-5 hours). Decreasing MIDs after 6 hours was corresponded with declining drug concentrations in the plasma.

Morphological changes in K_1 strain after exposure to plasma samples from Regimen I (ART alone) and regimen III (ART+PYR) showed complete inhibition by the light microscope. Ring and schizont stages showed abnormalities in both nuclei and cytoplasm as shown in Fig 1 a-d. However, more prominent changes were observed in the cytoplasm. Disorganization and vacuolization of cytoplasm as seen in the trophozoites and schizonts in this study accords well with ultrastructural changes in membranous structures reported for ART by observation with the electron microscope (Ellis *et al*, 1985). The less pronounced effect on nuclei was consistent with the finding of Gu *et al* (1983). It should also be noted that red cells harboring abnormal rings and trophozoites remained intact (Figs 1a, 1b), whereas most of those harboring abnormal immature or mature schizonts lysed (Fig 1d). Such findings were previously noted by Orjih *et al* (1996). Morphological changes observed with the light microscope clearly indicated that negative effects on parasite growth caused by plasma samples from the ART+PYR regimen (Regimen III) were caused by ART alone. This is because there was no evidence of morphological changes (fragile dispersed nuclei) caused by plasma containing PYR alone (Regimen II). This confirmed that PYR had no effect on the growth of K_1 strain in plasma samples after treatment with PYR alone or with ART+PYR. In addition, blood schizonticidal activities of plasma samples against K_1 strain (expressed as equivalent concentrations of DHA, shown in Fig 4 and summarized in Table 1) confirmed the lack of PYR activity in plasma from Regimen II (PYR alone) and Regimen III (ART+PYR).

By contrast, plasma samples from Regimen II (PYR alone) were found to inhibit growth of *P. falciparum* $T_{9\,94}$ clone, a pyrimethamine sensitive clone, in both RPMI and RPS media with highest MID in the latter (Figs 3b, 3e). In general, the MID patterns coincided with drug concentration-time profiles (Tan-ariya *et al,* 1998). The MID pattern observed in RPMI for Regimen III (ART+PYR) was likely the result of the sum effect of ART and PYR. The highest MIDs during the first 4 hours were due to both ART and PYR, whereas those from 6 hours-14 days were mostly due to PYR only (Figs 3a-c). An additive effect of ART and PYR was recognized in the combined regimen since most of the samples showed MID at 1:16 from 0.5 hour-4 hours. During this period, MIDs of plasma from Regimen II (PYR alone) were mostly 1 : 8 and only a few samples from Regimen I (ART alone) showed MIDs at 1:16. Prolonged activity of the ART+PYR regimen after 18 hours was directly due to PYR activity alone as can be seen obviously in Figs 3b and 3c. Examination of dead parasites during the first 4 hours revealed characteristic effects of ART. Increased ART activity during this period might have resulted from the additive activity from PYR. However, the activity after 6 hours till and up to 14 days was due to PYR only, as substantiated by microscopic observations.

When compared with RPMI medium (Fig 3b), a significant enhancement of PYR activity against *P. falciparum* was found in RPS medium (Fig 3e). This confirmed the requirement of an appropriate

Table 1

Blood schizonticidal activity (expressed as DHA_{eq} concentrations) of plasma after regimens of ART and PYR singly or combined against K_1 strain of *P. falciparum*.

	Regimen I (ART alone)	Regimen II (PYR alone)*	Regimen III (ART+PYR)
RPMI:			
A_{max} (nmol/l)	732 (351-5,440)	-	829 (500-3,520)
t_{Amax} (h)	3 (1.5-4)	-	2.25 (1.5-3)
t_{lag} (h)	0.25 (0.25-0.25)	-	0.25 (0.25-0.25)
AUA (nmol.h/l)	2,919 (745-15,471)	-	3,689 (2,311-12,195)
RPS:			
A_{max} (nmol/l)	742 (350-4,050)	-	867 (431-3,520)
t_{Amax} (h)	3 (1.5-4)	-	2.75 (1.5-4)
t_{lag} (h)	0.25 (0.25-0.25)	-	0.25 (0.25-0.25)
AUA (nmol.h/l)	3,564 (720-12,403)	-	3,347 (1,730-9,208)

*No activity was observed with plasma after the PYR regimen.

Table 2

Blood schizonticidal activity (expressed as DHA_{eq} concentrations) of plasma regimens of ART and PYR singly or combined against $T_{9.94}$ clone *P. falciparum*.

	Regimen I (ART alone)	Regimen II (PYR alone)	Regimen III (ART+PYR)
RPMI:			
A_{max} (nmol/l)	825 (397-3,500)	1 (0.84-1.48)	1,130 (703-2,980)[a]
t_{Amax} (h)	3 (2-4)	1.25 (1-4)	2.5 (1.5-4)
t_{lag} (h)	0.25 (0.25-0.25)	0.25 (0.25-0.5)	0.25 (0.25-0.25)
AUA (nmol.h/l)	3,003 (1,151-10,643)	39.9 (25.2-68.9)	8,413 (4,600-12,537)[b]
RPS:			
A_{max} (nmol/l)	935 (397-2,000)	9.9 (5.6-15.6)	6,935 (1,330-13,400)[c]
t_{Amax} (h)	3 (2-4)	1.25 (1-4)	2.75 (1.5-4)[d]
t_{lag} (h)	0.25 (0.25-0.25)	0.25 (0.25-0.25)	0.25 (0.25-0.25)
AUA (nmol.h/l)	3,618 (1,406-5,597)	334 (82.3-733.3)	128,397 (39,274-197,901)[e]

[a] Significantly different from PYR alone (p = 0.0039, 95% C.I. 896, 2,244)

[b] Significantly different from ART alone (p = 0.0039, 95% C.I. 2,271, 6,213) and PYR alone (p = 0.0078, C.I. 5,649, 10,947)

[c] Significantly different from ART alone (p = 0.0078, 95% C.I. 512, 1,612) and PYR alone (p = 0.0078, C.I. 3,711, 10,565)

[d] Significantly different from ART alone (p = 0.027, 95% C.I. 0, 3) and PYR alone (p = 0.026, C.I. 0,2)

[e] Significantly different from ART alone (p = 0.0078, 95% C.I. 1,735, 4,547) and PYR alone (p = 0.0078, C.I. 832,687, 164,498)

culture medium when assessing the sensitivity of malarial parasites to antifolate inhibitors. Similar to the results using RPMI medium, the MID pattern of plasma from Regimen III (ART+PYR) observed in RPS medium seemed to result from the sum effect of the two drugs (Fig 3f). Of interest was the

observation that MIDs of 1 or 2 of these samples from 1 hour - 4 hours were as high as 1 : 128, which was higher than that for Regimen I (ART alone) (1:32) and Regimen II (PYR alone) (1:64). This finding indicated some synergistic effects between ART and PYR. [Microscopic observations showed that the presence of these two drugs resulted in complete inhibition, and that ART exerted its inhibitory effect before PYR]. Dead rings and dead schizonts were characteristic of the action of ART. At the same time, the action of PYR in plasma samples was found only with diluted samples. Most dead parasites were schizonts which showed the characteristics of death due to PYR. After the ART had dissipated (after 6 hours), all parasites were inhibited by PYR. The rapid onset of action by ART seen in this study confirmed results from previous reports by Gu *et al* (1983) and Geary *et al* (1989).

When blood schizonticidal activities were expressed as molar equivalent activity of DHA, it was found that the plasma activity patterns and MID patterns for all regimens coincided. With K_1 strain, no PYR activity was detectable in plasma for either Regimen II (PYR alone) or Regimen III (ART+PYR) though relatively higher activity was observed with Regimen III than with Regimen I (ART alone) (Figs 4a, 4b and Table 1). It was found that RPS gave higher activity than RPMI with Regimen III (ART+PYR) samples against $T_{9.94}$ clone (Figs 5a, 5b and Table 2). An obvious synergistic effect of ART and PYR could be seen in RPS by reference to A_{max} and AUA. Significantly higher MIDs and blood schizonticidal activities (as DHA equivalents) were seen during the first 4 hours after administration of ART+PYR, possibly due partly to an increased C_{max} for PYR as recognized from a pharmacokinetic study (Tan-ariya *et al,* 1998, In press).

Our results did not reveal any antagonistic effect from the combination of ART and PYR as previously reported (Chawira and Warhurst, 1987; Chawira *et al*, 1987). On the contrary, the combination seemed to work synergistically against the PYR sensitive clone $T_{9.94}$ of *P. falciparum*. Although this synergistic effect was not observed with the highly PYR resistant strain K_1, neither was any antagonistic effect evident. A significant difference between this study and those previously reporting antagonism was that antimalarial activity in this study was evaluated from drug-containing plasma collected from volunteers, whereas the previous studies were carried out using drug solutions. Although no precise explanation can be offered to account for the different findings, some pharmacokinetic interaction between ART and PYR in *vivo* may be responsible. For example, ART may increases the C_{max} of PYR. In addition, results from tests using K_1 strain should be taken carefully because of its high degree of resistance to PYR. In this respect, K_1 strain is probably not a good representative of the *P. falciparum* isolates normally found in patients. More studies are needed using fresh isolates from infected individuals.

In vivo studies on the co-administration of artemisinin or ART with PYR have led to contradictory reports. Some groups of investigators have reported synergism (Li *et al*, 1984; Cai *et al*, 1981, 1985) or potentiating effects whereas others have reported antagonistic effects (Naing *et al*, 1988). Differences in drug dosages, routes of administration and parasite drug susceptibility may have led to these contradictory results. A report on *P. berghei* in a mouse model also claimed antagonistic effects between ART and PYR (Chawira *et al*, 1987). However, this effect in *P. berghei* might be partly explained by the presence of high percentages of infected reticulocytes during the course of treatment. This results from the predilection of the parasite for young erythrocytes (Garnham, 1966). A study by Waki *et al* (1987) has shown that *P. berghei* in reticulocytes is less sensitive to artemisinin and its derivatives than in *P. berghei* normocytes. This phenomenon may complicate interpretation of the results in the mouse model.

A preliminary clinical study of combined PYR and ART use in the treatment of uncomplicated falciparum malaria at the Thai-Myanmar border by Na-Bangchang *et al* (1996) revealed a rather promising clinical efficacy. The use of a 3-day combination regimen (300 mg ART plus 100 mg PYR on the first day, then 150 mg ART plus 50 mg PYR on the second and third days) gave a cure rate of up to 75%. This high cure rate was even obtained in areas where PYR-resistant *P. falciparum* had been documented.

The results of the present study and of Na-Bangchang *et al* (1996) are encouraging because they suggest a possible alternative drug treatment for *P. falciparum* in Thailand or in other endemic areas where *P. falciparum* is still sensitive to PYR, *eg* Africa. Appropriate doses of each drug and optimal combination ratios should be worked out using pharmocokinetic modeling before patients are treated (Karbwang J, personal communication).

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