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Anti-malarial effect of 1-(N-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one and green tea extract on erythrocyte-stage *Plasmodium berghei* in mice

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ABSTRACT

Objective: To examine the efficacy of 1-(N-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one (CM1) iron chelator and green tea extract (GTE) as anti-malarial activity in *Plasmodium berghei* (*P. berghei*) infected mice.

Methods: The CM1 (0–100 mg/kg/day) and GTE (0–100 mg (−)-epigallocatechin 3-gallate equivalent/kg/day) were orally administered to *P. berghei* infected mice for consecutive 4 days. Parasitized red blood cells (PRBC) were enumerated by using Giemsa staining microscopic method.

Results: CM1 lowered percentage of PRBC in dose-dependent manner with an ED_{50} value of 56.91 mg/kg, when compared with pyrimethamine (PYR) (ED_{50} = 0.76 mg/kg). GTE treatment did not show any inhibition of the malaria parasite growth. In combined treatment, CM1 along with 0.6 mg/kg PYR significantly inhibited the growth of *P. berghei* in mice while GTE did not enhance the PYR anti-malarial activity.

Conclusions: CM1 would be effective *per se* and synergize with PYR in inhibiting growth of murine malaria parasites, possibly by limiting iron supply from plasma transferrin and host PRBC cytoplasm, and chelating catalytic iron cstitutive in parasites' mitochondrial cytochromes and cytoplasmic ribonucleotide reductase. CM1 would be a promising adjuvant to enhance PYR anti-malarial activity and minimize the drug resistance.

1. Introduction

Malaria is a virulent infectious diseases associated with anemia in tropical and subtropical regions, in which *Plasmodium* spp. are the causative protozoans [1]. Chemotherapeutic drugs as chloroquine, pyrimethamine (PYR) and dihydroartemisinin (DHA) are used for treatment of human malaria infection based on the actions of β-hematin formation inhibitor, folate analogue and toxic peroxide metabolites, respectively [2,3]. Though these anti-malarial drugs are widely used to combat the malaria parasites, increasing drug resistance is a major problem in overcoming the pathogen [4]. Essentially, *Plasmodium* parasites use iron as a trace element for their metabolism and fast proliferation [5]. In *Plasmodium falciparum* (*P. falciparum*) infected patients, their serum levels of iron and transferrin were 19% and 44% respectively elevated whereas their serum transferrin saturation was 5% decreased when compared to non-malarial persons, due to increased synthesis of transferrin by the liver [6]. Meanwhile, non-transferrin bound iron was detected in plasma of *Plasmodium vinckei* infected mice [7]. Iron chelators such as deferoxamine (DFO), deferiprone (DFP) and deferasirox (DFX) have been reported in suppressing growth of malaria parasites successfully [8–11]. Some chemicals and medicinal plants have potential for anti-malarial activity [12–17].

1-(N-Acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one (CM1) is a novel orally active bidentate iron chelator with similar binding affinity constants (log β_3 and pFe^{III} values) to

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DFP and is more lipophilic [18]. Interestingly, the compound was able to remove hepatic ferritin-bound iron and decrease iron overload effectively in ferrocene-fed β-knock-out thalassemic mice [19]. IC_{50} value of the CM1 for growth of *P. falciparum* in cultured cells was 35.14 μmol/L when compared with those of DFO, green tea extract (GTE), DFX and DFP (14.09, 21.11, 44.71 and 58.25 μmol/L, respectively) [20]. Tea (*Camellia sinensis*) leaves are enriched with polyphenols, including (−)-epicatechin, (−)-epicatechin 3-gallate, (−)-epigallocatechin, (−)-epigallocatechin 3-gallate (EGCG), (+)-catechin and (−)-gallocatechin [21]. Among them, EGCG, which is the most abundant catechin, exerts effective antioxidant, iron-chelating and anti-malarial activity [22–24]. The goals of this study were to examine CM1 and GTE *per se*, and their combined treatments with PYR to inhibit growth of *Plasmodium berghei* (*P. berghei*) in mice.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO) (density 1.10 g/mL) for cell culture was purchased from Santa Cruz Biotechnology, Inc. Texas, USA. Standard EGCG (>95% purity) and Giemsa staining solution were purchased form Sigma–Aldrich Chemicals Company, St. Louis, MO, USA. RPMI-1640 (Gibco® Invitrogen) incomplete medium and phosphate buffered saline were purchased from Life Technologies, CA, USA. Deionized water (DI) was locally made by using the Milli®-Q Water Purification System, Distillation Equipment (Merck Millipore, Darmstadt, Germany). Chemicals and reagents are analytical grade and the highest pure.

2.2. Anti-malarial drugs

PYR and DHA were kindly provided by Dr. Chairat Uthaipibull at National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Ministry of Science and Technology, Thailand.

2.3. Iron chelators

DFO (Desferal®, molecular weight = 657) was purchased from a local drug store at Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. DFP (GPO-L-One®, molecular weight = 139) and DFX (Exjade®, molecular weight = 373) were kindly donated by the Research and Development Institute, Government Pharmaceutical Organization, Ministry of Public Health, Bangkok, Thailand. CM1 (molecular weight = 266, 98% purity) was kindly supplied by Dr. Kanjana Pangjit, PhD, College of Medicine and Public Health, Ubol Ratchathani University, Ubol Ratchathani, Thailand [18].

2.4. GTE

A big batch of GTE powder was prepared from fresh tea leaves according to the method of Srichairatanakool *et al.* [21]. Antioxidant activity and high EGCG content (24%, w/w) of the GTE preparation has not been changed for up to 1 year when it is kept in a plastic bottle in the dark at 4 °C.

2.5. Animals

Female mice (wild type, C57BL/6 strain), at the age of 4 weeks, with an approximate weight of 25 g, were purchased from Thalassemia Research Center, Institute of Molecular Bioscience, Mahidol University Salaya Campus, Bangkok, Thailand. They were maintained in polyethylene cages in the Animal House of Medical Faculty, Chiang Mai University under a controlled condition (12-h day/12-h night light cycle, 25 °C and 40%–70% humidity) and supplied with normal chow pellet diet and tap water *ad libitum*. Study protocol was approved by the Ethical Committee for Animal Experimentation, Faculty of Medicine, Chiang Mai University, Thailand and followed with International Guidelines for the Human Use of Animals in Experimental Studies (Reference Number-42/2556).

2.6. Murine malaria parasites

P. berghei (ANKA strain) infected red blood cells (PRBC) maintained in a 2 mL cryogenic vial in a liquid nitrogen tank (−80 °C) were thawed by placing in 37 °C water bath for 2–3 min. After that, the mice were injected intraperitoneally (*i.p.*) with 0.5 mL of the PRBC. Mouse heart blood was collected in a lithium heparin tube and diluted the infected blood (1%–10% parasitemia) in 200 μL of phosphate buffered saline to achieve 1×10^6 PRBC aliquots for further passage of the infection and experiments [22].

2.7. Drug-susceptibility testing of *P. berghei*

2.7.1. Single drug treatment

Stock PYR solution was freshly prepared in 100% DMSO. Stock CM1 and GTE solution were prepared in DI. The doses of drugs (mg/kg) were adjusted to the weight of mice by diluting with DMSO (at a final concentration of 20%) or DI for every dose. Blood obtained from *P. berghei* infected mice (10%–30% parasitemia) was diluted in RPMI-1640 incomplete medium to make the blood suspension (1×10^7 PRBC) and injected intraperitoneally. The mice (n = 5) were orally administered by gavage with the tested compounds (PYR 0–5 mg/kg, CM1 0–100 mg/kg, and GTE 0–100 mg of EGCG equivalent/kg) on Day 0, 1, 2 and 4. Their anti-malarial activity was then evaluated by using the Peter's 4-day suppressive test [25]. Non-treatment mice (n = 5) were given an equal volume of 20% DMSO or DI. Tails' venous blood samples were collected for enumerating parasites using Giemsa staining microscopic method. Percentage of suppression and parasite growth were calculated using the following formulae:

$$\%\ \text{Suppression} = \frac{P_n - P_t}{P_n} \times 100$$

$$\%\ \text{Parasite growth} = \frac{P_t}{P_n} \times 100$$

where P_n is the percentage of parasitemia in non-treatment group and P_t is the percentage of parasitemia in treatment group.

Maximal 100% parasite growth was normalized from the mean parasitemia of the non-treatment group and 0% parasite growth was normalized from the mean parasitemia of the treatment group with the maximal drug concentration. Dose-response curve of either suppression or parasite growth, and ED_{50} value were made.

2.7.2. Combined drug treatment

Study procedure of the combined drug treatment was similar to that of the single drug treatment, in which the PYR concentration was fixed at 0.6 mg/kg, while the CM1 and GTE doses were varied in the range of 0–100 mg EGCG equivalent/kg. Blood was collected for enumerating parasites using Giemsa staining microscopic method. Percentage of suppression and parasite growth were calculated using the formulae as described above.

2.8. Statistical analysis

Data were analyzed by using IBM SPSS statistics 20 program software and presented as mean ± SEM. Statistical significance was determined by using student's *t*-test or ANOVA test, where $P < 0.05$ was considered significant different.

3. Results

3.1. Single drug susceptibility test of P. berghei

PYR used as the reference drug clearly showed anti-malarial activity against *P. berghei* growth with an ED_{50} of 0.76 mg/kg (95% *CI* = 0.62–0.92 mg/kg). In comparison, CM1 which is our lead iron-chelating compound also showed anti-malarial activity against *P. berghei* growth with an ED_{50} of 56.91 mg/kg (95% *CI* = 47.98–67.50 mg/kg) (Figure 1). Unexpectedly, neither doses of GTE treatment inhibited *P. berghei* growth in the infected mice (Figure 2). The percentage of parasitemia was increased maximally to 158.41% of the control by GTE treatment (12.5 mg EGCG equivalent/kg) ($P < 0.000\,1$). Thereafter, the parasite growth was declined on the opposite way of increasing doses of GTE.

3.2. Combined drug susceptibility test of P. berghei

As shown in Table 1, percent *P. berghei* growth of the PYR (0.6 mg/kg) treatment group was far lower than that of non-treatment group ($P < 0.05$). Most importantly, combined PYR (0.6 mg/kg) + CM1 (12.5–100 mg/kg) treatment inhibited the *P. berghei* growth in mice, depending on the doses of GTE, which statistical significance was found at 50 and 100 mg EGCG equivalent/kg/day. Combined PYR (0.6 mg/kg) + GTE (12.5–100 mg/kg) treatment did not enhance efficacy of the PYR anti-malarial activity when compared with the PYR treatment alone (Table 2).

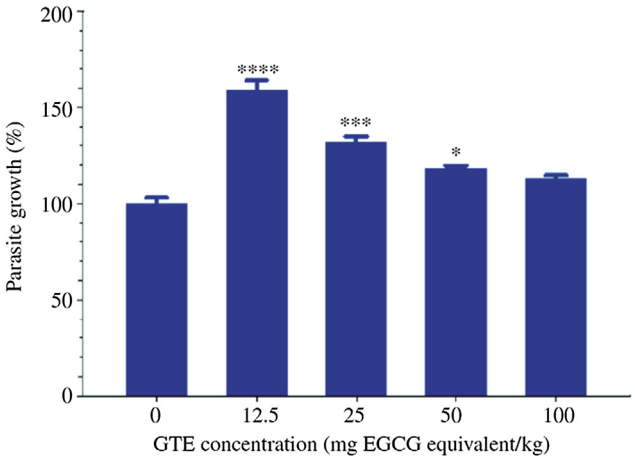

Figure 2. Dose-response GTE treatment in *P. berghei* infected mice. Data obtained from two independent triplicate experiments were expressed as mean ± SEM. $^{*}P < 0.05$; $^{***}P < 0.001$ and $^{****}P < 0.000\,1$ when compared with non-treatment group.

Table 1

Combined treatment of PYR with CM1 in *P. berghei* infected mice.

Concentration (mg/kg)	Parasite growth (%)
0 PYR + 0 CM1	100.0 ± 17.5
0.6 PYR + 0 CM1	23.4 ± 9.0*
0.6 PYR + 12.5 CM1	20.2 ± 5.6
0.6 PYR + 50.0 CM1	12.0 ± 5.7#
0.6 PYR + 100.0 CM1	8.8 ± 3.1#

Data obtained from two independent triplicate experiments were expressed as mean ± SEM. *: $P < 0.05$ when compared with non-treatment group; #: $P < 0.05$ when compared with 0.6 mg/kg PYR treatment group.

Table 2

Combined treatment of PYR with GTE in *P. berghei* infected mice.

Concentration (mg/kg)	Parasite growth (%)
0 PYR + 0 GTE	100.0 ± 17.5
0.6 PYR + 0 GTE	23.4 ± 9.0*
0.6 PYR + 12.5 GTE	15.1 ± 2.1
0.6 PYR + 50.0 GTE	19.4 ± 6.3
0.6 PYR + 100.0 GTE	17.6 ± 2.8

Data obtained from two independent triplicate experiments were expressed as mean ± SEM. $^{*}P < 0.05$ when compared with non-treatment group.

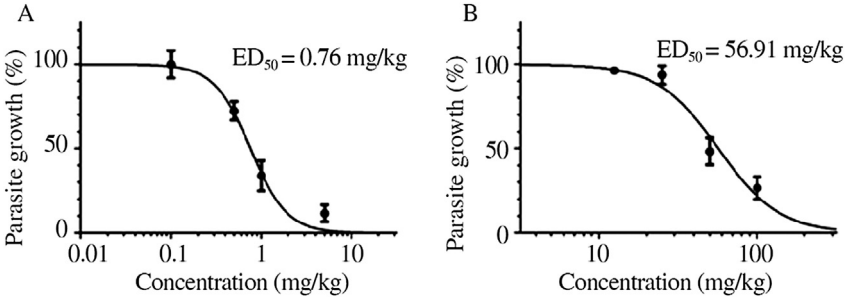

Figure 1. Dose-response PYR (A) and CM1 (B) treatments in *P. berghei* infected mice. Data obtained from two independent triplicate experiments were expressed as mean ± SEM.

4. Discussion

In their growth and development, fast dividing cells like malaria parasites require large amounts of iron for ribonucleotide reductase (RR)-catalyzed DNA replication and heme biosynthesis [26–29]. Iron chelators used for treatment of thalassemia-related iron overload to achieve negative iron balance which can inhibit parasite growth. Green tea has attractive anti-inflammatory, anti-microbial, cancer chemopreventive, anti-trypanosomal and anti-plasmodial activities [30–33]. Our *in vitro* study showed degree of inhibition of *P. falciparum* growth was DFO > GTE > CM1 > DFX > DFP, possibly due to more lipophilicity of CM1 than DFP and DFX to penetrate host RBC membrane and parasite plasma membrane readily [19].

According to leaky membrane of PRBC, we believe that CM1 would readily penetrate host RBC membrane to remove intracellular iron in the cytosol and iron-storage protein called ferritin, leading to iron depletion. Alternatively, CM1 may specifically withhold iron from any of several essential iron-dependent parasite enzymes, involved in CO_2 fixation, mitochondrial electron transport, pyrimidine synthesis and RR activity for DNA synthesis. RR catalyzes the conversion of ribose to deoxyribose for DNA synthesis and is the most studied target of iron chelator. In combined treatment, CM1 synergized anti-malarial activity with PYR to inhibit growth of *P. berghei* in mice, consistently with the result of dose–response inhibition of *P. falciparum* growth [20]. In this event, PYR will compete with plasmodial dihydrofolate reductase in the folate-metabolism pathway and DNA synthesis whereas CM1 chelator may interact directly on the iron catalytic site on RR molecule. Alternatively, CM1 would compete with the malaria parasite siderophore to limit the uptake of extracellular iron and/or deplete iron stuff persisting in host RBC cytosolic pool and ferritin [19]. Burte *et al.* have found that plasma level of hepcidin was lower in children with cerebral malaria and severe malaria anemia than in milder anemia children [34]. Recently, we have found hepcidin gene was upregulated in GTE-fed mice (unpublished data).

Furthermore, GTE was more effective in removing intracellular labile iron pools than CM1 in concentration-dependent manner, suggesting that a major constituent like EGCG shows pretty iron-chelating activity besides anti-oxidation [23]. In this study, we have found that GTE at low doses did not decrease the percentage of PRBC in *P. berghei* infected mice; inversely, they increased. Consistently, Francischetti *et al.* have reported that green tea EGCG did not improve survival of *P. berghei* infected mice [35]. Interestingly, EGCG, (−)-epicatechin 3-gallate, caffeoylquinic acid and rosmarinic acid from green tea potentiated anti-*P. falciparum* activity of artemisinin but did not interfere with the folate pathway [36,37]. Digitonin synergistically increases toxicity of EGCG on survival of plasmodium sporozoites [38]. It is postulated that GTE would have anti-hemolytic activity to maintain survival of circulating RBCs, so the PRBC were not burst to release the persisting merozoites at the end of erythrocyte stage [39]. Degradation of host RBC hemoglobin to hemozoin in parasites' food vacuoles promotes reactive oxygen species (ROS) generation. The immune system produced ROS in response to the bursting of PRBC and the release of merozoites. Taken together, antioxidant GTE has to diminish ROS production in *P. berghei* infected mice and consequently results in promoting rather than inhibiting malaria parasite growth.

In conclusion, CM1 could inhibit murine malaria parasite growth and synergize the anti-malarial activity with PYR in dose-dependent manner. Possible mechanisms included interfering uptake of exogenous iron, depleting cellular iron, and interacting with functional iron in parasite cells. For effective treatment and prophylaxis, pharmacokinetics and optimal doses of CM1 need to be investigated urgently in subjects with malaria infection.

Conflict of interest statement

We declare that we have no conflict of interest.

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