INTRODUCTION

Malaria is the major cause of mortality and morbidity in the tropical and subtropical regions in the world. An estimated 300-500 million persons suffer from malaria every year and more than 1million die each year. Majority of these cases and deaths particularly those in children occur in Sub Saharan Africa. Unlike some of the other acute diseases such as encephalitis, meningitis, and most of the chronic diseases, patients of severe malaria can recover completely without any long term effects if treated promptly and correctly. Therefore rationalization and standardization of treatment of cases of severe or uncomplicated malaria at different levels of health care is important. It has several advantages deaths can be reduced by effective use of standard treatment procedures. Patients who require hospitalization and those who need intensive care can be identified promptly and treated before they die or develop complications. The adoption of this approach of standard management can reduce the mortality and morbidity from malaria (WHO, 2004).

Approximately 800 – 1,000 malaria cases are admitted to Bangkok Hospital for Tropical Diseases annually. These include *P. falciparum* (51%), *P. vivax* (46%), mixed infections of *P. falciparum* and *P. vivax* (2%), few cases of *P. malariae* and occasional cases of *P. ovale*. Admitted patients are all treated with antimalarial regimens and most of them are enrolled for clinical trial (Faculty of Tropical Medicine, 2004).

Resistance to antimalarial drugs is increasing nearly everywhere in the tropical world, confounding global attempts to "Roll Rack Malaria" (Nosten and Brasseur, 2002) Southeast Asia has the most resistant malaria parasites in the world, which has limited treatment options in this region (WHO, 2001). In Thailand, treatment of acute uncomplicated falciparum malaria is becoming more difficult because of increasing resistance to all antimalarial drugs, except the artemisinin derivatives (Wilairatana *et al*, 2002). To combat the further spread of resistance, it is generally accepted that combinations of antimalarial drugs that include an artemisinin derivative should be used, and, if possible, that preparations should be formulated in a single tablet (Hien *et al*, 2004).

The artemisinin derivatives (artesunate and a recently developed, dihydroartemisinin which is short acting but powerful drug) has been studied extensively in the treatment of falciparum malaria in Thailand, are well tolerated. Their main drawback is that conventional

courses (3-5 days) are associated with high rate of recrudescence, typically >25 %. In addition, there is the risk that parasite resistance will develop when antimalarial drugs are used alone (Warhurst, 1999). Because artemisinin derivatives are now the first-line treatment for multidrug-resistant falciparum malaria in many tropical countries, the appearance of artemisinin-resistant *Plasmodium falciparum* would have serious implications. The development of suitable combinations of an artemisinin compound with a second drug is therefore a priority (WHO, 2001). At present, artesunate has been registered by Thai FDA for use in the treatment of falciparum malaria.

Mefloquine is another antimalarial drug, which is better tolerated than quinine and can be administered during a day, but resistance to mefloquine has developed when used alone. Furthermore, in Thailand where multidrug resistance is encountered, a high dose (25 mg/kg) of mefloquine is recommended for use as a combination with other short acting antimalarial drugs (Nosten *et al*, 1991). Recently, clinical trials of artesunate combination with mefloquine has proved effective and well tolerated (Looareesuwan *et al*, 1992; Looareesuwan *et al*, 1994; Looareesuwan *et al*, 1996;Price *et al*, 1997), therefore this regimen has been chosen for treatment of multidrug resistant falciparum malaria in Thailand. However, some patients can not tolerate adverse effects of mefloquine.

Piperaquine phosphate (1,3-bis[1-(7-chloro-4'-quinolyl)-4'-piperazinyl]) phosphate) replaced chloroquine as the recommended treatment for *Plasmodium falciparum* malaria in China in 1978 and was used extensively for mass prophylaxis and treatment. Reported adverse events are generally similar to those observed with chloroquine, although pruitus is uncommon. (Tropical Medicine Institute, 2003). Piperaquine proved to be effective and well tolerated, and no cross-resistance with chloroquine was observed (Chen *et al*, 1982). More recently, piperaquine has been used as part of short-course artemisinin based combination oral therapies designed to have a high cure rate, to have few side effects, and to reduce malaria transmission (Denis *et al*, 2002; Davis *et al*, 2005).

Artekin® (compound dihydroartemisinin) a combination of dihydroartemisinin 40mg, piperaquine 320mg per tablet (Batch No. 20011204 Mfg. 120401 Exp. 120403 supplied free of charge by Holleykin Pharmaceutical Co. Ltd., Guangzhou, China is claimed for high effective. In addition, this combination is well tolerated and convenient for use (3 days treatment). This compound has been on clinical trials and proved safe and well tolerated in

China, Vietnam, Laos, Cambodia and else where (Karunajeewa *et al*, 2003; Hien *et al*, 2004). We propose here a clinical trial of Artekin® *vs* artesunate and mefloquine (a standard regimen for treatment of multidrug resistant falciparum malaria in Thailand) at the Bangkok Hospital for Tropical Diseases to determine the safety, tolerability, and efficacy.

MATERIALS AND METHODS

Study site and recruitment procedures

All patients who fulfilled inclusion criteria (acute uncomplicated falciparum malaria, either male or female; if female, pregnancy test has to be negative before enrolment to the study, positive asexual forms of *P. falciparum* in blood smear, weight more than 40 kg and age more than 14 years, ability to take oral medication, agreement to stay in the hospital for at least 28 days). Informed consent to the study was obtained from patients, or their guardians, before enrolment to the study. The patients were admitted to the Bangkok Hospital for Tropical Diseases for 28 days to exclude reinfection and to assess the safety and efficacy of Artekin® and artesunate plus mefloquine. We excluded severe malaria according to WHO criteria (WHO, 2000), severe vomiting not allowing oral medication, pregnancy or lactating female, significant concomitant systemic diseases (for example systemic bacterial infections, liver and/or kidney insufficiencies, chronic disease or severe malnutrition), diseases requiring therapy except malaria, ingestion of other antimalarials in the past 14 days or presence of urine sulphonamides or 4-aminoquinolones. Clinical evaluation including neurological examination focused on brain stem, cerebellar function, muscle strength in all limb, extraocular and facial muscle strength, deep tendon reflexes, and finger-to-nose tests, and also parasite count were performed 12 hourly until negative then daily for 28 days. Malaria parasite count per microliter was obtained by calculation against the white blood cell count for a thick film. Geometric mean parasites were used as a standard method. Blood films were considered negative if no parasites were seen in 200 oil-immersion microscopic fields. Fever clearance time was taken as the period from the start of treatment until the oral temperature decreased to 37.5 °C and remained below this temperature for the next 48 hours. Side effects were defined as signs and symptoms that occurred or became more severe after treatment started. Cure rate at day 28 (cured patients/evaluable patients X 100%) was defined as the absence of parasite recrudescence during 28 days of follow-up. If there is RI, RII, or RIII failure (World Health Organization, 1973), standard antimalarial drugs of the hospital will be

given. Adverse events will also be treated as standard procedures at the Bangkok Hospital for Tropical Diseases. This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Study drug administration

An open randomized clinical trial of Artekin® *vs* Artesunate-Mefloquine was conducted at the Bangkok Hospital for Tropical Diseases, Mahidol University. Upon admission to the ward, patients were randomly treated at ratio of 1:2 into groups A:B as follows:

Group A: **AM:** Artesunate (4mg/kg/day) was given by oral route once a day for 3 days together with Mefloquine 8mg/kg/day for 3days

Group B: **Artekin**: Artekin® (2 mg/kg/day of dihydroartemisinin and 15 mg/kg/day piperaquine) was given by oral route once a day for 3 days (Artekin® 1 tab contains DHA 40mg + Piperaquin 320mg)

All patients were treated symptomatically as indicated (e.g. intravenous fluid and antipyretics.) according to the standard practice in the hospital. In cases of RI, RII, or RIII responses (WHO, 1973), other antimalarial drugs (e.g. quinine plus tetracycline for falciparum malaria and chloroquine followed by primaquine for vivax malaria) were used as indicated. Patients who vomited within one hour after drug administration were redosed.

Monitoring for safety

Patients were physically examined and adverse reactions during the study were recorded with the date and time at which they occurred and disappeared. Adverse effects were assessed on the basis of non-suggestive questioning by the study investigators. These include gastrointestinal, central nervous, cardiovascular, dermatological effects, as well as other changes possibly attributable to the study drugs. Routine blood investigations (hematology and biochemistry), and urinalysis were performed prior (Day 0) and weekly for 4 weeks of the study period.

Statistical analysis

Statistical analysis was performed by using the Analyze It Add Ins Excel for Windows. All the *P-values* reported are from 2-tailed test and the statistically significance level was set at 0.05. The distribution of data was assessed for normality using the Schapiro-Wilks test. Data were expressed, as means and SD. Two statistical tests were performed. We used chi-square analyses to test differences between 2 groups of the qualitative variables and independent t-test to test the difference between 2 groups of the quantitative variables on demographics and baseline laboratory data. (Tabachnick *et al,* 2001)

RESULTS

A total of one hundred and eighty patients were enrolled in this trial. All pregnancy tests in female patients were negative. Around 90% of patients completed the study as planed. Demographic clinical data and pretreatment laboratory characteristics are shown in Table 1.There were 135 male and 45 female patients aged 14 to 65 years old participated in this trial. There were no significant differences in the distribution of demographic, clinical and laboratory data between the two treatment groups.

At enrolment, patient in both treatment groups showed common malaria symptoms such as headache, asthenia, fatigue, fever, nausea, vomiting, myalgia and anorexia. Most clinical manifestations present on admission gradually disappeared during the first few days of treatment and coincided with high fever. Some baseline laboratory parameters were affected by disease status. However, they all returned to normal within 1-2 weeks.

Nineteen (6 and 13 patients in each group) patients did not complete the 28-days follow up due to social reasons not related to adverse effects. Thus, 161 patients out of 180 patients (89.5%) completed the 28-day study. No patients were deteriorated in clinical or biochemical changes after treatment in both groups. Parasitologic and clinical responses are shown in Table II. All patients in this study showed a prompted response to both antimalarial regimens (Figure 1). The cure rates at 28 days of follow-up were 100% and 99% respectively for the both treatment groups. There were no significant differences in fever clearance time and parasite clearance time between both treatment groups. No patients had RII or RIII failures. Only one patient in Artekin® treatment group had recrudescence on day 21 of study

period. The patient who drug failed to clear parasitemia was given the rescue antimalarial chemotherapy according to the hospital's standard regimen. Therefore, all patients had parasitologically negative at the time discharged from our hospital.

Means time for parasite clearances in each treatment group was fast, however there were no statistically significant different differences between the two treatment-groups [39.6 ± 13.7 hours and 35.0 ± 16.2 hours in group 1 and 2 respectively, (p=0.72)]. The parasites were all cleared from peripheral blood smears within 84 hours. Similarly, there was no statistically significant difference between the fever clearance times of both treatment groups (p=0.67).

No death occurred. No patients had vomiting related to the drugs. There were no major adverse effects and no neurologic or neuropsychiatric manifestations during treatment and during the 28-day follow-up period. Some minor symptoms such as nausea, headache, and dizziness occurred in group A (4, 3, 2 patients) and in group B (5, 4, 4 patients) respectively. However, these signs and symptoms could not be differentiated from malaria symptoms as they were disappeared between 1-4 days after treatment and while fever subsided. In addition, there was no serious adverse event reported during the study.

DISCUSSION

In Thailand, *plasmodium falciparum* is resistant to chloroquine and there has been a decline in sensitivity to mefloquine (Brockman *et al*, 2000). The use of the artemisinin derivatives has been central to successful malaria control efforts in Thailand, Vietnam and Cambodian (Denis *et al*, 2002; Hien *et al*, 2004; Looareesuwan *et al*, 1997). Artemisinin derivatives are potent, rapidly acting antimalarials that can reduce parasitemias by more than 90% within 24 hours in uncomplicated malaria cases. However, the rate of recrudescence within 28 days when used alone can be as high as 10-25 % depending upon dosage, duration of treatment, and severity of disease (Hien *et al*, 1991; Li *et al*, 1994). These drugs are often combined with other long acting antimalarials such as mefloquine, (in this study combined with piperaquine, to improve efficacy and compliance). The rationale using of the combination is as standard treatment of multidrug treatment for taberculosis, patients with HIV and most cases of cancers. The rapid killings of parasitemias of artemisinin derivatives is accelerate the therapeutic response, prevent dangerous early treatment failures in case of high grade resistance,

reduce the parasite biomass and reduce gametocyte transmission (Looareesuwan et *al*, 1999). The benefit of adding appropriate and suitable long action drug is prevent recrudescence by killing residual parasites, reduce the chance of a resistant mutant surviving and in addition the long acting antimalarial might protect the artemisinin derivative in low transmission areas. Combined administration of artemisinin derivatives and mefloquine in different dosages and duration had been studied in uncomplicated malaria in many countries. This combination is now a standard treatment for multidrug resistant falciparum malaria in Thailand (Wilairatana *et al*, 2002). However, some disadvantages of using artesunate-mefloquine might be seen (e.g. some patients could not tolerate mefloquine).

The combination of DHA and Piperaquine in the form of Artekin® was developed as an alternative to established combinations, such as artesunate-mefloquine, primarily to reduce treatment costs and toxicity. Our hospital based study has shown that a combination of dihydroartemisinin and piperaquine, is an effective and well tolerated by Thai adults with acute uncomplicated *plasmodium falciparum* malaria. Like a previous study (Denis et al, 2002; Hien et al, 2004) most of the patients whose treated by Artekin® in this study improved clinically and were parasite negative on the blood smear by the third day of treatment. The present study, all patients responded satisfactorily to the both treatment regimens. As well as, the present study shows a high total cure rate (99-100%) in the both groups. Artekin® showed similar cure rate to the standard treatment (artesunate-mefloquine). However at present, it has remained unclear whether the improve cure rate due to synergistic effect of the synergy dihydroartemisinin and piperaquine. There was no fatal patient in the study. Comparing with 3-day combination of artesunate-mefloquine treatment, Artekin® is given only in 3 days with 3 doses. The shorter period of Artekin® is now on clinical trial and might be better and have high chance of complete treatment course and improved compliance. This combination may serve as alternative regimens for treatment of uncomplicated falciparum malaria. The Artekin® has more advantage on these issues and more importantly since the drug was produced as a fix combination, less duration of treatment and possibly lower cost than artesunate-mefloquine. Other long acting drugs combined with artemisinin derivatives are under invention.

In conclusion, the results of this study indicate that Artekin® is effective and well-tolerated. Artekin® may be an alternative treatment to the standard combination of artesunate- mefloquine in treatment of multidrug resistant uncomplicated falciparum malaria

such as in Thailand. However, additional and more studies in special groups (in children, pregnant women, and field trials) and pharmacokinetic studies to guide rational dosing regimens are needed in order to get more informations of Artekin® in general practice.

ACKNOWLEDGEMENTS

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REFERENCES

Brockman A, Price RN, van Vugt M, *et al.* Plasmodium falciparum antimalarial durg susceptibility on the northwestern border of Artesunate Thailand during five years of extensive Artesunate-mefloquine use. *Trans R Soc Trop Med Hyg* 2000; 94: 537-44.

Chen L, Qu FY, Zhou YC. Field observations of the antimarial piperaquine. *Chin Med J* 1982; 95: 281-6.

Davis TM, Hung TY, Sim IK, *et al.* Piperaquine: a resurgent antimalarial drug. *Drugs* 2005; 65(1): 75-87.

Denis MB, Devis TM, Hewitt S, *et al.* Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated falciparum malaria. *CID* 2002; 35: 1469-76.

Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Annual report. 2004; 80.

Hien TT, Dolecek C, Mai PP, *et al.* Dihydroartemisinin-piperaquine againt multidrug-resistant Plasmodium falciparum malaria in Vietnam: randomized clinical trial. *Lancet* 2004; 363: 18-22.

Hien TT, Tam DT, Cuc NT, Arnold K. Comparative effectiveness of artemisinin suppositories and oral quinine in children with acute falciparum malaria. *Trans R Soc Trop Med Hyg* 1991; 85: 201-11.

Karunajeewa H, Lim C, Hung TY, *et al.* Safety evaluation of fixed combination piperaquine plus dihydroartemisinin (Artekin®) in Cambodian children and adults with malaria. *Br J Clin Pharmacol* 2003; 57(1): 93-99.

Li GQ, Guo XB, Fu LC, *et al.* Clinical trials of artemisinin and its derivatives in the treatment of malaria in China. *Trans R Soc Trop Med Hyg* 1994; 88: S5-6.

Looareesuwan S, Kyle DE, Viravan C, *et al.* Treatment of patients with recrudescent falciparum malaria with a sequential combination of artesunate and mefloquine. *Am J Trop Med Hyg* 1992; 47: 794-9.

Looareesuwan S, Vanijanonta S, Viravan C, *et al.* Randomised trial of mefloquine alone, and artesunate followed by mefloquine for the treatment of acute uncomplicated falciparum malaria. *Ann Trop Med Parasitol*, 1994; 88: 131-6.

Looareesuwan S, Viravan C, Vanijanonta S, *et al.* Comparative clinical trial of artesunate followed by mefloquine in the treatment of acute uncomplicated falciparum malaria : two and three-day regimens. *Am J Trop Med Hyg* 1996; 54: 210-3.

Looareesuwan S, Wilairatana P, Chokejindachai W, *et al.* A randomized, double-blind, comparative trial of a new oral combination of artemether and benflumetol (CGP 56697) with mefloquine in the treatment of acute *Plasmodium falciparum* malaria in Thailand. *Am J Trop Med Hyg* 1999; 60: 238-43.

Looareesuwan S, Wilairatana P, Vanijanonta S, *et al.* Monotherapy with artesunate for uncomplicated falciparum malaria : A comparison of 5-day and 7-day regimens. *Acta Tropica* 1997; 67: 197-205.

Nosten F, Brasseur P. Combination therapy for malaria. *Drug* 2002; 62: 1315-29.

Nosten F, Kuile F, Chongsuphajaisiddhi T, *et al.* Mefloquine- resistant falciparum malaria on the Thai-Burmese border. *Lancet* . 1991; i : 1140-3.

Price RN, Nosten F, Luxemburger C, *et al.* Artesunate-mefloquine treatment of 1967 patients with multi-drug resistant falciparum malaria. *Trans R Soc Trop Med Hyg* 1997; 91: 574-7.

Tabachnick BG, Fidell LS. Using Multivariate Statistics. 4th ed. USA.: Allyn&Bacon. 2001: 41-5.

Tropical Medicine Institute, Guangzhou University of Traditional Chinese Medicine. Documentation of Artekin for new drug registration. Guangzhou, China: Guangzhou University of Traditional Chinese Medicine. 2003.

Warhurst DC. Drug resistance in *Plasmodium falciparum* malaria. *Infection* 1999; 27: S55-8.

Wirlairatana P, Krudsood S, Chalermrut K, *et al.* An open randomized clinical trial of Artecom *vs* Artesunate-Mefloquine in the treatment of acute uncomplicated falciparum malaria in Thailand. *Southeast Asian J Trop med Public Health* 2002; 33: 519-24.

World Health Organization. Advances in malaria chemotherapy. *WHO Tech Rep Ser* 1973; 529: 30-5.

World Health Organization. Antimalarial drug combination therapy:report of a WHO Technical Consultation. Geneva: WHO, April 2001; 35.

World Health Organization. Guidelines for treatment of severe malaria in hospitals. Available at: HTTP://MOSQUITO.WHO.INT/DOCS/SM_SEARO.PDF (accessed May 2005)

World Health Organization. Management of severe malaria: A practical handbook. 2nd ed. Geneva: WHO, 2000.

Table I Clinical and laboratory characteristics of study groups before treatment.

	Group A	Group B

	(n = 60)	(n = 120)
Male/Female	49/11	86/34
Age (yr)		
Mean (SD)	26.5 (10.6)	24.3 (8.5)
Range	14-65	14-58
Mean (SD) height in cm	161.5 (9.3)	160.5 (8.1)
Mean (SD) weight in kg	55.6 (10.9)	51.8 (9.7)
Fever[Mean(SD)]		
Duration before admission (days)	5.6 (5.3)	5.3 (4.4)
Highest fever before treatment (°C)	38.2 (0.9)	38.3 (1.0)
No. of patients with:		
Splenomegaly	3	5
Hepatomegaly	12	19
Urine positive for drugs*	0	0
First malaria attack	29	32
Geometric mean parasites		
count (per μl)	4,645	3,759
Range high	102,500	190,860
Low	13	17
Laboratory data (mean [SD])		
Packed cell volume (%)	35.8 (5.1)	36.0 (6.0)
WBC count (per μl)	6,239 (4,593)	5,579 (1,776)
Blood urea (mmol/L)	14.2 (7.0)	15.3 (7.2)
Serum creatinine (umol/L)	0.8 (0.2)	0.9 (0.2)
Total bilirubin (umol/L)	1.4 (0.9)	1.5 (1.1)
Serum AST	38.1 (22.6)	42.7 (52.7)
Serum AAT	39.8 (34.4)	38.6 (31.8)
Albumin (mg/L)	3.7 (0.5)	3.6 (0.5)
Alk PO_4	134.9 (56.4)	140.5 (100.4)

WBC = white blood count
AST, AAT = aspartate and alamine aminotransferases (U/L)
Alk PO_4 = alkaline phosphatase (U/L)
*Sulphonamides and 4-aminoquinolones

Table II Therapeutic responses

	Group A (n = 60)	Group B (n = 120)
No. of patients dropout	6	13
No. of patients with 28 days follow up	54	107
No. (%) cured at 28 days	54 (100%)	106 (99%)
Recrudescence on days	-	21
Fever clearance time (hours)		
Mean (SD)	25.2 (28.4)	24.8 (24.7)
Range	4-100	4-124
Parasite clearance time (hours)		
Mean (SD)	39.6 (13.7)	35.0 (16.2)
Range	11-84	4-74

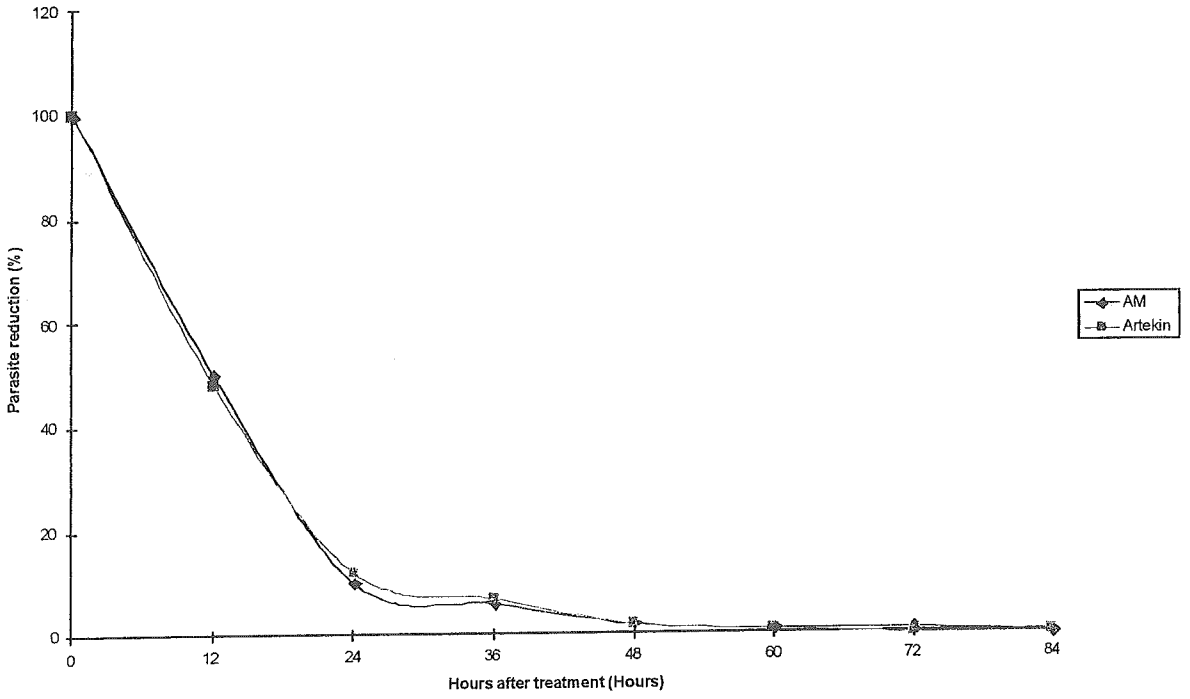

Figure 1 Percentage of malaria parasite reduction after treatment

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Plasmodium falciparum: The fungal metabolite gliotoxin inhibits proteasome proteolytic activity and exerts a plasmodicidal effect on *P. falciparum* ☆

Toshimitsu Hatabu [a,*], Masaki Hagiwara [a], Nao Taguchi [a], Masayuki Kiyozawa [b], Mamoru Suzuki [c], Shigeyuki Kano [d], Kumiko Sato [a]

[a] *Gunma University School of Health Sciences, 3-39-15 Showa-machi, Maebashi, Gunma 371-8514, Japan*
[b] *Nihon Hiryo Co., Ltd., 559-3 Tozaki, Fujioka, Gunma 375-0011, Japan*
[c] *Gunma University, 4-2 Aramaki, Maebashi, Gunma 371-8510, Japan*
[d] *Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*

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Abstract

The in vitro antimalarial activity of the fungal metabolite gliotoxin (GTX) was evaluated, and its mechanism of action was studied. GTX showed plasmodicidal activity against both *Plasmodium falciparum* chloroquine-resistant strain K-1 and chloroquine-susceptible strain FCR-3. GTX cytotoxicity was significantly lower against a normal liver cell line (Chang Liver cells). The intracellular reduced glutathione level of parasitized and of normal red blood cells was not affected by GTX treatment. However, GTX decreased the chymotrypsin-like activity of parasite proteasomes in a time-dependent manner. The results of this study indicate that GTX possesses plasmodicidal activity and that this effect is due to inhibition of parasite proteasome activity, suggesting that GTX may constitute a useful antimalarial therapy.

Index Descriptors and Abbreviations: Plasmodium falciparum; Gliotoxin; Proteasome inhibitor; pRBCs, parasitized red blood cells; RBCs, red blood cells; GTX, gliotoxin; GSH, reduced glutathione

1. Introduction

Since the report of chloroquine (CQ)-resistant *Plasmodium falciparum* in the 1950s, the spread and persistence of drug-resistant malaria has continued to pose problems for malaria therapy. Multidrug resistant *falciparum* malaria parasites have been reported frequently in the Thai-Myanmar border area (Wongsrichanalai et al., 2002). Considering the increasing prevalence of drug-resistant falciparum malaria, the development of new antimalarial drugs is an urgent issue.

Gliotoxin (GTX) is a member of the epipolythiodioxopiperazine class of fungal metabolites and possesses a diverse range of biologic activities including antimicrobial, antifungal, and antiviral activities, but is lethal to rodents at relatively low concentrations (LD_{50} in mice and rats: 25–50 mg/kg) (Taylor, 1971; Waring and Beaver, 1996). GTX has also been reported to induce apoptotic cell death in numerous cell types. An important property of GTX is its ability to go through a redox cycle in the presence of an appropriate reducing agent. Hydrogen peroxide produced by GTX during the redox cycle in a cell-free system directly damages plasmid and cellular DNA (Eichner et al., 1986). GTX thus acts as a redox-active protein and has been shown to form mixed disulfides with accessible thiol residues on proteins or to induce further oxidative modification

☆ Plasmodicidal activity of fungal metabolite gliotoxin.

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

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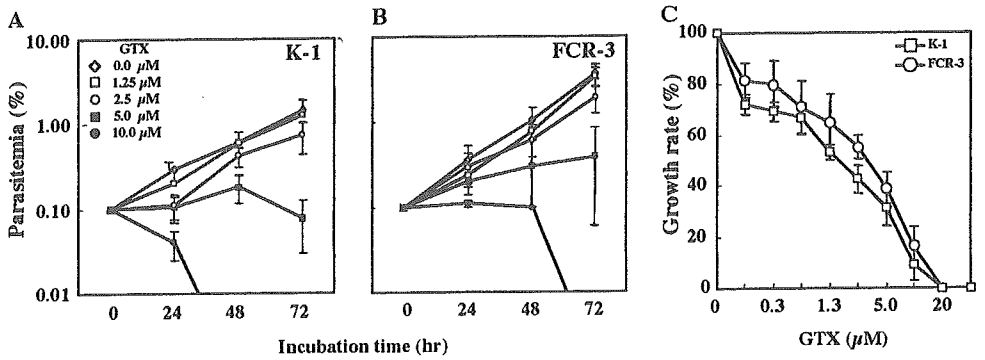

Fig. 1. Plasmodicidal effect of gliotoxin (GTX). GTX was added to cultures of chloroquine (CQ)-resistant strain K-1 (A) or CQ-susceptible strain FCR-3 (B) at a concentration of 10 μM (filled circles), 5 μM (filled squares), 2.5 μM (open circles), 1.25 μM (open squares), or 0.0 μM (open diamonds). Parasitemia (%) was determined every 24 h. (C) Concentration-dependent effect of GTX on inhibition of K-1 (squares) and FCR-3 (circles) maturation in vitro. Results are presented as means ± SEM. Parasitemia of the K-1 strain treated with 10 μM GTX at 48 and 72 h are both 0%, and that of the FCR-3 strain treated with 10 μM GTX at 72 h is also 0%.

(Bernardo et al., 2003). Recently, it was reported that GTX efficiently inhibits proteasome catalytic activity (Kroll et al., 1999).

In eukaryotic cells, most proteins in the cytoplasm and nucleus are degraded not within lysosomes but within proteasomes. The roles of proteasomes in protein turnover are involved in specific cellular functions, including programmed inactivation of mitotic cyclins, transcription factors, and transcriptional regulators; elimination of mutated or damaged proteins; and antigen presentation. These functions are tightly regulated (Zwickl and Baumeister, 2002).

Lactacystin, a proteasome inhibitor, has been reported to inhibit the growth of *P. falciparum* and to be effective against CQ-resistant parasites (Certad et al., 1999). Intracellular amastigote–trypomastigote transformation is also reported to be prevented by lactacystin treatment (de Diego et al., 2001). GTX has been reported to inhibit the growth of *Toxoplasma gondii* by inhibiting proteasome activity (Paugam et al., 2002). In this study, the plasmodicidal effect of GTX was evaluated in relation to effects on reactive oxygen species and intracellular proteasome activity.

2. Materials and methods

2.1. Parasite and cell culture

CQ-resistant *P. falciparum* strain K-1 and CQ-susceptible strain FCR-3 were grown asynchronously, according to the modified method of Trager and Jensen (1976), in RPMI 1640 (pH 7.4) medium supplemented with 10% human A serum, 25 mM Hepes, 25 μg/ml gentamicin (Sigma–Aldrich, St. Louis, MO), 24 mM sodium bicarbonate, and human O type red blood cells (RBCs) in disposable sterile culture dishes under a controlled atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C.

The human normal liver cell line, Chang liver cells, was kindly provided by Dr. Takeaki Nagamine (Gunma University School of Health Sciences, Gunma, Japan). Cells were grown continuously in complete Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml)–streptomycin (10 ml/L; Gibco-BRL, Grand Island, NY) under 5% CO_2 at 37 °C.

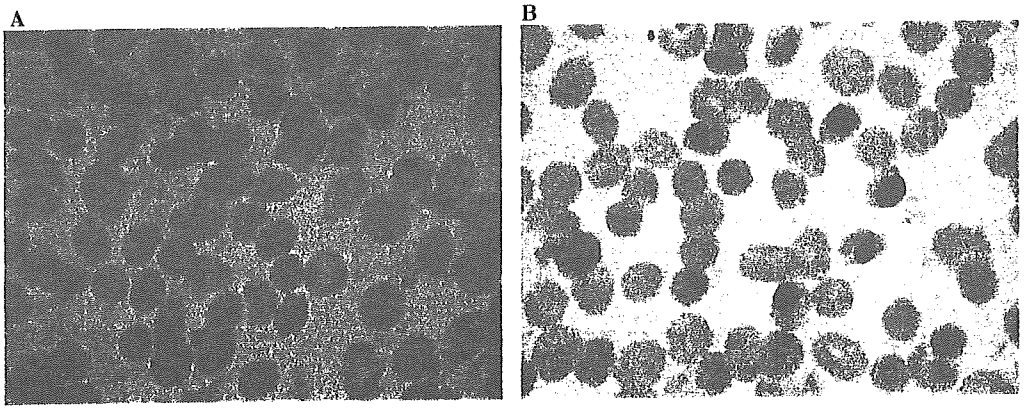

Fig. 2. Morphology of the Giemsa-stained K-1 strain after 24 h of incubation with GTX. The parasites were first synchronized at the ring stage and then cultivated with 10 μM of GTX (A) and drug-free condition (B) for 24 h. Note the degenerative changes of the parasites in the GTX-treated culture (arrows in A). Magnification, 1000×.

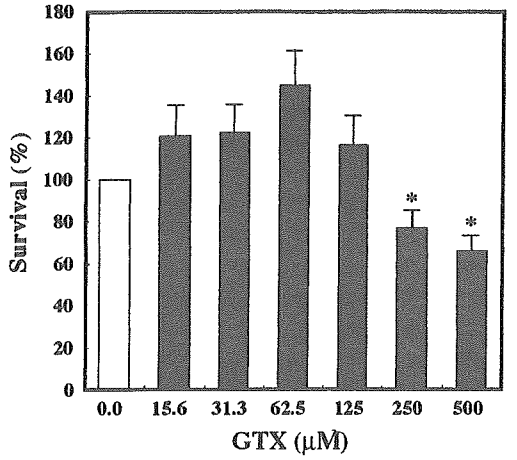

Fig. 3. Cell mortality after treatment with GTX for 72 h. The cytotoxic effect of GTX on Chang liver cells was evaluated by LDH release colorimetric assay (CytoTox-ONE). Results are presented as means ± SEM. $*P < 0.05$.

2.2. Evaluation of in vitro plasmodicidal effect of GTX

RPMI 1640 medium was supplemented with GTX in solution at final concentrations ranging from 0.156 to 10.0 μM. GTX-supplemented medium was changed every 24 h. Five hundred microliters of parasitized-RBC (pRBC) suspension was placed into each well of a 24-well culture plate (Corning Incorporated, Corning, NY) at a hematocrit of 5% and initial parasitemia of 0.1%. Thin-smear specimens were made every 24 h and stained with Giemsa solution, and parasitemia was evaluated by counting the number of pRBCs in 3000 RBCs. Morphologic changes of the parasites in response to drug treatment were monitored by observing Giemsa-stained thin-smear specimens under light microscopy.

2.3. Determination of GTX IC_{50}

pRBCs were synchronized with 5% D-sorbitol for 15 min at room temperature as previously described (Taguchi et al., 2004). After three washes in RPMI 1640 medium, pRBCs were resuspended in RPMI 1640 medium (pH 7.4) supplemented with 10% human O serum, Hepes (25 mM), gentamicin (25 μg/ml), and sodium bicarbonate. Synchronous pRBCs showing approximately 2% parasitemia were seeded onto 24-well cell culture plates as described above. Twenty microliters of GTX solution was added to each well at final concentrations ranging from 0.156 to 40.0 μM. Six wells per plate served as GTX-untreated controls to monitor parasite growth. After 24 h of incubation, the control wells were checked for parasite growth. When parasites in control wells became schizonts, the culture plates were removed from the incubator, and thin-smear specimens were made from the contents of each well and stained with Giemsa solution. We counted RBCs in control smears for a total of 50 schizonts. The effect of GTX on parasite growth was evaluated by comparing the number of schizonts in the same number of RBCs as was counted in the control cultures. Growth inhibition (%) was calculated as follows: (test well schizont count/control well schizont count) × 100. The GTX concentration that inhibited parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba et al., 2001).

2.4. Toxicity of GTX in Chang liver cells

To assess GTX cytotoxicity in a mammalian cell line, Chang liver cells were analyzed with a lactate dehydrogenase

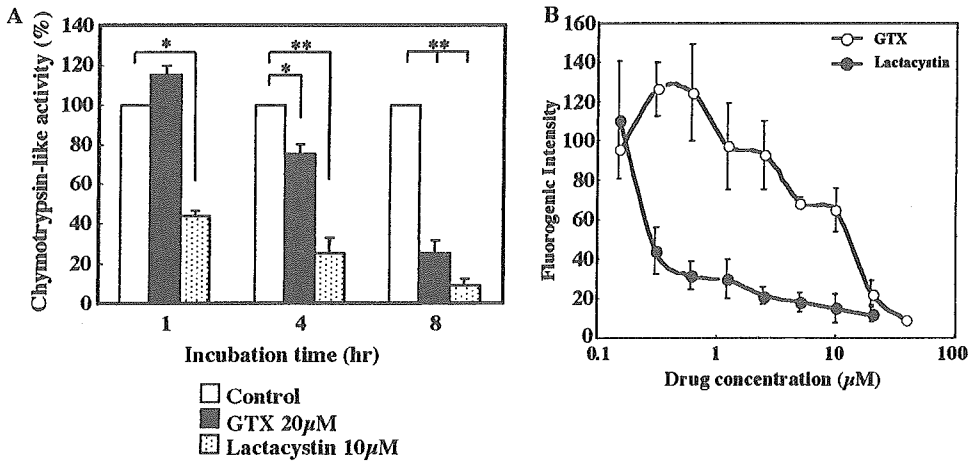

Fig. 4. GTX inhibits chymotrypsin-like activity of *P. falciparum* proteasomes in time- and concentration-dependent manner. (A) Chymotrypsin-like activity was measured on crude parasite extracts after addition of GTX together with the fluorogenic peptide, succinyl-Leu-Leu-Var-Tyr-4-methylcoumarinyl-7 amide. Activities are presented relative to GTX-untreated control (open columns, 100% activity). Data are presented as means ± SEM of three independent experiments. Filled and hatched columns indicate GTX-treated (20 μM) and lactacystin-treated (10 μM) parasites as positive controls. $*P < 0.05$, $**P < 0.01$. (B) Proteasomes isolated from the parasites, plus lactacystin (open circle) and GTX (closed circle) at concentrations, were incubated with fluorogenic substrate succinyl-Leu-Leu-Var-Tyr-4-methylcoumarinyl-7 amide to measure chymotrypsin-like activity. Each point is the mean of the fluorescences of triplicate wells ± SEM.

release colorimetric assay (CytoTox-ONE 96 Assay Kit; Promega, Madison, WI) as described previously (Hatabu et al., 2005). In brief, Chang liver cells were seeded at 1×10^5 cells/ml in 96-well culture plates (Corning Costar) and incubated at 37 °C for 72 h in DMEM supplemented with GTX. After incubation, cells were washed three times with Hanks solution (Sigma–Aldrich), and 100 μl of reaction reagent was added to each well and incubated at 37 °C for 10 min. After the reaction was terminated, fluorescence levels were determined with a multi-well plate reader (CytoFluor; Perseptive Biosystems, Framingham, MA) at an excitation wavelength of 530 nm and an emission wavelength of 580 nm.

2.5. Measurement of reduced glutathione levels in pRBCs and normal RBCs

Intracellular reduced glutathione (GSH) levels were measured according to the method of Hissin and Hilf (Hissin and Hilf, 1976). In brief, cell suspensions were supplemented with various concentrations of GTX (2.5–20 μM). After a 24-h incubation, cells were counted with a hemocytometer. After a wash in PBS, homogenization buffer (4:1 mixture of 0.1 M sodium phosphate, 5 mM EDTA buffer, pH 8.0, and 25% metaphosphoric acid) was added. Cell suspensions were homogenized on ice with a sonic wave homogenizer for 40 s. Homogenates were centrifuged (21,000*g*, 30 min, 4 °C), and supernatants were collected for analysis. After addition of *o*-phthalaldehyde (0.1% in methanol), GSH levels were determined with a multi-well plate reader (CytoFluor) at an excitation wavelength of 360 nm and an emission wavelength of 420 nm. Standards contained 0–2 μg GSH/ml.

2.6. Enzymatic assay of P. falciparum proteasome activity

Chymotrypsin-like activity of *P. falciparum* proteasomes was assessed by the fluorometric method of Gonzalez et al. (1999). In brief, GTX-treated and control pRBCs containing trophozoite/schizont-stage parasites were harvested and incubated in 0.05% saponin (Sigma–Aldrich) on ice for 30 min. After a PBS wash, homogenization buffer was added as described above. Cell suspensions were homogenized on ice with a sonic wave homogenizer for 40 s. Homogenates were centrifuged (21,000*g*, 30 min, 4 °C), and supernatants were collected for analysis.

Chymotrypsin-like activity of 20S proteasomes was measured with the use of the fluorogenic substrate *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (Suc-LLVY -amc; Peptide Institute, Osaka, Japan). Lactacystin was purchased from the same source. A concentration of 10 μM of lactacystin was used as a positive control. Mean values of three experiments are shown.

2.7. Statistical analysis

Data presented are the means ± SEM values of at least three independent experiments. Student's *t* test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Plasmodicidal activity of GTX in CQ-resistant and CQ-susceptible P. falciparum

To confirm the plasmodicidal activity of GTX, CQ-resistant *P. falciparum* strain K-1, and CQ-susceptible strain FCR-3 were included in medium containing GTX for 3 days, and parasite growth was monitored by determining the level of parasitemia (Figs. 1A and B). Growth of both strains was inhibited in a concentration-dependent manner by GTX, the parasites being degenerative (Fig. 2). In the presence of 5 μM GTX, the growth of both strains was inhibited but not completely. In the presence of 10 μM GTX, the growth of both parasite strains was completely inhibited. However, CQ-resistant strain K-1 looked a bit more susceptible to GTX than CQ-susceptible strain FCR-3 by the graph.

Dose–response curves for GTX in K-1 and FCR-3 are shown in Fig. 1C. IC_{50} values for GTX were 3.60 ± 0.97 μM for K-1 and 4.17 ± 1.08 μM for FCR-3. K-1 seemed to be a little more susceptible to GTX, but there was no significant difference between IC_{50} values of CQ-resistant and CQ-susceptible parasites ($P > 0.05$).

3.2. GTX toxicity in Chang liver cells

Toxicity of GTX in Chang liver cells was also evaluated (Fig. 3). Although the survival of Chang liver cells was ≈70% in response to ⩾250 μM GTX, survival was not affected by ⩽125 μM GTX.

3.3. GTX effect on intracellular proteasome activity

To determine whether GTX inhibits proteasome-associated proteolytic activity, we tested the effect of 20 μM GTX on chymotrypsin-like activity (Fig. 4A). GTX decreased chymotrypsin-mediated proteolysis of Suc-LLVY-amc by ≈25% after 4 h treatment compared to untreated controls ($P < 0.05$). After 8 h GTX treatment, 74% inhibition of Suc-LLVY-amc activity was observed compared to untreated controls ($P < 0.01$). When tested on proteasomes isolated from parasites, GTX showed inhibitory activity that paralleled its plasmodicidal activity (Fig. 4B). Thus, GTX decreased chymotrypsin-like activity of parasite proteasomes in a time- and concentration-dependent manner.

To study another plasmodicidal mechanism of GTX, intracellular GSH content was determined. Intracellular GSH levels of both pRBCs and RBCs were not affected by treatment with GTX (data not shown).

4. Discussion

The results of this study indicate that GTX possesses plasmodicidal activity and is effective against *P. falciparum*

CQ-resistant strain K-1 and CQ-susceptible strain FCR-3. GTX has a multitude of effects, many of which are due to proteasome inhibition. The results also indicate that GTX inhibits chymotrypsin-like activity of parasite proteasomes. Many parasites showed degenerative changes after GTX treatment, as determined by light microscopy. Gonzalez et al. (1999) reported inhibition of normal development to the schizont stage by treatment with another proteasome inhibitor, lactacystin. Morphologic changes in their report were similar to those observed in this study.

GTX acts as a redox-active protein, and in cell-free experiments, it has been shown to form mixed disulfides with accessible thiol residues on proteins or to induce further oxidative modification (Bernardo et al., 2003). Kweon et al. (2003) reported that GTX induces apoptosis in hepatic stellate cells by forming reactive oxygen species and that the formation of reactive oxygen species by GTX is concentration-dependent. However, our data indicate that intracellular GSH levels are not altered, and there was no difference in GSH levels between pRBCs and RBCs in response to 10 μM GTX, suggesting that the plasmodicidal activity of GTX is not induced by oxidative damage. These findings suggest that GTX primarily targets parasite proteasomal chymotrypsin-like proteolytic activity.

Proteasome inhibitors, including lactacystin, are candidates for antimalarial therapy (Gantt et al., 1998). Taylor (1971) reported that GTX was lethal to rodents at relatively low concentrations (LD_{50} in mice and rats: 25–50 mg/kg). However, the present study showed that GTX exerted approximately 25-fold lower cytotoxicity on normal liver cells than on parasites, indicating that GTX may be a promising treatment for drug-resistant malaria.

In conclusion, GTX showed an inhibitory effect on plasmodial development in vitro by inhibitinparasite proteasome activity, and selective inhibitors of parasite proteasomes may be useful antimalarial agents.

References

Bernardo, P.H., Brasch, N., Chai, C.L., Waring, P., 2003. A novel redox mechanism for the glutathione-dependent reversible uptake of a fungal toxin in cells. Journal of Biological Chemistry 278, 46549–46555.

Certad, G., Abrahem, A., Georges, E., 1999. Cloning and partial characterization of the proteasome S4 ATPase from *Plasmodium falciparum*. Experimental Parasitology 93, 123–131.

de Diego, J.L., Katz, J.M., Marshall, P., Gutierrez, B., Manning, J.E., Nussenzweig, V., Gonzalez, J., 2001. The ubiquitin-proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling. Biochemistry 40, 1053–1062.

Eichner, R.D., Al Salami, M., Wood, P.R., Mullbacher, A., 1986. The effect of gliotoxin upon macrophage function. International Journal of Immunopharmacology 8, 789–797.

Gantt, S.M., Myung, J.M., Briones, M.R., Li, W.D., Corey, E.J., Omura, S., Nussenzweig, V., Sinnis, P., 1998. Proteasome inhibitors block development of *Plasmodium* spp. Antimicrobial Agents and Chemotherapy 42, 2731–2738.

Gonzalez, J., Bai, G., Frevert, U., Corey, E.J., Eichinger, D., 1999. Proteasome-dependent cyst formulation and stage-specific ubiquitin mRNA accumulation in *Entamoeba invadens*. European Journal of Biochemistry 264, 897–904.

Hatabu, T., Takada, T., Taguchi, N., Suzuki, M., Sato, K., Kano, S., 2005. Potent plasmodicidal activity of heat-induced reformulation of deoxycholate-amphotericin B (Fungizone) against *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy 49, 493–496.

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.

Kroll, M., Arenzana-Seisdedos, F., Bachelerie, F., Thomas, D., Friguet, B., Conconi, M., 1999. The secondary fungal metabolite gliotoxin targets proteolytic activities of the proteasome. Chemistry and Biology 6, 689–698.

Kweon, Y.O., Paik, Y.H., Schnabl, B., Qian, T., Lemasters, J.J., Brenner, D.A., 2003. Gliotoxin-mediated apoptosis of activated human hepatic stellate cells. Journal of Hepatology 39, 38–46.

Paugam, A., Creuzet, C., Dupouy-Camet, J., Roisin, P., 2002. In vitro effects of gliotoxin, a natural proteasome inhibitor, on the infectivity and proteolytic activity of *Toxoplasma gondii*. Parasitology Research 88, 785–787.

Taguchi, N., Hatabu, T., Yamaguchi, H., Suzuki, M., Sato, K., Kano, S., 2004. *Plasmodium falciparum* : selenium-induced cytotoxicity to *P. falciparum*. Experimental Parasitology 106, 50–55.

Taylor, A., 1971. The toxicology of sporidesmis and other epipolythiodioxopiperazines. Microbiology and Toxins 7, 337–376.

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

Waring, P., Beaver, J., 1996. Gliotoxin and related epipolythiodioxopiperazines. Genetic Pharmacology 27, 1311–1316.

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

Zwickl, P., Baumeister, W., 2002. The proteasome-ubiquitin degradation pathway. Current Opinion of Tropical Microbiology and Immunology 268, 1–209.

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Down-regulation of tight junction mRNAs in human endothelial cells co-cultured with *Plasmodium falciparum*-infected erythrocytes

Pannapa Susomboon [a,b], Yaowapa Maneerat [b], Paron Dekumyoy [c], Thareerat Kalambaheti [d], Moritoshi Iwagami [e], Kanako Komaki-Yasuda [e], Shin-ichiro Kawazu [e], Noppadon Tangpukdee [a], Sornchai Looareesuwan [a], Shigeyuki Kano [e,*]

[a] *Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchavithi Road, Bangkok 10400, Thailand*
[b] *Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchavithi Road, Bangkok 10400, Thailand*
[c] *Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchavithi Road, Bangkok 10400, Thailand*
[d] *Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchavithi Road, Bangkok 10400, Thailand*
[e] *Department of Appropriate Technology Development and Transfer, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan*

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Abstract

To understand the mechanism of sequestration in the microvasculature of patients with falciparum malaria, we examined the patterns of expression of mRNAs for adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and tight junction molecules (occludin, vinculin, and ZO-1) in human umbilical vein endothelial cells (HUVECs) co-cultured with *Plasmodium falciparum*-parasitized red blood cells (PRBCs) in vitro. The PRBCs were collected from patients with uncomplicated, severe, or cerebral malaria (CM). Patterns of mRNA expression in HUVECs co-cultured with PRBCs were examined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Levels of mRNAs for all the three adhesion molecules increased with increased culture time within 3 h, regardless of the source of the PRBCs. In contrast, the patterns of mRNA expression for the tight junction molecules varied between the different co-cultures. When HUVECs were cultured with PRBCs from uncomplicated malaria patients, levels of mRNAs for tight junction molecules increased according to the culture time. HUVECs co-cultured with PRBCs from severe malaria patients showed no change in the mRNAs levels during 3 h of observation. When HUVECs were cultured with PRBCs from CM patients, levels of mRNAs for tight junction proteins decreased according to the culture time. Although the mechanisms underlying these phenomena are not clear, our results suggest that PRBCs can alter expression of tight junction proteins in endothelial cells at the site of sequestration and thereby influence disease severity.

Keywords: *Plasmodium falciparum*; HUVEC; PRBC; Adhesion molecule; Tight junction molecule

1. Introduction

Approximately 40% of the world's population lives in malaria endemic areas, which are distributed across 100 countries in tropical and subtropical regions. Malaria infection results in 300–500 million clinical cases and 1.5–2.7 million deaths annually, with approximately 1 million deaths occurring in children less than 5 years of age. Approximately 90% of the cases and deaths occur in tropical Africa. *Plasmodium falciparum* is responsible for the majority of severe clinical cases of malaria, which occur primarily in young children, non-immune adults, and pregnant women [1].

The most serious complication of malaria is cerebral malaria (CM). It is a diffuse reversible encephalopathy characterized by an altered level of consciousness, focal neurologic findings, and seizures. Sequestration of *P. falciparum*-parasitized red blood cells (PRBCs) to the microvasculature, predominantly that in the brain, heart, lungs, and submucosa of the small intestine, may be directly related to the pathogenesis of the disease. In vitro studies have shown that sequestration is

* Corresponding author. Tel.: +81 3 3202 7181x2877; fax: +81 3 3202 7287.
E-mail address: kano@ri.imcj.go.jp (S. Kano).

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Table 1
Patient characteristics at time of admission per disease severity and isolate

Patient's severity	Uncomplicated malaria		Severe malaria		Cerebral malaria	
Isolate no.	MP 058	AA 863	NAC 42	NAC 44	AQ 1133	AQ 1142
Age (years)	38	20	18	21	50	45
Sex	M	F	F	F	F	M
RBC ($\times 10^6$/μl)	5.55	4.48	3.09	3.9	3.55	4.87
Hb (g/dl)	13.1	11.9	7.2	8.2	10.4	15.5
Hematocrit (%)	41	35	22	26	31	45
WBC ($\times 10^3$/μl)	3.5	3.8	12.6	7.5	14.3	7.1
Plt ($\times 10^4$/μl)	15.2	25	28	38	11	40
BUN (mg/dl)	7	8.2	171	17.1	65	55.1
Cr (mg/dl)	0.8	0.77	4.71	0.65	1.9	2.4
Albumin (g/dl)	4.3	3.9	2.7	3.1	2.9	2.6
Total bilirubin (mg/dl)	0.7	0.6	2.59	7.1	7.1	11.76
AST (U/L)	14	53	284	73	175	156
ALT (U/L)	13	59	106	66	208	102
Parasite count (/μl)	44,400	98,560	954,810	561,600	188,150	735,370
Schizont presented	No	No	Yes	Yes	Yes	Yes
Acute renal failure	No	No	Yes	No	No	No
Jaundice	No	No	Yes	Yes	Yes	Yes
Blood transfusion (no. of unit transfusion)	No	No	Yes (2)	Yes (2)	No	No
Respiratory distress with endotracheal tube insertion	No	No	Yes	No	Yes	Yes
Glasgow coma score	15	15	13	15	8	7
Fever clearance time (h)	48	56	144	24	96	116
Parasite clearance time (h)	40	51	73	50	88	164

RBC, red blood cell; Hb, hemoglobin; WBC, white blood cell; Plt, platelet; BUN, blood urea nitrogen; Cr, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

mediated by the interaction of a parasite protein, *P. falciparum* membrane protein-1 (PfEMP-1), which is located on knob-like protrusions on the surface of PRBCs, with receptors on the host endothelium [2]. Many host adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1 or CD54), thrombospondin (TSP) [3,4], CD36 [5], chondroitin sulfate A (CSA) [6,7], vascular cell adhesion molecule 1 (VCAM-1), and endothelial leukocyte adhesion molecule 1 (ELAM-1) or E-selectin [8], bind PRBCs.

However, there is evidence that endothelial junctions are disrupted in patients with CM [9]. Immunohistochemistry of post-mortem tissue from Vietnamese adults and Malawian children with CM showed loss of endothelial-cell junctional proteins ZO-1, occludin, and vinculin, most notably in vessels containing sequestered PRBCs [9–11]. Therefore, the pathogenesis caused by sequestration in CM may be related to expression of adhesion molecules and tight junction molecules in endothelial cells.

To examine whether *P. falciparum* isolates from patients can alter expression of mRNAs for adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and tight junction molecules (occludin, vinculin, and ZO-1) in endothelial cells, we co-cultured human umbilical vein endothelial cells (HUVECs) with PRBCs from patients with malaria of different severities.

Table 2
Sequences of oligonucleotide primers and probes used for TaqMan RT-PCR

Gene	Amplicon length (bp)	Oligonucleotide sequence (5′-3′)		GenBank accession no.
ICAM-1	90	Forward	CATAGAGACCCCGTTGCCTAAA	NM_000201
		Reverse	TGGCTATCTTCTTGCACATTGC	
		Probe	TACACCTTCCGGTTGTTCCCAGGCA	
VCAM-1	124	Forward	ATGACCTTCATCCCTACCATTGA	NM_001078
		Reverse	CATTGACATAAAGTGTTTGCGTACTCT	
		Probe	TGATGACATGGAATTCGAACCCAAACAA	
E-selectin	122	Forward	CCACTGGGAAACTTCAGCTACAA	NM_000450
		Reverse	GCTGGAATAGGAGCACTCCATT	
		Probe	CCAAGCAGCATGGAGACCATGCA	
Occludin	77	Forward	CCCATCTGACTATGTGGAAAGA	NM_002538
		Reverse	AAAACCGCTTGTCATTCACTTTG	
		Probe	TTGACAGTCCCATGGCATACTCTTCCAATG	
Vinculin	136	Forward	GCATCGTCTGGCTAATGTTATGAT	NM_014000
		Reverse	TGCTCGTGCCTGAGGACTCT	
		Probe	CTGTCAGCTGGTCCACTCGGTCACA	
ZO-1	144	Forward	CACCTTTTGATAATCAGCACTCTCA	NM_003257
		Reverse	CTCTAGGTGCCTGTTCGTAACGT	
		Probe	TTGAAGAGCCAGCCCCTCTGTCTTACG	