Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Parasitology 106 (2004) 50–55
Experimental Parasitology
www.elsevier.com/locate/yexpr

Plasmodium falciparum: selenium-induced cytotoxicity to *P. falciparum*

Nao Taguchi,[a] Toshimitsu Hatabu,[a,*] Haruyasu Yamaguchi,[a] Mamoru Suzuki,[b] Kumiko Sato,[a] and Shigeyuki Kano[c]

[a] *Gunma University School of Health Sciences, 3-39-15 Showa-machi, Maebashi 371-8514, Japan*
[b] *Gunma University Graduate School of Medical Sciences, 3-39-22 Showa-machi, Maebashi 371-8511, Japan*
[c] *Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan*

Received 4 September 2003; received in revised form 15 January 2004; accepted 23 January 2004

Abstract

The in vitro antimalarial activity of sodium selenite (NaSe) was investigated and the mechanism of its action was studied. NaSe had antimalarial activity against both the chloroquine-susceptible strain FCR-3 and chloroquine-resistant strain K-1 of *Plasmodium falciparum*. The shrunken cytoplasm of the parasite was observed in a smear 12 h after treatment with NaSe. Co-treatment with copper sulfate ($CuSO_4$) in culture did not affect the antimalarial activity of NaSe, but NaSe cytotoxicity against the mammalian cell line Alexander was decreased significantly. The intracellular reduced glutathione level of parasitized red blood cells was decreased significantly by treatment with NaSe, and the decrease was consistent with their mortality. Treatment with NaSe had a strong inhibitory effect on plasmodial development, and NaSe cytotoxicity to human cells was decreased by co-treatment with $CuSO_4$. These results suggest that co-treatment with NaSe and $CuSO_4$ may be useful as a new antimalarial therapy.

Index Descriptors and Abbreviations: Plasmodium falciparum; antiplasmodial activity; sodium selenite; pRBCs, parasitized red blood cells; RBCs, red blood cells; GSH, reduced glutathione; NaSe, sodium selenite; $CuSO_4$, copper sulfate

1. Introduction

Drug resistance in malaria is an important public health concern. *Plasmodium falciparum* has developed resistance to most of the commonly used antimalarial drugs including chloroquine, sulfadoxine–pyrimethamine, and mefloquine (Wongsrichanalai et al., 2002). Therefore, a search for new antimalarial drugs is very important.

Selenium is an essential trace element in mammals. Numerous epidemiological and experimental studies have found an anticarcinogenic activity of selenium (Clark et al., 1996; Combs and Gray, 1998; Comstock et al., 1992; Siwek et al., 1994; Spallholz, 1994; Spyrou et al., 1996; Thompson et al., 1994). Recently, it has been argued that the anticarcinogenic activity of selenium depends on its ability to generate superoxide, which is known for its DNA-damaging activity and induction of apoptosis (Lu et al., 1994).

There are some preliminary experiments suggesting that oxidative stress is the cause of selenium cytotoxicity and growth inhibition. Some investigators reported that the superoxide anion was generated by the reaction of sodium selenite (NaSe) with reduced glutathione (GSH) in a cell-free system (Davis and Spallholz, 1996). Other investigators reported that the cytotoxicity of selenium was decreased by co-treatment with copper sulfate ($CuSO_4$) (Jensen, 1975). But the mechanisms of these beneficial reactions are not known.

Since the reactive oxygen species is known to have the toxic effects for the malaria parasite and NaSe generate the superoxide and consume the intracellular GSH, we have evaluated the antimalarial effect of NaSe and investigated its interaction with intracellular GSH for development of new antimalarial drug to resolve drug resistance problem.

* Corresponding author. Fax: +81-27-220-8915.
E-mail address: hatabu@health.gunma-u.ac.jp (T. Hatabu).

0014-4894/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2004.01.005

2. Materials and methods

2.1. Parasites and cells

Chloroquine-susceptible *P. falciparum* strain FCR-3 and chloroquine-resistant *P. falciparum* strain K-1 were grown asynchronously, following the modified method of Trager and Jensen (1976), in RPMI 1640 medium supplemented with 10% human B serum, 25 mM Hepes, 25 μg/ml gentamicin (Sigma–Aldrich, St. Louis, MO), sodium bicarbonate, and human type O red blood cells (RBCs) in disposable sterile dishes under a controlled atmosphere of 5% CO_2 at 37 °C.

The human hepatocellular carcinoma cell line Alexander (Alex cells) was a kind gift of Dr. Takeaki Nagamine, Gunma University School of Health Sciences. Alex cells were grown continuously in complete Dulbecco's modified Eagle's medium (DMEM; BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 U/ml, 10 ml/liter; BRL, Grand Island, NY) under a 5% CO_2 atmosphere at 37 °C.

2.2. Investigation of in vitro plasmocidal effect of NaSe

Since several studies have shown effective anticarcinogenic activity of NaSe between 10 and 100 μM (Batist et al., 1986; Caffrey and Frenkel, 1992; Davis et al., 1998), RPMI 1640 medium was supplemented with either a 10 or 100 μM NaSe solution that was sterilized by filtration through a 0.22-μm filter. The NaSe-supplemented medium was changed every 24 h. Five hundred microliters of a parasitized-RBCs (pRBCs) suspension was placed in each well of a 24-well flat bottom culture plate (Corning Costar, New York, NY) at a hematocrit of 5% and an initial parasitemia of 0.1%. Thin-smeared specimens stained with Giemsa solution were made every 24 h and parasitemia was evaluated by counting the parasites in 3000 RBCs. Morphological changes of the parasites were monitored by observing Giemsa-stained thin-smeared specimens 2, 4, 12, and 24 h after treatment.

2.3. Determination of IC_{50} of NaSe against parasite

The pRBCs were synchronized with 5% D-sorbitol for 15 min at room temperature as described by Lambros and Vanderberg (1979) and then were washed three times with RPMI 1640 medium by centrifugation at 400*g* for 5 min. After washing, the pRBCs were resuspended in RPMI 1640 medium supplemented with 10% human B serum, 25 mM Hepes, 25 mM gentamicin, and sodium bicarbonate. Synchronous pRBCs having about 2% parasitemia were placed in 24-well cell culture plates as described above. Twenty microliters of NaSe solution was added to each well to give a series of concentrations from 10 to 100 μM. Six wells per plate served as NaSe-free controls to monitor parasite growth. After 24 h of incubation under a 5% CO_2 atmosphere at 37 °C, the control wells were checked for parasite growth. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from the contents of each well. We first counted RBCs in the control smears until we encountered a total of 50 schizonts. The effect of NaSe on parasite growth was evaluated by comparing the number of schizonts in the same number of RBCs as was counted in the control cultures. The growth inhibition effect (%) was calculated as follows: (test well schizont count/control well schizont count) × 100. The NaSe concentration inhibiting parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba et al., 2001).

2.4. Detection of hemolysis caused by treatment with NaSe and $CuSO_4$

Hemolytic level was determined by measuring the hemoglobin that eluted into the medium with the SLS-hemoglobin method (Hemoglobin B test Wako, Wako Pharmaceutical, Osaka, Japan). Briefly, after exposure of pRBCs or RBCs to 40 μM NaSe that completely inhibited parasite growth and 2.5–40 μM $CuSO_4$, as described above, samples were centrifuged at 1000*g* for 5 min at 20 °C, and the supernatant was collected and analyzed. These Se:Cu ratios were shown to decrease NaSe toxicity against carcinoma cells (Shen et al., 2001).

2.5. Cytotoxicity of NaSe and $CuSO_4$ to P. falciparum and Alex cells

Synchronized FCR-3 parasitized erythrocytes having about 2% parasitemia were cultured in 24-well cell culture dishes. Each well, containing 500 μl of a 5%—hematocrit RBC suspension was supplemented with a 100% growth inhibition (IC_{100})—concentration of NaSe and various concentrations of $CuSO_4$ (2.5–40 μM). The remaining procedures were the same as described above. The growth inhibition effect was calculated as shown above. Morphological changes of the parasites in these conditions were monitored by observing Giemsa-stained thin-smeared specimens at 2, 4, 12, and 24 h after treatment.

To assess NaSe and $CuSO_4$ cytotoxicity to a mammalian cell line, the same experiment was carried out with Alex cells. Alex cells were seeded at 0.5×10^6 cells/ml in 24-well culture plates and incubated (37 °C, 5% CO_2) 24 h in 1 ml of DMEM supplemented with NaSe (40 μM) and $CuSO_4$. After the incubation period, detached cells were collected; attached cells were rinsed in the wells once with phosphate-buffered saline (PBS, pH 7.4) and then trypsinized to remove them from the solid

matrix. Cells were then collected by centrifugation (10 min, 830*g*, 25 °C). Cell viability was assessed by trypan blue exclusion.

2.6. GSH measurement in pRBCs, normal RBCs, and Alex cells

Intracellular GSH measurement was done according to Hissin and Hilf's method (Hissin and Hilf, 1976). Briefly, after treatment with NaSe and $CuSO_4$, cells were counted with a hemocytometer. After washing with PBS, homogenization buffer (4:1 mixture of 0.1 M sodium phosphate, 0.005 M EDTA buffer, pH 8, and 25% metaphosphoric acid) was added. All suspensions were homogenized on ice with a sonic wave homogenizer for 40 s. The homogenate was centrifuged (30 min, 21000*g*, 4 °C), and the supernatant was collected for analysis. After addition of fluorescent agent *o*-phthalaldehyde (0.1% in methanol), GSH level was determined with a multi-well plate reader (CytoFluor, Perspective Biosystem, Framingham, MA) at an excitation wavelength of 360 nm and an emission wavelength of 420 nm. Standards contained 0–2 μg GSH/ml.

2.7. Data analysis

Data are presented as means ± SEM from at least three sets of independent experiments. Student's *t* test was used for statistical analyses. A P value <0.05 was considered statistically significant.

3. Results

3.1. The plasmocidal activity of NaSe to chloroquine-susceptible and -resistant P. falciparum

To confirm the plasmocidal activity of NaSe, chloroquine-susceptible and -resistant *P. falciparum* were exposed to medium containing either 10 or 100 μM NaSe for 3 days and growth inhibition was monitored by determining the parasitemia (Fig. 1). In the presence of 10 μM NaSe, the growth of chloroquine-susceptible FCR-3 caused less parasitemia (1.14 ± 0.34%) than in the control culture (2.41 ± 0.30%, $P < 0.0005$, Fig. 1A). The growth of chloroquine-resistant K-1 also caused less parasitemia (0.20 ± 0.06%) than in the control culture (1.29 ± 0.01%, $P < 0.005$, Fig. 1B). In the presence of 100 μM NaSe, the growth of both strains was inhibited completely.

Results of the in vitro drug susceptibility tests using NaSe are shown in Fig. 1C. The IC_{50} values for NaSe were 21.26 ± 0.94 μM for FCR-3 and 15.50 ± 1.60 μM for K-1. There was no significant difference between IC_{50} values of chloroquine-susceptible FCR-3 and -resistant K-1 ($P > 0.05$). No intact parasites were observed after

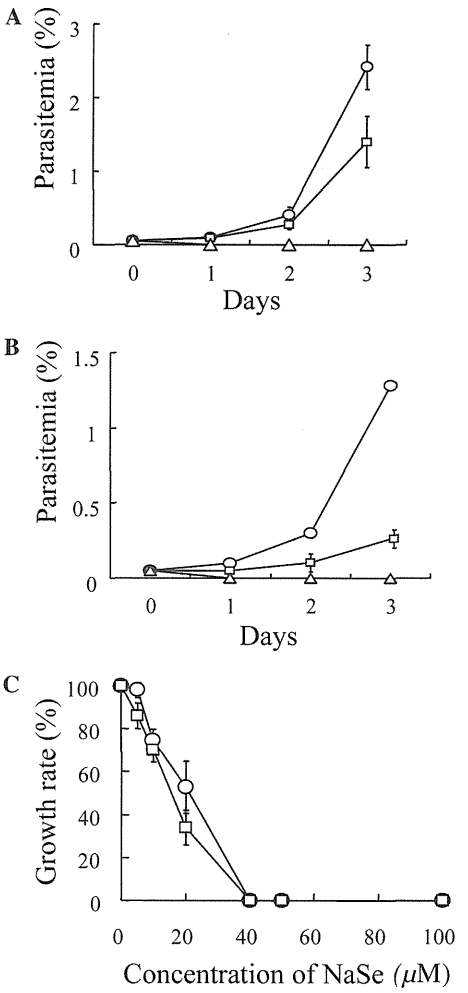

Fig. 1. Antimaralial effect of NaSe. NaSe was added to cultures of chloroquine-susceptible strain FCR-3 (A) and chloroquine-resistant strain K-1 (B) at concentrations of 100 μM (triangle), 10 μM (square), and 0 μM (circle). Parasitemia (%) was determined every 24 h. (C) Dose-dependent effect of NaSe on the inhibition of FCR-3 (circle) and K-1 (square) maturation in vitro. K-1 has less sensitivity than does FCR-3. Results are presented as means ± SEM. SE bars smaller than symbols are not shown.

treatment with 40 μM NaSe. For this reason, 40 μM NaSe was applied in the following experiments.

3.2. Effect of $CuSO_4$ on NaSe toxicity to Alex cells or FCR-3

The effect of $CuSO_4$ in combination with NaSe on parasite growth was investigated. Although the mortality of the Alex cells was 30.01 ± 10.00% upon exposure to medium containing 40 μM NaSe, the mortality of Alex cells was decreased by co-treatment with $CuSO_4$ (Fig. 2). In the presence of either 2.5 or 40 μM $CuSO_4$, the susceptibility of the parasites to NaSe was not affected. Mortality of parasites was higher (50.0 ± 15.9%) than that of Alex cells (0.13 ± 1.50%) upon exposure to 20 μM $CuSO_4$ only.

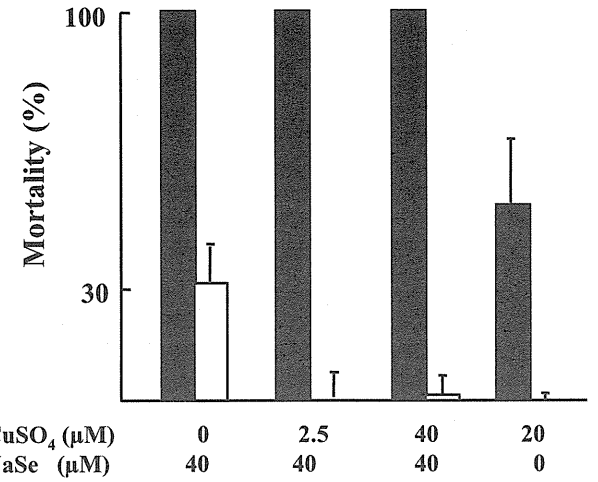

Fig. 2. Cell mortality after treatment with NaSe and $CuSO_4$ for 24 h. Trypan blue exclusion was used to assess mortality of Alexander cells (white bar). The effect of sodium selenite on *P. falciparum* growth was evaluated by comparing the number of schizonts in test erythrocytes with the number in the control cultures (black bar). The results are presented as means ± SEM.

3.3. Hemolysis caused by NaSe and $CuSO_4$

To detect hemolysis as an index of cytotoxicity, the concentration of hemoglobin in pRBCs and RBCs culture media was determined. No hemolysis was detected in the pRBCs culture medium in the presence of 40 μM NaSe only or of 20 μM $CuSO_4$ only. In the presence of 40 μM NaSe with 40 and 20 μM $CuSO_4$, the concentration of hemoglobin was significantly higher (6.53 ± 0.29 g/dl) than in the control culture (0.37 ± 0.05 g/dl, $P < 0.005$) (Table 1). At $CuSO_4$ concentrations less than 10 μM, the concentration of hemoglobin ranged from 0.53 ± 0.05 to 0.69 ± 0.22 g/dl, as compared to the control level of 0.37 ± 0.05 g/dl. These values were not significantly different ($P > 0.05$). Similar results were observed in RBCs culture.

Table 1
Lysis of pRBCs or RBCs induced by NaSe and $CuSO_4$

Concentration of NaSe/Cu (μM)	Hemoglobin (g/dl)[a]	
	pRBCs	RBCs
40/40	6.53 ± 0.29	12.08 ± 0.23
40/20	1.97 ± 0.15	4.16 ± 0.77
40/10	0.53 ± 0.05	0.63 ± 0.13
40/5	0.57 ± 0.08	0.56 ± 0.06
40/2.5	0.59 ± 0.13	0.73 ± 0.13
40/0	0.69 ± 0.22	0.59 ± 0.00
0/20	0.61 ± 0.19	0.50 ± 0.06
0/0	0.37 ± 0.05	0.49 ± 0.05

[a] Each value represents mean ± SEM.

3.4. Morphological changes of parasites exposed to NaSe and $CuSO_4$

Fig. 3 shows the morphological changes of the parasites when treated with 40 μM NaSe and 2.5 μM $CuSO_4$. In the control culture, only intact ring-form parasites were present after 4 h (Fig. 3A). Four hours after treatment with 40 μM NaSe and 2.5 μM $CuSO_4$, a small number of shrunken parasites were observed (Fig. 3B). In the presence of 40 μM NaSe and no $CuSO_4$, similar morphological changes of the parasites were observed (Fig. 3C). Twelve hours after treatment, most of the parasites were early and late trophozoites in culture with 0 μM NaSe and 0 μM $CuSO_4$ (Fig. 3D). Intact parasites were undetectable and almost all the parasites were shrunken after treatment with 40 μM NaSe and 2.5 μM $CuSO_4$ (Fig. 3E) and with 40 μM NaSe and 0 μM $CuSO_4$ (Fig. 3F).

3.5. Changes of intracellular GSH content after treatment with NaSe and $CuSO_4$

To evaluate the intracellular redox condition after treatment with NaSe and $CuSO_4$, intracellular GSH contents were determined. As shown in Fig. 4, intracellular GSH levels decreased significantly after a single treatment with 40 μM NaSe in pRBCs (33.2%), RBCs (27.5%), and Alex cells (36.2%), as compared with control GSH content ($P < 0.05$). $CuSO_4$ could prevent the depletion of intracellular GSH contents in Alex cells, even at 2.5 μM, but not in pRBCs and RBCs.

4. Discussion

Selenium is an essential trace element in humans. It exists in a number of forms with differing valence states, some of which have antineoplastic activity. The usual form of selenium used for supplementation is either selenite or selenomethionine; both have been given in doses up to 200 μg without toxicity. We studied the plasmocidal activity of NaSe against chloroquine-susceptible and -resistant *P. falciparum* strains. Our results showed that both *P. falciparum* strains were susceptible to the cytotoxic effects of NaSe and that the susceptibility to NaSe-induced cytotoxicity was unaffected by co-treatment with $CuSO_4$. In contrast, no cytotoxic effect of NaSe was found in Alex cells upon co-treatment with $CuSO_4$. These results suggest a selective inhibition of plasmodia by NaSe and $CuSO_4$. However, in considering the use of NaSe in antimalarial treatment, it will be a critical issue to select the appropriate dose to optimize both the plasmocidal effect and minimize adverse effects to host cells.

Growth inhibition by NaSe of all parasite developmental stages in vitro is obviously different from the

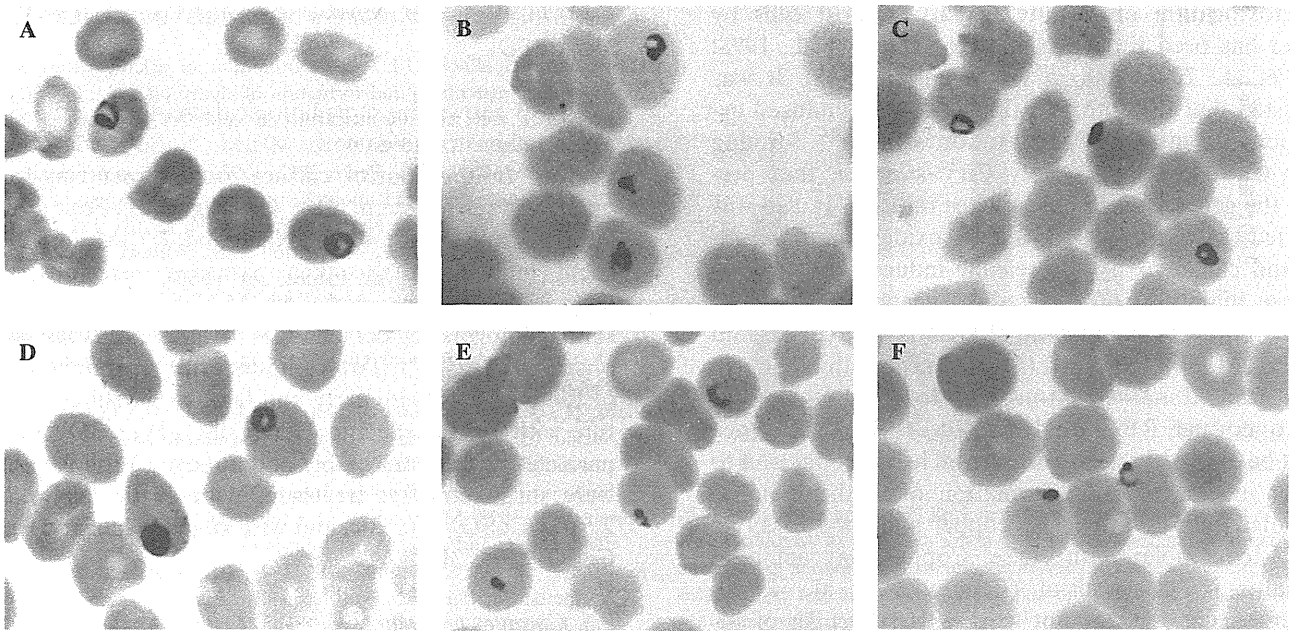

Fig. 3. Light micrographs of Giemsa-stained blood smears. (A) The parasite culture without NaSe or $CuSO_4$ for 4 h, (B) with 40 μM NaSe and 2.5 μM $CuSO_4$ for 4 h, (C) with 40 μM NaSe and 0 μM $CuSO_4$ for 4 h, (D) without NaSe or $CuSO_4$ for 12 h, (E) with 40 μM NaSe and 2.5 μM $CuSO_4$ for 12 h, and (F) with 40 μM NaSe and 0 μM $CuSO_4$ for 12 h (magnification, 1000×).

chloroquine effect on growth (data not shown). The shrunken and pyknotic appearance of parasites was observed by light microscopy (Fig. 3). Similar morphological changes of the parasites have been observed in owl monkeys infected with *P. falciparum* and treated with the Qhinghaosu derivative artemether (Kawai et al., 1993). Qhinghaosu derivatives, including artemether, are sesquiterpene lactone endoperoxide antimalarials and act through the generation of free radicals that alkylate parasite proteins (Meshnick et al., 1989). It is also well known that NaSe-induced cell death is induced by oxidative stress associated with selenite metabolism and that selenite metabolism is very sensitive to cell GSH (Davis and Spallholz, 1996; Seko and Imura, 1997; Shen et al., 2000). Morphological changes shown in this study and these facts indicate that the plasmocidal action induced by NaSe is mediated by mechanisms similar to those associated with Qhinghaosu derivatives.

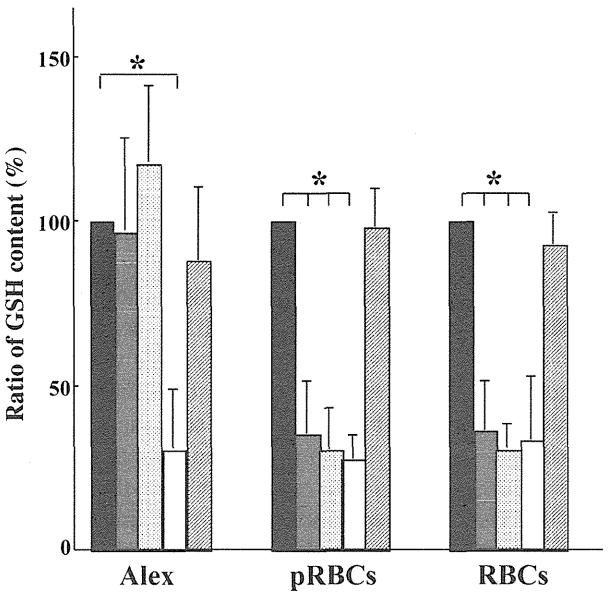

Fig. 4. The ratio of intracellular GSH content in Alexander (Alex) cells, pRBCs, and RBCs after treatment with NaSe and $CuSO_4$ to that in control cells. Results are presented as means ± SEM. The black filled column represents the control ratio for cells without NaSe and $CuSO_4$. The gray shaded column represents the ratio after treatment with 40 μM NaSe and 40 μM $CuSO_4$, the spotted column represents the ratio with 40 μM NaSe and 2.5 μM $CuSO_4$, the open column represents the ratio with 40 μM NaSe and 0 μM $CuSO_4$, and the hatched column represents the ratio with 0 μM NaSe and 20 μM $CuSO_4$. *$P < 0.05$.

No different morphological changes of the parasites were observed between those treated with NaSe alone and with NaSe and $CuSO_4$. Treatment of human cells with NaSe and $CuSO_4$ is considered beneficial. The copper cation did not block selenite-induced oxidative stress in erythrocytes, as it does in other cells (Davis et al., 1998; Shen et al., 2001). Our data have also shown the prevention of GSH consumption by $CuSO_4$ in Alex cells. Several in vitro studies have reported that selenite treatments lead to a decline of intracellular GSH level, and co-treatment with copper prevents that decrease (Davis et al., 1998; Shen et al., 2000, 2001). However, in RBCs, GSH consumption by NaSe was not affected by $CuSO_4$. These results suggest that parasites might be killed in the same or a similar way as that seen after a single treatment with NaSe.

Detoxification of selenite toxicity in host cells by copper has been shown in in vitro (Davis et al., 1998; Shen et al., 2001) and in vivo (Jensen, 1975). It was suggested that the detoxification of selenite-induced cytotoxicity is caused by complexation with Cu^{2+}, forming GSSe–Cu^{2+}–SeSG or simply Cu^{2+}-selenide, that prevents the generation of oxygen-free radicals. It also was reported that Cu^{2+} acts as a superoxide dismutase mimic and can detoxify the selenite-induced cytotoxicity because inhibition of free radical generation was observed at a Cu:Se molar ratio of less than 1:1 (Davis and Spallholz, 1996). However, the treatment with NaSe and $CuSO_4$ caused marked cytotoxicity to *P. falciparum* but not to normal RBCs and Alex cells. This difference might be due to charge transfer from hemoglobin iron to copper in erythrocytes. Formation of methemoglobin prevented charge transfer to copper and took away its ability to complex with NaSe. Copper may act as a generator of free radical (Fernandes et al., 1988). Therefore, $CuSO_4$ may not work as a scavenger of superoxide or an inhibitor of the conversion of selenite to selenium by consumption of GSH in RBCs.

In conclusion, NaSe has a strong inhibitory effect on in vitro plasmodial development and is devoid of cytotoxicity towards human cells if there is co-treatment with $CuSO_4$. Co-treatment with NaSe and $CuSO_4$ may be a useful antimalarial regimen.

Acknowledgments

The authors thank Dr. Hiroshi Koyama and Dr. Hiroyuki Oku for their valuable comments.

References

Batist, G., Katki, A.G., Klecker Jr., R.W., Myers, C.E., 1986. Selenite-induced cytotoxicity of human leukemia cell: interaction with reduced glutathione. Cancer Research 46, 5482–5485.

Caffrey, P.B., Frenkel, G.D., 1992. Selenite cytotoxicity in drug resistant and nonresistant human ovarian tumor cells. Cancer Research 52, 4812–4816.

Clark, L.C., Combs Jr., G.F., Turnbull, B.W., Slate, E.H., Chalker, D.K., Chow, J., Davis, L.S., Glover, R.A., Graham, G.F., Gross, E.G., Krongrad, A., Lesher Jr., J.L., Park, H.K., Sanders Jr., B.B., Smith, C.L., Taylor, J.R., 1996. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. The Journal of the American Medical Association 276, 1957–1963.

Combs Jr., G.F., Gray, W.P., 1998. Chemopreventive agents: selenium. Pharmacology and Therapeutics 79, 179–192.

Comstock, G.W., Bush, T.L., Helzlsouer, K., 1992. Serum retinol, beta-carotene, vitamin E, and selenium as related to subsequent cancer of specific sites. American Journal of Epidemiology 135, 115–121.

Davis, R.L., Spallholz, J.E., 1996. Inhibition of selenite-catalyzed superoxide generation and formation of elemental selenium (Se^0) by copper, zinc, and aurintricarboxylic acid (ATA). Biochemical Pharmacology 51, 1015–1020.

Davis, R.L., Spallholz, J.E., Pence, B.C., 1998. Inhibition of selenite-induced cytotoxicity and apoptosis in human colonic carcinoma (HT-29) cells by copper. Nutrition and Cancer 32, 181–189.

Fernandes, A., Mira, M.L., Azevedo, M.S., Manso, C., 1988. Mechanisms of hemolysis induced by copper. Free Radical Research Communications 4, 291–298.

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74, 214–226.

Inaba, H., Ohmae, H., Kano, S., Faarado, L., Boaz, L., Leafasia, J., Suzuki, M., 2001. Variation of incubation time in an in vitro drug susceptibility test of *Plasmodium falciparum* isolates studied in the Solomon Islands. Parasitology International 50, 9–13.

Jensen, L.S., 1975. Modification of a selenium toxicity in chicks by dietary silver and copper. The Journal of Nutrition 105, 769–775.

Kawai, S., Kano, S., Suzuki, M., 1993. Morphologic effects of artemether on *Plasmodium falciparum* in *Aotus trivirgatus*. American Journal of Tropical Medicine and Hygiene 49, 812–818.

Lambros, C., Vanderberg, J.P., 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. Journal of Parasitology 65, 418–420.

Lu, J., Kaeck, M., Jiang, C., Wilson, A.C., Thompson, H.J., 1994. Selenite induction of DNA strand breaks and apoptosis in mouse leukemic L1210 cells. Biochemical Pharmacolgy 47, 1531–1535.

Meshnick, S.R., Tsang, T.W., Lin, F.B., Pan, H.Z., Chang, C.N., Kuypers, F., Chiu, D., Lubin, B., 1989. Activated oxygen mediates the antimalarial activity of qinghaosu. Progress in Clinical and Biological Research 313, 95–104.

Seko, Y., Imura, N., 1997. Active oxygen generation as a possible mechanism of selenium toxicity. Biomedical and Environmental Sciences 10, 333–339.

Shen, C.L., Song, W., Pence, B.C., 2001. Interactions of selenium compounds with other antioxidants in DNA damage and apoptosis in human normal keratinocytes. Cancer Epidemiology Biomarkers and Prevention 10, 385–390.

Shen, H., Yang, C., Liu, J., Ong, C., 2000. Dual role of glutathione in selenite-induced oxidative stress and apoptosis in human hepatoma cells. Free Radical Biology and Medicine 28, 1115–1124.

Siwek, B., Bahbouth, E., Serra, M.A., Sabbioni, E., de Pauw-Gillet, M.C., Bassleer, R., 1994. Effect of selenium compounds on murine B16 melanoma cells and pigmented cloned pB16 cells. Archives of Toxicology 68, 246–254.

Spallholz, J.E., 1994. On the nature of selenium toxicity and carcinostatic activity. Free Radical Biology and Medicine 17, 45–64.

Spyrou, G., Bjornstedt, M., Skog, S., Holmgren, A., 1996. Selenite and selenate inhibit human lymphocyte growth via different mechanisms. Cancer Research 56, 4407–4412.

Thompson, H.J., Wilson, A., Lu, J., Singh, M., Jiang, C., Upadhyaya, P., el-Bayoumy, K., Ip, C., 1994. Comparison of the effects of an organic and an inorganic form of selenium on a mammary carcinoma cell line. Carcinogenesis 15, 183–186.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug-resistant malaria. Lancet Infectious Diseases 2, 209–218.

THE PHARMACOKINETICS OF ORAL DIHYDROARTEMISININ AND ARTESUNATE IN HEALTHY THAI VOLUNTEERS

K Na-Bangchang[1], S Krudsood[2], U Silachamroon[2], P Molunto[2], O Tasanor[1], K Chalermrut[2], N Tangpukdee[2], O Matangkasombut[3], S Kano[4] and S Looareesuwan[2]

[1]Faculty of Allied Health Sciences, Thammasat University (Rangsit Campus), Pathum Thani; [2]Faculty of Tropical Medicine, Mahidol University, Bangkok; [3]Faculty of Pharmacy, Rangsit University, Pathum Thani, Thailand; [4]Research Institute, International Medical Center of Japan, Japan

Abstract. The pharmacokinetics of oral dihydroartemisinin (DHA) following the dose of 2 and 4 mg/kg body weight dihydroartemisinin (Twisinin™, T-2 Program, Thailand) and 4 mg/kg body weight oral artesunate (AS; Guilin Pharmaceutical Works, Guangxi, China) were investigated in 20 healthy Thai volunteers (10 males, 10 females). All formulations were generally well tolerated. Oral DHA was rapidly absorbed from gastrointestinal tract with marked inter-individual variation. The pharmacokinetics of DHA following the two dose levels were similar and linearity in its kinetics was observed. Based on the model-independent pharmacokinetic analysis, median (95% CI) values for C_{max} of 181 (120-306) and 360 (181-658) ng/ml were achieved at 1.5 hours following 2 and 4 mg/kg body weight dose, respectively. The corresponding values for $AUC_{0-\infty}$, $t_{1/2z}$, CL/f and V_z/f were 377 (199-1,128) *vs* 907 (324-2,289) ng.h/ml, 0.96 (0.70-1.81) *vs* 1.2 (0.75-1.44) hours, 7.7 (4.3-12.3) *vs* 6.6 (3.1-10.1) l/kg, and 90.5 (28.6-178.2) *vs* 6.6 (3.1-10.1) ml/min/kg, respectively (2 *vs* 4 mg/kg dose). Oral AS was rapidly biotransformed to DHA, which was detectable in plasma as early as 15 minutes of AS dosing. Following 4 mg/kg dose, median (95% CI) value for C_{max} of 519 (236-284) ng/ml was achieved at 0.7 (0.25-1.5) hours. $AUC_{0-\infty}$, and $t_{1/2z}$ were 657 (362-2,079) ng.h/ml, 0.74 (0.34-1.42) hours, respectively. C_{max} of DHA following oral AS were significantly higher, but total systemic exposure was greater following oral DHA at the same dose level (4 mg/kg body weight). There was no significant sex difference in pharmacokinetics of DHA.

INTRODUCTION

Malaria is a leading cause of mortality and morbidity in developing areas of the world, and remains a major public health problem in endemic regions (Berman *et al,* 2001). Resistance to available drugs is increasing, and therefore creating a need for new drugs that are well tolerated and simple to use. In the face of this ominous situation, artemisinin and derivatives (artesunate, arteether, and dihydroartemisinin) have lately become a renewed hope for combating the emerging generations of resistant malaria (Hein and White, 1993; Harinasuta and Karbwang, 1994; McIntosh and Olliaro, 1998). These artemisinin drugs have different physicochemical properties and are available in a variety of formulations that influence their routes of administration and dosage regimens (de Vries and Dien, 1996; van Agtmael *et al*, 1999; Navaratnam *et al*, 2000).

Artesunate (AS) is a water-soluble hemisuccinate derivative of artemisinin that is widely used in the treatment of both uncomplicated (oral formulation) and severe falciparum malaria (intravenous or suppository formulation) (White, 1994; Barradell and Fitton, 1995; de Vries and Dien, 1996; Looareesuwan *et al,* 1996; Newton *et al,* 2003). Dihydroartemisinin (DHA), a reduced lactol derivative, is the main acting blood schizontocidal metabolite of the semisynthetic artemisinin derivatives, with activity 2-5 fold that of the parent drugs (Basco and Le Bras, 1993). DHA is the chemical intermediate in the production of AS and other semisynthetic artemisinin derivatives (Lin *et al,* 1987), as well as their principal active metabolite (Lee and Hufford, 1990). Although DHA is not sufficiently water-soluble

Correspondence: Dr Sornchai Looareesuwan, Faculty of Tropical Medicine, 420/6 Rajvithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2247-1688; Fax: 66 (0) 2245-7288
E-mail: tmslr@mahidol.ac.th

to be formulated as an intravenous injection, it is cheaper to produce than other aremisinin derivatives. The production of this drug is simple with the high yield. Since AS is rapidly deesterified to DHA (Yang *et al*, 1985; Batty *et al*, 1998a; b; Zhao *et al*, 1988), it may be equally acceptable to administer DHA itself. DHA is currently in clinical use as formulated tablets/capsules or suppositories. Pharmacokinetic and bioavailability data for DHA, when given as an oral or suppository formulation, have been reported (Yang *et al*, 1985; Zhao *et al*, 1988; Na-Bangchang *et al*, 1997; 1998a, b; 1999; Batty *et al*, 1998a,b; Hung *et al*, 1999; Binh *et al*, 2001; Ilett *et al*, 2002). The objective of the present study was to describe the pharmacokinetics and tolerability of the two oral doses of a new oral formulation of DHA (Twisinin™, T-2 Program, Thailand) in healthy Thai volunteers. This was performed in comparison with oral formulation of AS (Guilin Pharmaceutical Works, Guangxi, China) which has been registered for clinical treatment of uncomplicated falciparum in Thailand.

MATERIALS AND METHODS

Subjects

Twenty healthy male and female Thai volunteers, aged between 20 and 35 years, weighing 46.7 to 59 kg, who were residents of the Bangkok area, participated in the study. Inclusion criteria included: non-lactating and non-pregnant (females), no significant abnormal findings on history or examination, particularly liver, kidney, cardiovascular diseases or peripheral neuropathy, no history of antimalarial drug ingestion in the preceding three months, and no other drugs or medications ingested in the preceding week. None was a smoker or alcohol drinker nor was on regular medication. Written informed consent for participation was obtained from all the volunteers before initiation of the study. The study was approved by the Ethics Committees of the Faculty of Tropical Medicine, Mahidol University and the Ministry of Public Health, Thailand.

At enrollment, a medical history was taken, including a full physical examination; each volunteer had a thorough physical examination, routine laboratory investigations, plain chest x-ray, urinalysis, and a 12-lead electrocardiogram (ECG).

Drug administration and study design

The trial design was a single randomized three-phases cross-over model. Study participants received, in random order, the following three study sessions: (i) a single oral dose of 2 mg/kg body weight DHA (Twisinin™: 50 or 100 mg per capsule, the T-2 Program, Thailand); (ii) a single oral dose of 4 mg/kg body weight DHA (Twisinin™: 50 or 100 mg per capsule; and (iii) a single oral dose of 4 mg/kg artesunate (AS: 50 mg per tablet; Guilin Pharmaceutical Works, Guangxi, China).

Compliance with all drug intake was under investigators' supervision. No food was allowed until 2 hours after drug intake. The washed-out period after each occasion was at least 2 days. Volunteers were hospitalized in the Bangkok Hospital for Tropical Diseases one day prior to, and on the day of pharmacokinetic study. No other concurrent drugs or alcohol were taken two weeks prior to, and during the study period.

Blood sample collection

Blood samples (5 ml each) were collected through an indwelling intravenous Teflon™ catheter, inserted into a forearm vein of the subject; the patency was maintained with sodium-heparinized saline. Samples for the assay of DHA, and/or AS were collected pre-dose, and at 15, 30, 45, 60, 90, and 120 minutes, and 3, 4, 6, and 8 hours after drug administration. Plasma samples were obtained through centrifugation within 10 minutes (1,500*g*, 15 minutes), and stored at -80°C until analysis.

Adverse reaction monitoring

The volunteers were physically examined and adverse reactions during the study were recorded with the date and time at which they appeared and disappeared. Adverse effects were assessed on the basis of non-suggestive questioning by the study investigators. These included gastrointestinal, central nervous, cardiovascular, and dermatological effects, as well as other changes possibly attributable to the study drugs. Routine blood investigations (hematology and biochemistry), and urinalysis were performed prior to and at the end of (2 days after last drug administration) the study.

Drug analysis

Concentrations of DHA and/or AS in the

plasma were determined by reductive mode high-performance liquid chromatography (HPLC-EC), according to the method of Na-Bangchang *et al* (1998b). The procedure involved the extraction of AS, DHA, and the internal standard - artemisinin (AN) with the mixture of dichloromethane and *tert*-methyl-butyl-ether (8:2, v/v). Chromatographic separation consisted of the mobile phase (acetonitrile: water containing 0.1 M acetic acid pH 4.8 = 45: 55%) running through the column (Nova-Pak™ C_{18}, 3.9 mm i.d. x 150 cm, 5 mm particle size). The average recoveries of AS, DHA-(α-anomer) 2 and 4 mg, and AN at the concentration range of 10-800 ng/ml were 81.9, 88.2, 101.1 and 84.3 %, respectively. The coefficients of variation (precision and repeatability) were below 10% for all three compounds at concentrations of 800, 400, 200, 500, and below 20% at concentration of 10 ng/ml. The limits of quantification for both AS and α-DHA in spiked plasma samples were 5 and 3 ng/ml, respectively.

Pharmacokinetic analysis

Pharmacokinetic parameters of DHA following the administration of a single oral dose of DHA or AS were calculated by model-independent (oral DHA, DHA as an active plasma metabolite of AS) and model-dependent (oral DHA) methods from plasma concentration-time data (Gibaldi, 1991).

Model-independent method. The time at which maximum plasma concentration occurred (t_{max}), and the maximum concentration (C_{max}) were obtained directly from concentration-time data. The area under the curve from zero time to the last observed time (AUC_{0-t}) was calculated by the linear trapezoidal rule for ascending data points and by the log-linear trapezoidal rule for descending data points. The area under the curve extrapolated from the last data point to infinity ($AUC_{t-\infty}$) was estimated by dividing the estimated concentration at the last data point with the elimination rate constant (λ_z). The total area under the curve ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + AUC_{t-\infty}$. The terminal elimination rate constant (λ_z) and half-life ($t_{1/2z}$) were estimated by log-linear regression of at least four last concentration-time data. The apparent total body clearance (CL/f) and apparent volume of distribution associated with terminal phase (V_z/f) were calculated as CL/f = dose/AUC and $V_z/f = (Cl/f)/\lambda_z$, respectively.

Model-dependent. To better characterize the absorption phase, a one-compartment open model with first order input and first-order elimination was fitted to the data by an iterative least squares curve fitting Program TopFit™. The observed concentrations were weighted as the reciprocal of the analytical variance. The adequacy of the pharmacokinetic models chosen was based on statistical methods to assess the validity of the models for describing the experimental data, *ie F*-ratio test, Akaike's information, Schwartz and Imbimbo criteria.

Statistical analysis

Statistical analysis of the data was performed with SPSS for Windows (SPSS Software, Gorichem, The Netherlands). The distribution of data was assessed for normality using the Schapiro-Wilks test. Data were expressed as medians with 95% CIs values.

The pharmacokinetics of DHA in healthy Thai volunteers following the administration of 2, 4 mg/kg body weight DHA, or 4 mg/kg body weight AS were compared using Kruskal Wallis test and Wilcoxon signed ranks test for non-normally distributed data. Comparison of pharmacokinetic parameters between sex (male, female) in each drug regimen was performed by Mann-Whitney *U*-test. Categorical data (adverse reactions) were analyzed by calculating chi-square with Yate's correction or by Fisher's exact test. Significance level for all tests was set at $\alpha < 0.05$.

RESULTS

Tolerability

All volunteers were healthy, verified by laboratory results, physical examination, and vital sign monitoring. Table 1 presents demographic and baseline laboratory (hematology/ biochemistry) data of the volunteers. Significant laboratory changes in some hematological or biochemical tests were noted at the end of the study (2 days after drug administration). Parameters which decreased at the end of the study included hemoglobin, hematocrit, total protein, albumin,

Table 1
Demographic and baseline laboratory data of 20 healthy Thai volunteers (10 males, 10 females); data are presented as median (95% CI) values.

	Male	Female
Age (y)	21 (20, 33)	23 (21, 35)
Body weight (kg)	53.7 (47.8, 59)	49.3 (46.7, 51.6)
Hematology		
Hemoglobin (mg/dl)	13.85 (11.8, 15.1)	12.7 (11.4, 13.8)
Hematocrit (%)	43 (36, 46)	39.5 (36, 42)
Red cells (x 10^{12} /l)	1.44 (4.57, 6.78)	4.67 (4.01, 6.41)
Platelets (x 10^9 /l)	233 (203, 310)	309 (192, 328)
White cells (x 10^9 /l)	7.7 (5.1, 9.8)	7.4 (4.5, 9.3)
PMN (%)	49 (37, 61)	49.5 (42, 62)
Lymphocyte (%)	36 (26, 55)	41 (27, 49)
Monocyte (%)	6 (0, 11)	6 (2, 7)
Eosinophil (%)	5 (2, 12)	3 (1, 13)
Biochemistry		
Direct bilirubin (mg/dl)	0.15 (0.1, 0.6)	0.165 (0.006, 0.25)
Total bilirubin (mg/dl)	0.685 (0.36, 2.4)	0.595 (0.32, 1.3)
Alkaline phosphatase (U/l)	64.5 (44, 112)	67.5 (44, 79)
SGOT (U/l)	22 (15, 50)	18 (16, 24)
SGPT (U/l)	20 (12, 98)	11 (7, 19)
Total protein (g/dl)	7.35 (6.3,7.9)	7.65 (7.2,8.2)
Albumin (g/dl)	4.75 (4.3, 4.9)	4.5 (4.2, 4.6)
Globulin (g/dl)	2.6 (2, 3.1)	3.0 (2.6, 3.4)
Creatinine (mg/dl)	0.9 (0.78, 1.2)	0.75 (0.65, 0.9)
BUN (mg/dl)	11.5 (8.3, 17.7)	9.95 (7, 13.8)
Glucose (mg/dl)	93 (80, 113)	86 (81, 115)
Sodium (mmol/l)	141.5 (139, 145)	142 (139, 144)
Potassium (mmol/l)	3.8 (3.6, 4.8)	4.15 (3.7, 4.6)
Chloride (mmol/l)	106 (104, 108)	106.5 (105, 108)
Bicarbonate (mmol/l)	24.5 (24, 26)	24.5 (24, 26)

globumin, while parameter which increased at the end of the study was platelet count. However, these values returned to normal within 2 weeks after the termination of the drugs. None of the volunteers complained of adverse reaction or drug-related effect during the study.

Pharmacokinetics

Median plots of plasma concentration-time profiles of DHA and/or AS following the administration of a single oral dose of 2 or 4 mg/kg body weight DHA, or 4 mg/kg body weight AS in 20 healthy Thai volunteers are shown in Figs 1a and 1b. Oral DHA was rapidly absorbed from gastrointestinal tract with marked inter-individual variation. In most cases, the drug was detectable in plasma within 15 minutes of dosing; it disappeared thereafter from systemic circulation within 3-8 hours. Oral AS was rapidly biotransformed to DHA, which was detectable in plasma as early as 15 minutes of AS dosing. Considerable inter-individual variation in plasma DHA concentrations following both oral formulations of DHA and AS was observed. Systemic exposure of AS itself was seen only during 15 minutes to 1 hour but with markedly low concentrations.

The pharmacokinetics of DHA (median and 95% CI) following a single oral dose administration of 2 or 4 mg/kg body weight DHA, or 4 mg/kg body weight AS in 20 healthy Thai volunteers, calculated based on model-independent and

Table 2
Pharmacokinetics of DHA (model-independent) following a single oral dose of 2 or 4 mg/kg body weight DHA (Twisinin™), or 4 mg/kg body weight AS (Guilin Pharmaceutical Works) in healthy Thai males (n=10) and females (n=10) data are presented as median (95% CI) values.

Pharmacokinetic Parameters	2 mg/kg DHA	4 mg/kg DHA	4 mg/kg AS
C_{max} (ng/ml)[a]	181 (120-306)	360 (181-658)	519 (236-284)
$AUC_{0-\infty}$ (ng.h/ml)[b]	377 (199-1,128)	907 (324-2,289)	657 (362-2,079)
t_{max} (h)[c]	1.5 (0.75-2.0)	1.5 (0.75-3.0)	0.7 (0.25-1.5)
λ_z (/h)[d]	1.009 (0.532-1.375)	0.802 (0.668-1.284)	1.301 (0.678-2.832)
$t_{1/2z}$ (h)[e]	0.96 (0.70-1.81)	1.2 (0.75-1.44)	0.74 (0.34-1.42)
V_z/f (l/kg)	7.7 (4.33-12.3)	6.6 (3.1-10.1)	-
CL/f (ml/min/kg)	90.5 (28.6-178.2)	72.2 (31.9-113.6)	-

[a]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.0001 (95% CI = 151-246); and between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.006 (95% CI = 202-430); and between 4 mg/kg body weight DHA and AS with p = 0.02 (95% CI = 9-235).
[b]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.00001 (95% CI = 425-689); and between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.0005 (95% CI = 98-420); and between 4 mg/kg body weight DHA and AS with p = 0.02 (95% CI = -481to -48).
[c]Significant difference between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.0002 (95% CI = -1 to -0.5); and between 4 mg/kg body weight DHA and AS with p = 0.00005 (95% CI = -14 to -0.5).
[d]No statistical test was performed.
[e]Significant difference between 2 mg/kg body weight DHA and 4 mg/kg body weight AS with p = 0.0097 (95% CI = -0.044 to -0.0009); and between 4 mg/kg body weight DHA and AS with p = 0.0005 (95% CI = -0.56 to -0.21).

Table 3
Pharmacokinetics of DHA (model-dependent) following a single oral dose of 2 or 4 mg/kg body weight DHA (Twisinin™) in healthy Thai males (n=10) and females (n=10); data are presented as median (95% CI) values.

Pharmacokinetic parameters	2 mg/kg DHA	4 mg/kg DHA
C_{max} (ng/ml)	144 (91-260)	283 (105-632)
$AUC_{0-\infty}$ (ng.h/ml)	415 (211-1,280)	919 (408-2,480)
t_{max} (h)	1.45 (0.81-2.2)	1.46 (0.81-2.2)
t_{lag} (h)	0.25 (0.11-0.25)	0.24 (0.19-0.25)
k_a (/h)	0.83 (0.51-2.59)	0.83 (0.42-3.84)
$t_{1/2a}$ (h)	0.83 (0.27-1.35)	0.84 (0.18-1.65)
k_e (/h)	0.82 (0.51-1.24)	0.81 (0.41-1.21)
$t_{1/2}$ (h)	0.83 (0.2-1.35)	0.86 (0.39-10.1)
V_c/f (l/kg)	4.8 (2.5-10.6)	5.2 (2.2-7.6)
CL/f (ml/min/kg)	82.4 (25.3-171.9)	68.6 (24.5-103.6)

[a]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.00001 (95% CI = 107 to 208).
[b]Significant difference between 2 and 4 mg/kg body weight DHA with p = 0.00001 (95% CI = 411 to 749).

model-dependent methods, are summarized in Tables 2 and 3, respectively. The fitting of the concentration-time curves of DHA either when given as oral DHA to a one-compartment model with first-order input and output yielded satisfactory results in all volunteers. Pharmacokinetics of DHA calculated using both methods were generally in good agreement. No significant absorption lag-time was observed from the time of drug administration until it was first detectable in the plasma. Large inter-individual variation among the pharmacokinetic parameters was noted, particularly with $AUC_{0-\infty}$ and CL/f as reflected by the values of coefficients of variation for both parameters (40-45%).

No significant difference was found in any of DHA pharmacokinetic parameters between male and female volunteers following the administration of either dose of DHA (2 or 4 mg/kg body weight), or as a single oral dose of 4 mg/kg body weight AS.

Model-independent analysis. Marked differ-

ences in the pharmacokinetic parameters of DHA were observed following the oral dose regimens of DHA and AS. Oral DHA at the dose level of 2 mg/kg body weight resulted in a significantly lower C_{max} and $AUC_{0-\infty}$ of DHA compared with 4 mg/kg body weight dose of DHA or AS. With respect to DHA, C_{max} increased proportionally with the dose with a median ratio of 2.3. C_{max} of DHA following the same dose of AS (4 mg/kg body weight) was significantly higher than that following DHA, but greater $AUC_{0-\infty}$ was achieved following DHA. In addition, t_{max} of DHA following AS was found to be significantly shorter than that following DHA at either dose level.

Model-dependent analysis. Pharmacokinetics of DHA following the two dose levels of DHA (2 and 4 mg/kg body weight) were generally comparable and consistent with the values calculated using model-independent method. Only two significant differences in dose-dependent pharmacokinetic parameters were noted; C_{max} and $AUC_{0-\infty}$ following the higher dose level were approximately double that seen in the model-independent analysis.

DISCUSSION

The pharmacokinetics of AS and DHA have been addressed in a few studies, with varying routes of administration, ethnicity of the subjects and disease states (Yang *et al,* 1985; Zhao *et al,* 1988; Na-Bangchang *et al,* 1997; 1998a; 1999; Batty *et al,* 1998a,b; Hung *et al,* 1999; Binh *et al,* 2001; Ilett *et al,* 2002). The concentration-time profiles of DHA following the administration of the oral doses of both DHA and AS observed in the present study were generally in accord with those previously reported. No marked sex diffrences in DHA pharmacokinetics was observed following either oral DHA or AS, which supports a previous report in healthy Vietnamese volunteers (Hung *et al,* 1999). The current formulation of oral DHA (Twisinin™) was rapidly absorbed from the gastrointestinal tract; C_{max} was attained at approximately 1-2 hours of dosing. The pharmacokinetic profile was generally well described by a one-compartment open model with first- order input and output, characterising the rapid absorption, distribution and elimination phase. Elimination half-life was estimated to be in the range of 0.8-1.5 hours. Little is known about the ultimate phase of dihydroartemisinin in human body. The *in vitro* studies using rat isolated perfused liver (IPRL) and microsomes have identified glucuronide conjugate as a sole and principal metablite of DHA (Maggs *et al,*1997). The extent of the hepatic extraction of oral DHA is unknown. Oral AS (Guilin Pharmaceutical Works, China) was almost immediately biotransformed to the active metabolite, DHA. Systemic exposure to AS itself was very low (C_{max} of less than 200 ng/ml in most cases) and was observed only during a short period, the first hour, after drug intake. In contrast, its plasma metabolite, DHA, attained a relatively high C_{max} within 0.25-1.5 hours of AS dosing. Distribution/elimination of this metabolite was also rapid. The apparent elimination half-life was estimated to be in the range of 0.4-1.4 hours.

The pharmacokinetics of DHA following a single oral dose of 2 or 4 mg/kg body weight DHA were generally similar. This was indicated by the comparable values of dose-independent pharmacokinetic parameters. Linearity of DHA kinetics was seen at these two dose levels, which was ascribed by the proportional increase in $AUC_{0-\infty}$ with the dose (mean $AUC_{0-\infty}$ ratio of 2.4). It appears that the bioavailability of the current formulation of oral DHA (Twisinin™) is markedly low (approximately 50%) when compared with the formulation produced by Guilin Pharmaceticals, China (Cotexin™: tablet) or Arenco nv, Belgium (Dihydroartemisinin™: capsule) (Na-Bangchang *et al,* 1997; Hung *et al,* 1999). In a previous study in healthy Thai volunteers following 300 mg of Dihydroartemisinin™ (Arenco-nv, Belgium), median (range) $AUC_{0-\infty}$ and C_{max} of 2,010 (636-4,079) ng.h/ml and 679 (307-1,000) ng/ml were achieved, respectively (Na-Bangchang *et al,* 1997). Furthermore, in a study in healthy Vietnamese volunteers following 240 mg of Cotexin™, median (range) $AUC_{0-\infty}$ and C_{max} of 1,867 (420-3,535) ng.h/ml and 466 (128-787) ng/ml were attained, respectively (Hung *et al,* 1999).

It was noted that the pharmacokinetics of DHA following oral DHA and AS doses showed noticeable differences in pharmacokinetic. The

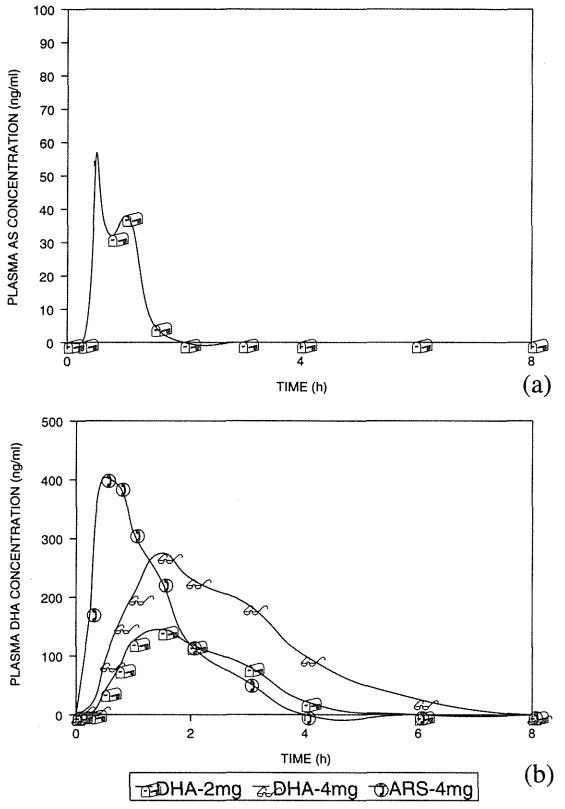

Fig 1–Median plasma concentration-time profiles of (a) artesunate (AS) following the dose of oral 4 mg/kg body weight oral AS, and (b) dihydroartemisin (DHA) following the doses of oral 2 and 4 mg/kg body weight oral DHA.

Disposition of DHA, when the drug was given as oral AS, was greatly influenced by the kinetics of the parent compound itself, *ie,* formation of DHA was rate-limited by kinetics (absorption, distribution, and metabolism) of AS. Higher C_{max} of DHA was attained at faster time following the oral AS, but total systemic exposure was higher following oral DHA given at the same dose level (4 mg/kg body weight). This may suggest that absorption of DHA from oral formulation may be more erratic but relatively complete compared with oral AS. DHA is poor water solubility, which means that it can only be administered orally or rectally. Previous data, however, showed that the bioavailability of orally administered DHA was only 45% relative to the DHA from intravenous AS (Binh *et al,* 2001).

In conclusion, the oral formulation of both AS and DHA were well tolerated. No clinically adverse reaction or drug-related effect was observed during the study. Nevertheless, adequate therapeutic plasma concentrations following the administration of the current formulation of oral DHA (Twisinin™) may not be guaranteed. This is of concern, especially in patients with malaria, whose absorption of the drug by the oral route may be erratic and incomplete.

ACKNOWLEDGEMENTS

The study was supported by the Thailand Tropical Diseases (T-2 Program) and partly by a Mahidol University Grant. We thank nursing staff of the Hospital for Tropical Diseases for their assistance during the clinical phase of the study.

REFERENCES

Barradell LB, Fitton A. Artesunate. A review of its pharmacology and therapeutic efficacy in the treatment of malaria. *Drugs* 1995; 50: 714-41.

Basco L, Le bras J. *In vitro* activity of artemisinin derivatives against African siolates and clones of *Plasmodium falciparum*. *Am J Trop Med Hyg* 1993; 49: 301-7.

Batty KL, Le AT, Ilett KF, *et al.* A pharmacokinetic and pharmacodynamic study of artesunate for vivax malaria. *Am J Trop Med Hyg* 1998a; 59: 823-7.

Batty KT, Thu LT, Davis TME, *et al.* A pharmacokinetic and pharmacodynamic study of intravenous vs oral artesunate in uncomplicated falciparum malaria. *Br J Clin Pharmacol* 1998b; 45: 123-29.

Berman JG, Egan A, Keusch GT. The intolerable burden of malaria: a new look at the numbers. *Am J Trop Med Hyg* 2001; 64 (suppl): 4-7i.

Binh TQ, Ilett KF, Batty KT, *et al.* Oral bioavailability of dihydroartemisinin in Vietnamese volunteers and in patients with falciparum malaria. *Br J Clin Pharmacol* 2001; 51: 541-6.

de Vries PJ, Dien TK. Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. *Drugs* 1996; 52: 818-6.

Gibaldi M. Biopharmaceutics and clinical pharmacokinetics. 4th ed. Philadelphia: Lea and Febriger, 1991: 14-23.

Harinasuta T, Karbwang J. Qinghaosu: a promising

antimalarial. *JAMA* 1994; 34: 7.

Hein TT, White NJ. Qinghaosu. *Lancet* 1993; 341: 603-8.

Hung LN, Na-Bangchang K, Thuy LTD, Anh TK, Karbwang J. Pharmacokinetics of a single oral dose of dihydroartemisinin in Vietnamese healthy volunetters. *Southeast Asian J Trop Med Public Health* 1999; 30: 11-6.

Ilett KF, Batty KT, Powell SM, *et al.* The pharmacokinetic properties of intramuscular artesunate and rectal dihydroartemisinin in uncomplicated falciparum malaria. *Br J Clin Pharmacol* 2002; 53: 23-30.

Lee IS, Hufford CD. Metabolism of antimalarial sesquiterpene lactones. *Pharmacol Ther* 1990; 48: 345-5.

Lin AJ, Klayman DL, Milhous WK. Antimalarial activity of new water-soluble dihydroartemisinin derivatives. *J Med Chem* 1987; 30: 2147-50.

Looareesuwan S, Wilairatana P, Andrial M. Artesunate suppository for treatment of severe falciparum malaria in Thailand. *Jpn J Trop Med Hyg* 1996; 24 (suppl 1): 13-5.

McIntosh HM, Olliaro P. Treatment of severe malaria with artemisinin derivatives. A systematic review of randomised controlled trials. *Med Trop* 1998; 58 (suppl): 61-2.

Maggs JL, Madden S, Bishop LP, O'Neil PM, Park BK. The rat biliary metabolites of dihydroartemisinin, an antimalarial endoperoxide. *Drug Metab Dispos* 1997; 25: 1200-04.

Na-Bangchang K, Congpuong K, Ubalee R, Thanavibul A, Tan-ariya P, Karbwang J. Pharmacokinetics and *ex vivo* antimalarial activity of sera following a single oral dose of dihydroartemisinin in healthy Thai males. *Southeast Asian J Trop Med Public Health* 1997; 28: 731-5.

Na-Bangchang K, Karbwang J, Congpoung K, Thanavibul A, Ubalee R. Pharmacokinetic and bioequivalence evaluation of two generic formulations of oral artesunate. *Eur J Clin Pharmacol* 1998a; 53: 375-6.

Na-Bangchang K, Congpoung K, Hung LN, Molunto P, Karbwang J. Simple high performance liquid chromatographic method with electrochemical detection for the simultaneous determination of artesunate and dihydroartemisinin in biological fluids. *J Chromatogr B* 1998b; 708: 201-7.

Na-Bangchang K, Tippawangkosol P, Thanavibul A, Ubalee A, Karbwang J. Pharmacokinetic and pharmacodynamic interactions of mefloquine and dihydroartmisinin. *Int J Clin Pharm Res* 1999; XIX: 9-17.

Navaratnam V, Mansor SM, Sit N, Grace JM, Li Q, Olliaro P. Pharmacokinetics of artemisinin-type of compounds. *Clin Pharmacokinet* 2000; 39: 255-70.

Newton PN, Angus BJ, Chierakul W, *et al.* Randomized comparison of artesunate and quinine in the treatment of severe falciparum malaria. *Clin Infect Dis* 2003; 37: 7-16.

van Agtmael MA, Eggelte TA, van Boxtel CJ. Artemisinin drugs in the treatment of malaria: from medicinal herbs to registered medication. *Trends Pharmacol Sci* 1999; 20: 199-205.

Wernsdorfer WH. Epidemiology of drug resistance in malaria. *Acta Trop* 1994; 56: 143-56.

White NJ. Clinical pharmacokinetics and pharmacodynamics of artemisinin and derivatives. *Trans R Soc Trop Med Hyg* 1994; 88 (suppl 1): S41-S43.

Yang SD, Ma JM, Sun JH, Chen DX, Song ZY. Clinical pharmacokinetics of a new effective antimalarial artesunate, a qinghaosu derivative. *Chin J Clin Pharmacol* 1985; 1: 106-9.

Zhao KC, Chen ZX, Lin BL, Guo XB, Li GQ, Song ZY. Studies on the phase 1 clinical pharmacokinetics of artesunate and artemether. *Chin J Clin Pharmacol* 1988; 4: 76-81.

APPLICATION OF REAL-TIME POLYMERASE CHAIN REACTION (PCR) ANALYSIS FOR DETECTION AND DISCRIMINATION OF MALARIA PARASITE SPECIES IN THAI PATIENTS

Yuko Katakai[1], Rachatawan Chiabchalard[2], Kanako Komaki-Yasuda[1,3], Shin-ichiro Kawazu[1], Pratap Singhasivanon[2], Srivicha Krudsood[2], Sornchai Looareesuwan[2] and Shigeyuki Kano[1]

[1]Department of Appropriate Technology Development and Transfer, Research Institute, International Medical Center of Japan, Tokyo, Japan; [2]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [3]Domestic research fellow, Japan Society for the Promotion of Science, Tokyo, Japan

Abstract. A TaqMan real-time PCR system was used to detect and discriminate the 4 species of human malaria parasites in clinical blood samples. A 150-base pair (bp) region of the small subunit ribosomal RNA (SSU rRNA) gene of each malaria parasite, including species-specific sequences to be detected by TaqMan probe, was used as a target for PCR analysis. The PCR method used universal primers and species-specific TaqMan probes for *Plasmodium falciparum, P. vivax, P. ovale,* and *P. malariae*. The detection threshold for the method, as determined with serial dilution of cultured *P. falciparum*-infected erythrocytes, was 5 parasite-infected erythrocytes per reaction. Fifty blood samples of falciparum malaria and a second set of 50 samples of vivax malaria, diagnosed by microscopic examination at the Hospital for Tropical Diseases, Mahidol University, Thailand, were analyzed by real-time PCR. In the 50 samples of microscopically-diagnosed falciparum malaria, 40 were regarded as *P. falciparum* single infection, 7 were *P. falciparum* and *P. vivax* mixed infections, and 3 were *P. vivax* single infection by real-time PCR. In the second set of 50 samples of microscopically diagnosed vivax malaria, all were considered *P. vivax* single infection by PCR. Neither *P. ovale* nor *P. malariae* infection was identified in the 100 blood samples. Real-time PCR analysis was shown to be more sensitive and accurate than routine diagnostic methods. Application and extension of the PCR method reported here will provide a powerful tool for further studies of malaria.

INTRODUCTION

Accurate diagnosis of malaria and identification of the *Plasmodium* species responsible are essential for optimal patient management and for understanding the epidemiology of malaria. Microscopic examination of blood smears is the standard method for diagnosis of malaria. Although this method is sensitive and specific, it is subjective and time-consuming, particularly for detecting a small number of parasites. In addition, it is often very difficult to identify mixed infections (Snounou *et al*, 1993a; Rubio *et al*, 2002).

Various alternative diagnostic methods have been developed. These include fluorescence microscopy (Kawamoto and Billingsley, 1992), concentration techniques, such as the quantitative buffy coat method (Petersen and Marbiah, 1994), and immunological antigen capture assays such as malaria rapid diagnostic tests (Tham *et al*, 1999; Wongsrichanalai *et al*, 2003). However, persistence of the antigens in the blood-streams of patients might result in false positive results in an antigen capture assay. Thus, use of more than one test is recommended to confirm diagnosis.

In this study, we applied real-time PCR with TaqMan technology (Bell and Ranford-Cartwright, 2002) to analyze malaria parasite infections in clinical blood samples.

Correspondence: Shigeyuki Kano, Department of Appropriate Technology Development and Transfer, Research Institute, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku, Tokyo 162-8655, Japan.
E-mail: kano@ri.imcj.go.jp

MATERIALS AND METHODS

Parasite culture

The FCR-3 strain of *P. falciparum* was cultured by the method of Trager and Jensen (1976) followed by synchronization with sorbitol treatment (Lambros and Vanderberg, 1979). When the parasites reached the ring stage, the culture was diluted serially, and blood samples containing 5×10^5 to 5×10^1 parasite-infected erythrocytes were prepared. Genomic DNA was extracted from the samples, and of the amount, 1/10 was used as a template for PCR. The minimum number of parasite-infected erythrocytes per reaction was 5.

Preparation of plasmid DNA

Approximately 150-bp of the small subunit ribosomal RNA (SSU rRNA) gene sequence was amplified from the parasite DNA with the universal

primers shown in Table 1. Genomic DNA served as the PCR template and was isolated from the clinical blood samples that contained *P. vivax, P. ovale*, or *P. malariae*, or from cultured blood for *P. falciparum*. The DNA fragment was cloned into the plasmid vector pCR® 2.1-TOPO® (Invitrogen Corp, Carlsbad, CA, USA), and the sequence was confirmed by cycle sequencing (Applied Biosystems, Foster City, CA, USA).

TaqMan PCR

The universal primers and TaqMan probes used are shown in Table 1. The TaqMan probes were labeled with a reporter dye, 5-carboxyfluorescein (FAM), and conjugated with minor groove binder (MGB) for increased sensitivity and decreased background fluorescence (Kutyavin *et al*, 2000). The probes were designed with Primer Express software (Applied Biosystems) based on the SSU rRNA gene sequence (Genbank accession numbers of the 4 parasite species are shown in Table 1). The primers and probes were synthesized and HPLC purified by Applied Biosystems Japan (Tokyo, Japan).

Real-time PCR was performed according to the manufacturer's instructions (Applied Biosystems). Briefly, 5 µl template DNA, 300 nM forward primer, 300 nM reverse primer, 100 nM TaqMan MGB probe and 1x Universal Master Mix in a total reaction volume of 25 µl were amplified on ABI PRISM 7700 or 7900 Sequence Detection Systems (SDS) (Applied Biosystems). The following PCR conditions were used: optimization of AmpErase uracil-N-glycosylase (Applied Biosystems) activity at 50°C for 2 minutes, initial hot start at 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence signal was detected and analyzed with ABI PRISM SDS software (Applied Biosystems). Plasmid DNA in the range of 0.001fg -10pg was used as a quantification standard for the SDS calibration curve.

Clinical blood samples

One hundred blood samples were obtained from patients at the Hospital for Tropical Diseases, Mahidol University, Thailand, in 1997. The patients provided written consent, and this study was approved by the Ethics Committee of Mahidol University. The blood was collected with EDTA, and thin blood smears were prepared. The blood smears were examined microscopically by the laboratory staff of the hospital. *P. falciparum* was confirmed in 50 samples, and *P. vivax* was confirmed in a second set of 50 samples. The blood samples were stored at -20°C until DNA extraction.

DNA extraction from blood samples

The frozen blood samples were thawed on ice, and 250 µl were taken for DNA extraction. Five hundred µl DNAzol® BD reagent (Invitrogen) were mixed in, 200 µl isopropanol were added, and the reaction was incubated for 5 minutes at room temperature. The samples were centrifuged at 6,000*g* for 6 minutes to sediment the precipitated DNA. The DNA pellets were washed with DNAzol® BD reagent and then washed with 75% ethanol. After removing the ethanol, the DNA pellets were dissolved in 50 µl 8 mM NaOH and frozen at -20°C until use.

Table 1
Sequence of primers and oligonucleotide probes used for the detection of SSU rRNA genes of the malaria parasites.

Primers/probes	Sequence (5'→3')	Position[a]	Origin
Primer			
Forward	ACGATCAGATACCGTCGTAATCTT	1062-1085	M19172, Kimura *et al*, 1997
Reverse	GAACCCAAAGACTTTGATTTCTCAT	1180-1204	M19172, Kimura *et al*, 1997
Probe[b]			
Pf-probe	CATCTTTCGAGGTGACTT	1138-1155	M19172
Pv-probe	TCTCTTCGGAGTTTAT	1471-1486	X13926
Po-probe	TTTCCCCGAAAGGA	1152-1165	L48987
Pm-probe	AGAGACATTCTTATATATGAGTG	1173-1195	M54897

[a]Positions correspond to the 18S rRNA sequence.
[b]Pf = *P. falciparum;* Pv = *P. vivax;* Po = *P. ovale;* Pm = *P. malariae*

Statistical analysis

Nonparametric analysis was performed with the Mann-Whitney *U* test. p-values < 0.05 were considered to be statistically significant.

RESULTS

Specificity of PCR method

To test the specificity of the probes for the SSU rRNA genes of the 4 species of *Plasmodium*, PCR was performed with a species-specific gene fragment in the plasmid as a template. The amplification plot with the *P. falciparum*-specific probe is shown in Fig 1. Only signal from the *P. falciparum* plasmid DNA was detected, whereas signals from other species were not detected. The probe detected *P. falciparum* plasmid DNA in a concentration-dependent manner. The specificity of the probe was confirmed with the use of cultured parasite DNA as a template. Equivalent specificity of the probes for the other 3 parasite species was obtained with the use of species-specific genes in the plasmid as the template DNA (data not shown).

Sensitivity of PCR method

The sensitivity of the method was determined with a 10-fold dilution of cultured *P. falciparum*-infected erythrocytes. *P. falciparum* DNA could be detected in a reaction containing as few as 5 parasite-infected erythrocytes.

Application of PCR method to clinical blood samples

DNA extracted from patient blood was analyzed by real-time PCR (Table 2). Parasite DNA was detected in 100 blood samples. In 1 group of 50 samples, which were diagnosed as *P. falciparum* infection by microscopic examination, *P. falciparum* DNA alone was detected in 40 samples, and *P. vivax* DNA alone was detected in 3 samples. Both *P. falciparum* DNA and *P. vivax* DNA were found in 7 samples. In the second group of 50 samples, which were diagnosed as *P. vivax* infection by microscopic examination, *P. vivax* DNA alone was detected in all 50 samples. DNA of other parasite species was not detected in these samples.

DISCUSSION

Several techniques for the detection of malaria parasite DNA and RNA have been developed. These techniques, including standard PCR (Snounou *et al*, 1993a), nested PCR (Snounou *et al*, 1993b; Kimura *et al*, 1997; Rubio *et al*, 2002), and DNA probe assay (McLaughlin *et al*, 1993), are objective and have advantages in specificity and sensitivity. In the present study, we applied real-time PCR with TaqMan technology for the detection and discrimination of human malaria parasite species. An approximate 150-bp region of the SSU rRNA gene of *Plasmodium* (Waters and MacCutchan, 1989) was amplified with interspecies conserved universal primers and was detected with species-specific TaqMan MGB probes. In experiments with the plasmid-cloned SSU rRNA gene fragment as a template, probes specific for each parasite species only amplified DNA corresponding to each species, with no cross-reactivity with that of other species. In experiments with DNA from cultured *P. falciparum*-infected erythrocytes as the template, the detection and discrimination threshold for the PCR method was 5 parasite-infected erythrocytes per reaction. Equivalent sensitivity was determined for the 3 other parasite species. It has been reported that microscopy can routinely detect as few as 10-100 malaria parasites per μl of blood (Rubio *et al*, 2002).

Table 2
Comparison of real-time PCR and microscopic examination for diagnosis of malaria in blood samples from Thai patients.

Microscopy		Real-time PCR[b]				
		Pf	Pv	Pf + Pv	Po	Pm
Pf	50	40	3[a]	7	0	0
Pv	50	0	50	0	0	0
Total	100	40	53	7	0	0

[a] Numbers underlined indicate discrepant results between the 2 methods.
[b] Pf: *P. falciparum*; Pv: *P. vivax*; Po: *P. ovale*; Pm: *P. malariae*

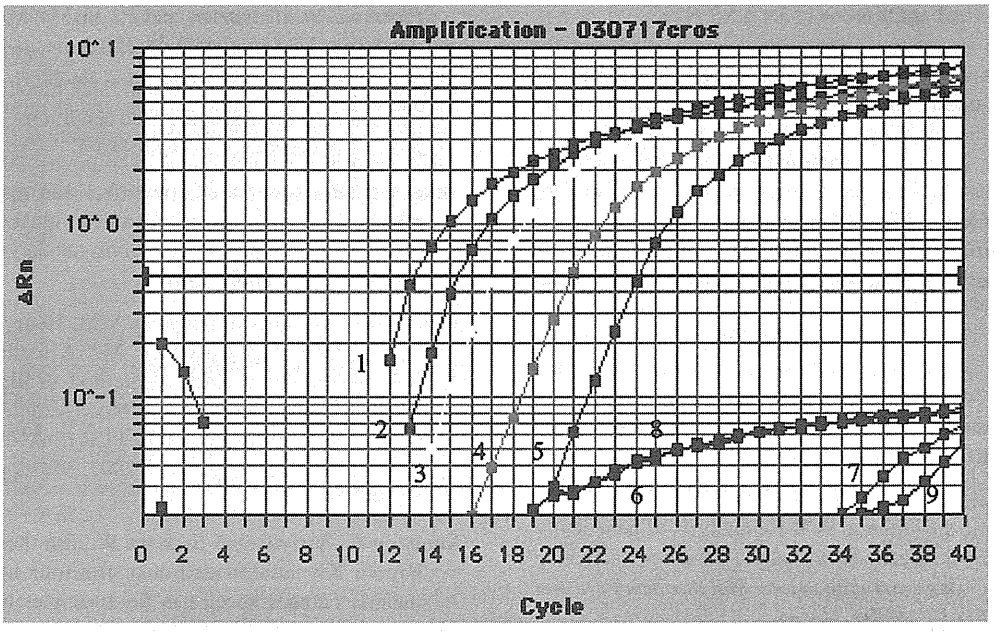

Fig 1- Amplification plot of fluorescence against cycle number for 4 SSU rRNA genes of human malaria parasites, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. ΔRn is relative fluorescence units. One hundred nM TaqMan MGB probes specific for *P. falciparum* and 300 nM universal primers for *Plasmodium* SSU rRNA gene were used. The templates were composed of plasmid DNA with a species-specific SSU rRNA gene. Each curve indicates the template used:
1-5 = plasmid DNA with the *P. falciparum* gene, 10 ng, 1ng, 100 pg, 10 pg, and 1 pg, respectively; 6 = plasmid DNA with the *P. vivax* gene, 10 pg; 7 = plasmid DNA with the *P. ovale* gene, 10 pg; 8 = plasmid DNA with the *P. malariae* gene, 10 pg; 9 = No template control.

Thus, the sensitivity of the real-time PCR method reported here is apparently higher than that of microscopic examination.

To evaluate the real-time PCR method, blood samples from Thai patients who were diagnosed by microscopy were utilized. Interestingly, comparison of methods for the 100 blood samples showed that the PCR method could detect mixed infection in 7% of the samples, whereas microscopic observation detected only a single infection for the same samples (Table 2). All samples diagnosed as mixed infection by PCR showed relatively low parasite counts by microscopy. Mean parasitemia in the group with *P. falciparum* single infection was 0.82%, whereas that in the group with mixed infection was 0.17% ($p<0.05$). It is known that *P. vivax* and *P. falciparum* in the ring/early trophozoite stage are difficult to distinguish, particularly when the level of parasitemia is low (Snounou *et al*, 1993b), and microscopic diagnosis may only be successfully performed when the late asexual stages of *P. vivax* are present in the sample (Rubio *et al*, 2002).

Recently, the real-time PCR method, with other systems, such as SYBR® Green I have been used in malaria research (Polanco *et al*, 2002; Cheesman *et al*, 2003; de Monbrison *et al*, 2003). However, nonspecific binding of SYBR® Green I to primer-dimers and spurious amplicons must be minimized by careful optimization of the reaction conditions (Bell and Ranford-Cartwright, 2002). In the present study, we found that real-time PCR with target-specific TaqMan MGB probes allowed for high-performance detection and discrimination of parasite DNA without complicated optimization procedures. Because this PCR method uses standard cycling conditions and reagent concentrations, it permits simultaneous assay of multiple targets. This method may be useful in monitoring the effectiveness of malaria chemotherapy in situations where drug-resistant strains are prevalent. Drug-resistant genes of the parasite, such as *pfcrt* and *pfmdr-1*, would be used as targets. Further application of the real-time PCR method with allele-specific probes for other genetic markers of malaria parasites, or for human genetic markers associated with susceptibility, may increase the efficacy of treatment as well as the prevention of this most globally prominent infectious disease.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant for International Health Cooperation Research (16C-1) from the Ministry of Health, Labor and Welfare of Japan, a Grant-in-Aid for Scientific Research (B) (16406012) from the Ministry of Education, Science, Sports and Culture of Japan, Mahidol University grant, and JICA-Mahidol University-Asian Center of International Parasite Control Project.

REFERENCES

Bell AS, Ranford-Cartwright LC. Real-time quantitative PCR in parasitology. *Trends Parasitol* 2002;18:337-42.

Cheesman SJ, de Roode JC, Read AF, Carter R. Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: technique validation and applications. *Mol Biochem Parasitol* 2003;131:83-91.

de Monbrison F, Raynaud D, Latour-Fondanaiche C, *et al.* Real-time PCR for chloroquine sensitivity assay and for *pfmdr1-pfcrt* single nucleotide polymorphisms in *Plasmodium falciparum*. *J Microbiol Methods* 2003;54:391-401.

Kawamoto F, Billingsley PF. Rapid diagnosis of malaria by fluorescence microscopy. *Parasitol Today* 1992;8:69-71.

Kimura M, Kaneko O, Liu Q, *et al.* Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. *Parasitol Int* 1997;46: 91-5.

Kutyavin IV, Afonina IA, Mills A, *et al.* 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res* 2000;28:655-61.

Lambros C, Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* 1979;65:418-20.

McLaughlin GL, Subramanian S, Lepers JP, Raharimalala L, Deloron P. Evaluation of a nonisotopic DNA assay kit for diagnosing *Plasmodium falciparum* malaria in Madagascar. *Am J Trop Med Hyg* 1993;48:211-5.

Petersen E, Marbiah NT. QBC and thick blood films for malaria diagnosis under field conditions. *Trans R Soc Trop Med Hyg* 1994;88:416-7.

Polanco JC, Rodriguez JA, Corredor V, Patarroyo MA. *Plasmodium vivax*: parasitemia determination by real-time quantitative PCR in *Aotus* monkeys. *Exp Parasitol* 2002;100:131-4.

Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M. Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg* 2002; 96(suppl 1): S199-203.

Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* 1993a;58:283-92.

Snounou G, Viriyakosol S, Zhu XP, *et al.* High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol* 1993b;61:315-20.

Tham JM, Lee SH, Tan TM, Ting RC, Kara UA. Detection and species determination of malaria parasites by PCR: comparison with microscopy and with ParaSight-F and ICT malaria Pf tests in a clinical environment. *J Clin Microbiol* 1999;37: 1269-73.

Trager W, Jensen JB. Human malaria species in continuous culture. *Science* 1976;193:673-5.

Waters AP, McCutchan TF. Rapid, sensitive diagnosis of malaria based on ribosomal RNA. *Lancet* 1989;17:1343-6.

Wongsrichanalai C, Arevalo I, Laoboonchai A, *et al.* Rapid diagnostic devices for malaria: field evaluation of a new prototype immunochromatographic assay for the detection of *Plasmodium falciparum* and non-falciparum *Plasmodium*. *Am J Trop Med Hyg* 2003;69:26-30.

Tropical Medicine and Health Vol. 32 No. 4, 2004, pp. 335-337

A PILOT FIELD TRIAL OF AN *IN VITRO* DRUG SUSCEPTIBILITY TEST USING THE ANAEROPACK® MALARIA CULTURE SYSTEM ON THE THAI-MYANMAR BORDER

TOSHIMITSU HATABU[1,2], SHIN-ICHIRO KAWAZU[1], SOMEI KOJIMA[3], PRATAP SINGHASIVANON[4], SRIVICHA KRUDSOOD[4], SORNCHAI LOOAREESUWAN[4], SHIGEYUKI KANO[1,*]

Accepted 10, September, 2004

Abstract: The AnaeroPack® malaria culture system with a portable thermostat incubator was evaluated in a field laboratory on the Thai-Myanmar border conducting *in vitro* drug susceptibility tests on blood samples from 5 Karen children infected with *P. falciparum*. Only one isolate was susceptible to chloroquine; the others were highly resistant. The IC_{50} value of an isolate was only resistant to mefloquine, whereas the values of the 3 patients who presumably showed recrudescence were slightly elevated in the susceptible ranges. These results suggested that chloroquine should no longer be used for *P. falciparum* malaria in this geographic area, and that mefloquine should be carefully monitored for its *in vivo* effectiveness. In this study, the AnaeroPack® malaria culture system with portable thermostatic incubator is a powerful and useful mobile tool, which aids in providing detailed evidence-based distribution data concerning of drug resistant malaria in the field.
Key words: AnaeroPack®, Drug susceptibility test, *Plasmodium falciparum*

INTRODUCTION

Since chloroquine-resistant *Plasmodium falciparum* was first reported in 1959 in Thailand, it has developed resistance to all commonly used drugs [1]. The spread of multi-drug resistant *P. falciparum* is now a major public health problem worldwide, with prophylactic and therapeutic implications [2]. Evidence-based detection of drug-resistant parasites is important for the accurate evaluation of susceptibility to antimalarial drugs. However, isolation of fresh parasites for *in vitro* drug susceptibility testing is often difficult in the field because of the precise experimental conditions needed for the test. *In vivo* susceptibility tests are frequently conducted, but they sometimes fail because of difficulties in following up the patients. In fact, it is sometimes difficult to determine whether an adequate drug concentration has been successfully achieved in the patients' blood. Therefore, *in vitro* tests are indispensable to determine the exact degree of resistance acquired by the parasites.

We previously reported that the AnaeroPack® gas system can be used for the continuous cultivation of both laboratory strains and fresh isolates of *P. falciparum* from patients [3]. This gas system is safer, simpler, and easier to use than the candle jar method. In this study, we evaluated the AnaeroPack® malaria culture system with a portable thermostat incubator in a field laboratory on the Thai-Myanmar border to conduct *in vitro* drug susceptibility tests on *P. falciparum*. The feasibility of the system and the results of the tests are described in this report.

The study was conducted at Rajanagarindra Tropical Disease International Center, a field laboratory center of the Faculty of Tropical Medicine, Mahidol University, located in Suan Phung, Rachaburi, about 200 km west of Bangkok. The area was inhabited by Karen as well as Thai people, and in 2001 more than 6,000 people were subjected to microscopic diagnosis of malaria in the center with about 1,000 positive cases found. Malaria is endemic throughout the year in this area with the peak season occurring around May and June. Mefloquine is currently the first drug of

[1] Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan.
[2] Gunma University School of Health Sciences, Maebashi, Gunma 371-8511, Japan.
[3] Asian Centre of International Parasite Control, Bangkok 10400, Thailand.
[4] Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
*Corresponding author:
Shigeyuki Kano,
Department of Appropriate Technology Development and Transfer, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan.
Tel: +81-3-3202-7181 (ext. 2877), Fax: +81-3-3202-7364, E-mail: kano@ri.imcj.go.jp