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Antimalarial properties of Goniothalamin in combination with chloroquine against *Plasmodium yoelii* and *Plasmodium berghei* growth in mice

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Abstract. Malaria is a disease which is still endemic and has become a disastrous scourge because of the emergence of antimalarial drug resistant *Plasmodium falciparum*. A new approach in addressing this is in developing a combination drug. This study is to show the enhancement of antimalarial properties, when single compound, goniothalamin combine with standard drug, chloroquine. Based on 4 Day Test, percentage of parasite growth on treated infected mice were determined. Oral treatment with 1 mg/kg BW of chloroquine on experimental mice suppressed 70% and 76.7% of both *Plasmodium yoelii* and *Plasmodium berghei*, respectively. The infection of *P. berghei* in mice was inhibited less than 50% by goniothalamin individual treatment at all doses in this study. About 27.8% and 18.5% inhibition of infection were observed in *P. yoelii* infected mice treated with 30 mg/kg and 60 mg/kg of goniothalamin respectively and the suppression exceed more than 50% at higher doses (90 and 120 mg/kg). Combination of 1 mg/kg chloroquine with either 30 mg/kg or 60 mg/kg of goniothalamin decreased the parasitemia of *P. yoelii* infected mice more than 90% and prolong the survival up to 100% after treatment. Similar treatment to *P. berghei* infected mice only shows about 60% reduction of parasitemia. The study findings showed that antimalarial property of goniothalamin was enhanced by combination with chloroquine at lower dose of each drug.

INTRODUCTION

Malaria is the world's most important tropical parasitic disease and is transmitted through the bite of female *Anopheles* mosquitoes. There are four species of the parasite that cause malaria in humans. Among these, *Plasmodium falciparum*, causes the majority of infections and can be fatal if left untreated. WHO estimated that 1.5 to 2.7 million deaths resulted by malaria infection in 2001 and most of the deaths occurred in children under five years old. Each year, 300 to 500 million new cases are detected globally. Nearly 40% of the world's population live in affected regions. Despite over a century of work to control or eradicate this disease, malaria continues to take its devastating toll, largely in developing nations. The emergence of insecticide resistant mosquitoes and drug-resistant malarial parasites has made the situation much worse (WHO, 2000).

In Malaysia, chloroquine resistant case was first reported in 1963 (Montgomery & Eyles, 1963). Subsequently, several chloroquine resistant cases have been reported in Sabah, West Malaysia (Clyde *et al.*, 1973). In addition, other drug resistant cases also have been detected. For example, the combination of sulfonamides - pyrimethamine (Dondero *et al.*, 1976; Hurwitz *et al.*, 1981; Onori, 1988; and sulfadoxine - pyrimethamine resistant (Black

et al., 1982; Ponnampalam, 1982). The most recent study reported by Lokman *et al*. (1996), revealed a widespread resistance of falciparum malaria to both chloroquine and sulfadoxine-pyrimethamine in endemic areas of Peninsular Malaysia.

Combination therapy of anti-malarial drugs refers to the simultaneous use of two or more blood schizontocidal drugs with independent mode of action and different biochemical targets in the parasite. The concept of combination therapy is based on the synergistic or additive potential of two or more drugs to improve therapeutic efficacy and also to delay the development of resistance to the individual components of the combination (WHO, 2000).

Plant remains an important source of medicine and health care remedies for most people. One of the Malaysian medicinal plants which has potential as an anti-malarial is *Goniothalamus scortechinii*, known locally as "Selada putih". *Goniothalamus* spp. are commonly found in tropical countries particular in Malaysia and Indonesia. It belongs to the Annonaceae family, which naturally grows as shrubs or small trees in Peninsular Malaysia. The plant exhibits a great deal of herbal potential that is locally and originally was used to treat fever and procure abortion (Burkill, 1935).

Bioassay guided fractionation of *G. scortechinii* have shown that extracts at different fractionation from the crude to the isolated compound, goniothalamin, exhibit an *in vitro* anti-plasmodial properties to malaria parasite, *P. falciparum*. The crude methanol extracts have been tested for their anti-plasmodial activity and cytotoxicity and showed that it has anti-plasmodial properties and minimal cytotoxicity to normal cell line (Siti Najila *et al.*, 2002).

These *in vitro* findings concluded that goniothalamin has potential for antimalarial chemotherapy development. Hence, this study aims to investigate the anti-malarial activity of goniothalamin in combination with standard anti-malarial drug chloroquine *in vivo*.

MATERALS AND METHODS

Experimental animals

ICR mice for the experiment were obtained from Animal Unit, Medical Research Resource Center, Institute for Medical Research. The use of the laboratory animals was approved by the Institutional Animal Care and Used Committee (IACUC) of Institute for Medical Research, Kuala Lumpur (ACUC/KKM 4/ 2004). The mice were about 3 to 4 weeks old with average weight about 30 g. The mice were acclimatized to the animal room for 5 days prior to experiment. They were fed with standard mice feed and given enough drinking water supplemented with 1 part per million (1 ppm) *p*-aminobenzoic acid (PABA) (Sigma, USA). Mice were maintained at room temperature (22 ± 2ºC).

Parasites

The parasites used in the study were *P. berghei* (ANKA strain) MRA 311 and *P. yoelii* MRA 312. The isolates were obtained from American Type Culture Collection, Malaria Research Reference Reagent Resource Center (MR4).

Chemicals

Goniothalamin was provided by Dr. Khozirah Shaari, University Putra Malaysia, Serdang. It was isolated from the roots of *G. schortechinii*. The white crystallized powder was dissolved in 100% dimetyl sulfoxide (DMSO) (Fluka, Netherland) as a stock. Chloroquine diphosphate were purchased from Sigma, USA [Code no: 50-63-5] EC No. 200-055-2.

Infection of donor mice

Cryopreserved specimen of *P. berghei* or *P. yoelii* was taken from liquid nitrogen. The vial was thawed in the water bath at 37ºC and transferred into a 15-ml centrifuge tube and centrifuged at 1800 rpm for 10 minutes. The supernatant was discarded and two volumes of washing solution (16% mannitol in 0.9% sodium chloride) was added to the

pellet. The blood was mixed by pipetting and centrifuged at 1800 rpm for 10 minutes. The supernatant was discarded and 1 ml of 0.9% sodium chloride solution was added. Two hundred microlitres of the blood suspension containing 2 x 10^6 *P. berghei* infected red blood cells were intraperitoneally inoculated into each mouse. The parasitemia of donor mice were microscopically monitored daily by making thin blood smears, stained with 10% Giemsa at 100 times magnification.

Preparation of goniothalamin (test drug), chloroquine (standard drug), and combination

Chloroquine was dissolved in water while goniothalamin was dissolved in DMSO to obtain a stock concentration of 0.3 g/ml, followed by dilution with water to a final concentration of 1 to 3 % of DMSO. The drug or compound was further diluted to the doses required for the experiment. Doses used for chloroquine treatment are 1, 3, 10 and 30 mg/kg body weight (BW). For goniothalamin, 30, 60, 90 and 120 mg/kg BW were used. For combination formula, 1 mg/kg BW of chloroquine was combined with both 30 mg/kg BW and 60 mg/kg BW of goniothalamin.

The *in vivo* study

The *in vivo* study was carried out according to standard protocol following the "4 Day Test" (Peters, 1975). Each *P. berghei* or *P. yoelii* infected mice consisted of the control group (only infected with parasite), test groups, treated with chloroquine, goniothalamin, and combination of goniothalamin with chloroquine. Each group consisted of minimum of 5 mice. On day 0, each mouse was injected with 200 µl volume delivering 2 x 10^6 infected red blood cells intravenously. Two hours after inoculation, each mouse was orally treated with 200 µl of goniothalamin (Goniothalamin group) and chloroquine (Chloroquine group). The control mice were given 200 µl of distilled water orally. For combination treatment group, the mice were treated with 200 µl of goniothalamin and 200 µl of chloroquine while its control group, the mice were given 400 µl of water.

The treatment was repeated for the next 3 days for all the 4 groups of experimental animal. On day 4, thin blood smear was made and stained with 10% Giemsa and examined under the light microscope with 100 times magnification. Percentage of parasitemia was counted based on infected erythrocytes calculated per 1000 erythrocytes.

Survival of mice after treatment

Number of dead mice was recorded daily from all the study groups to determine the average survival time of the infected mice after treatment. A similar procedure was carried out for the *P. yoelii* strain.

Data analysis

All the results were analyzed by using ANOVA with multiple comparison tests (Tukey's test).

RESULTS

Treatment of *P. berghei*- and *P. yoelii*-infected mice with each individual and combination of goniothalamin: chloroquine

Treatment of *P. berghei* infected mice with 1 mg/kg BW of chloroquine reduced the parasitemia of infected mice for 76.7%. As mentioned above, 100% suppression of infection were also observed in *P. berghei* infected mice treated with higher dose of chloroquine (Table 1).

P. berghei infected mice treated with goniothalamin showed a mild suppression of infection as compared to the similar treatment in *P. yoelii* infected mice (Table 1). Treatment of *P. berghei* infected mice with combination formula (1 mg/kg BW chloroquine with 30 mg/kg BW and 60 mg/kg BW of goniothalamin) showed more than 50% reduction of parasitemia (Table 1). However, by statistical analysis, the reduction of parasitemia by both combination treatment was not significant as compared with both chloroquine and goniothalamin treatment alone (Figure 1).

Treatment of *P. yoelii* infected mice with 1 mg/kg of chloroquine showed a reduction of parasitemia for 70% ($p<0.05$) while at

higher doses (3, 10 and 30 mg/kg BW), a hundred percent suppression was achieved (Table 1). Meaning, the ED90 of chloroquine fell between 1 to 3 mg/kg BW. Treatment of *P. yoelii* infected mice with goniothalamin at 30 mg/kg BW and 60 mg/kg BW suppressed 18.5 to 27.8 percent of infection. However, at doses of 90 mg/kg BW and 120 mg/kg BW, the suppression of infection was achieved at 56.2% and 63.5%, respectively (Table 1).

Table 1. The effect of chloroquine and goniothalamin to *P. berghei* and *P. yoelii* growth in mice

Experimental conditions		Percentage of parasitemia reduction (%)	
		P. berghei	*P. yoelii*
Chloroquine (n=5)	1 mg/kg	76.7 ± 3.7	70.0 ± 20.9
	3 mg/kg	100	100
	10 mg/kg	100	100
	30 mg/kg	100	100
Goniothalamin (n=5)	30 mg/kg	24.0 ± 8.9	27.8 ± 16.8
	60 mg/kg	23.8 ± 14.3	18.5 ± 16.0
	90 mg/kg	38.9 ± 25.8	56.2 ± 12.9
	120 mg/kg	26.8 ± 8.7	63.5 ± 17.6
Combination of 1 mg/kg chloroquine with:			
Goniothalamin (n=10)	30 mg/kg	61.3 ± 26.3	95.4 ± 4.6
	60 mg/kg	69.3 ± 23.1	95.4 ± 4.2

The results are presented in mean value ± S.D.

Treatment of *P. yoelii* infected mice with combination of chloroquine: goniothalamin at dose of 1 mg/kg BW of chloroquine with 30 mg/kg BW and 60 mg/kg BW of goniothalamin showed a significant increase in parasitemia reduction (>90% inhibition) (Table 1 & Figure 2).

Survival time of *P. yoelii* and *P. berghei* infected mice after treatment

After treatment of infected mice with the drugs on day 3 post infection, they were left without treatment and the survival time of each group of treated mice was recorded. Both *P. yoelii* and *P. berghei* infected mice treated with 1 mg/kg of chloroquine were survived for average of 12 to 13 days post infection as compared to untreated infected mice which survived until 7 to 8 days respectively (Table 2). *P. yoelii* infected mice treated with 30 to 120 mg/kg BW of goniothalamin only survived until 7 to 10 days post infection while *P. berghei* infected

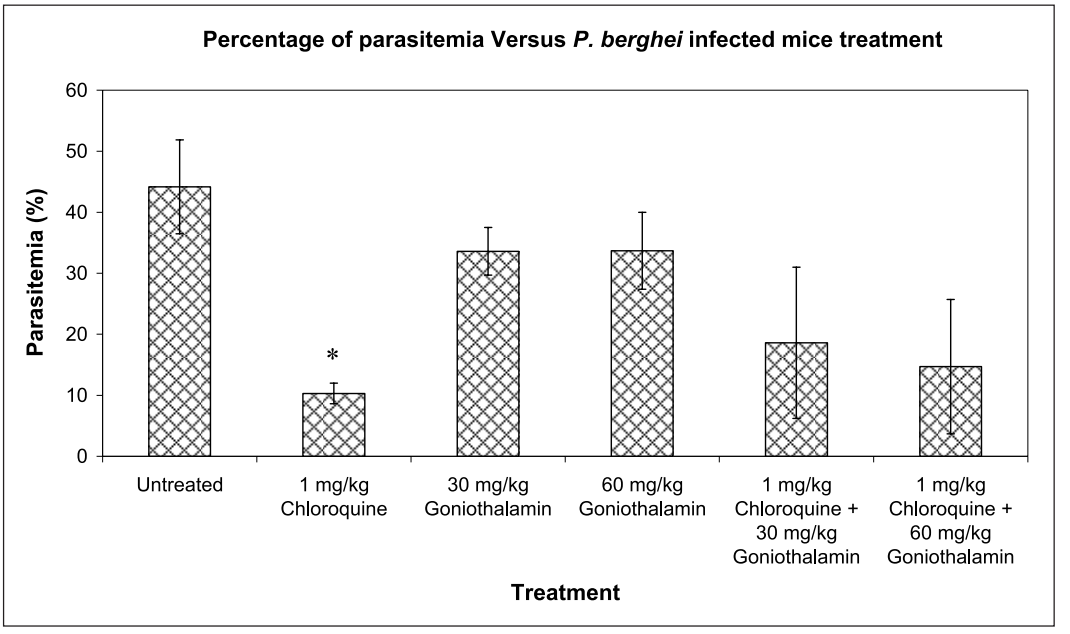

Figure 1. *P. berghei* infected mice were treated with single drug and combination drug (chloroquine:goniothalamin) at specified dosage by oral administration. The results showed that combination treatment (chloroquine:goniothalamin) slightly inhibited the *P. berghei* growth in mice. The results are in mean value ± S.D (n=10). * $p<0.05$ significant when compared with untreated mice.

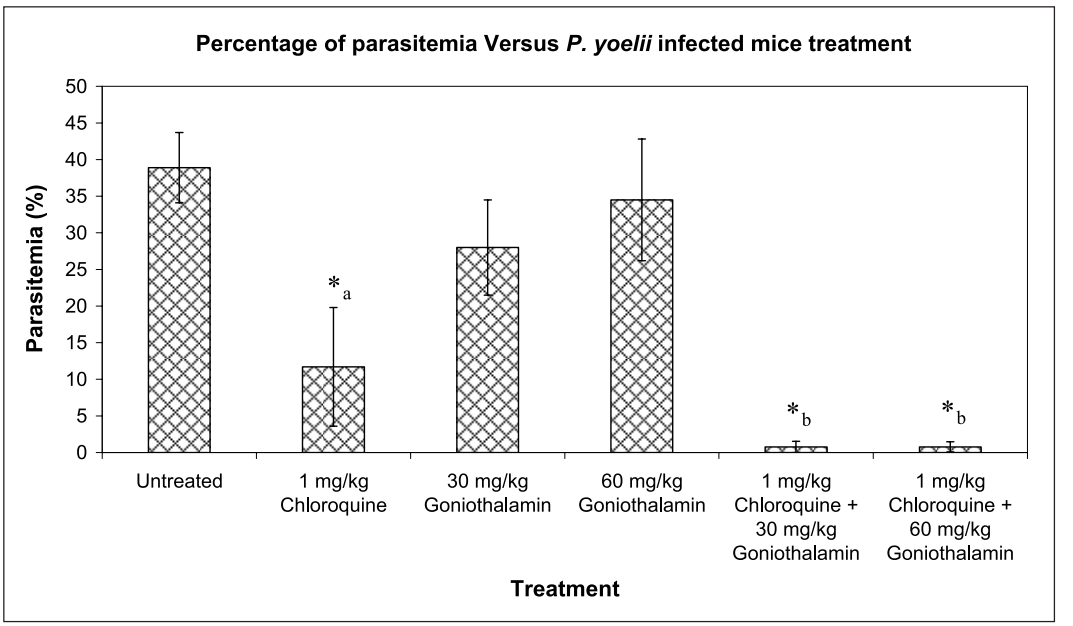

Figure 2. *P. yoelii* infected mice were treated with single drug and combination drug (chloroquine:goniothalamin) at specified dosage by oral administration. Results showed that both combination of chloroquine:goniothalamin significantly reduced the parasitemia of *P. yoelii* infected mice ($p<0.05$) as compared with chloroquine and goniothalamin treatment. The results are in mean value ± S.D (n=10). $*_a$ $p<0.05$ when compared with untreated mice. $*_b$ $p<0.05$ when compared with both single drug (chloroquine and goniothalamin).

Table 2. The effect of goniothalamin to *P. berghei*- and *P. yoelii*-infected mice survival time

Experimental conditions		Survival time (days)	
		P. berghei	*P. yoelii*
Control (distilled water)		8.0 ± 1.9	7.0 ± 1.3
Chloroquine	1 mg/kg	11.8 ± 1.8	12.8 ± 4.6
Goniothalamin	30 mg/kg	7.8 ± 1.5	6.8 ± 1.6
	60 mg/kg	8.6 ± 1.7	6.4 ± 0.9
	90 mg/kg	9.0 ± 2.3	7.6 ± 0.5
	120 mg/kg	9.6 ± 1.7	9.8 ± 2.9

The test samples were orally given once daily for 4 days (Day 0- Day 3). The results are presented in mean value ± S.D (n=5).

Table 3. The effect of combination treatment (goniothalamin:chloroquine) to *P. berghei*- and *P. yoelii*-infected mice survival time

Experimental conditions		Survival time (days)	
		P. berghei	*P. yoelii*
Control (distilled water)		7.3 ± 1.3	10.0 ± 0.8
Chloroquine 1 mg/kg		11.8 ± 1.8	12.8 ± 4.6
Combination of 1 mg/kg chloroquine with:			
Goniothalamin	30 mg/kg	9.6 ± 3.5	20.2 ± 2.7 *
	60 mg/kg	9.7 ± 4.2	18.6 ± 4.4 *

The test samples were given orally once daily for 4 days (Day 0- Day 3). The results presented are in mean value ± S.D. (n=10)
* $p<0.05$ significant when compared with chloroquine treatment.

mice treated with 30 to 120 mg/kg BW of goniothalamin survived around 8 to 10 days respectively (Table 2).

However, combination (chloroquine: goniothalamin) treatment, *P. yoelii* infected mice survived for 19 to 20 days post infection ($p<0.05$) (Table 3) as compared to chloroquine treatment which exhibited shorter survival time as mentioned above. *P. berghei* infected mice treated with combination formula show a shorter survival time as compared to control drug (chloroquine) treated infected mice (Table 3).

DISCUSSION

The study on goniothalamin, the compounds isolated from the *G. scortechinii*, was shown to have anti-plasmodial activity to *P. falciparum* resistant isolate Gombak A and sensitive strain D10 *in-vitro*. Recent investigation in mouse model has shown that goniothalamin at dose of 120 mg/kg BW suppressed *P. berghei* infection to more than 90% (unpublished data).

Both combination treatment showed potent antimalaria properties in mice infected with *P. yoelii*. The differences of suppression between *P. yoelii* and *P. berghei* could be due to their ability to invade mature or immature erythrocytes and their degree of synchronism (Ancelin *et al*., 2003) and schizogony cycle (Laudau & Gautret, 1998). The combination treatment was potent in inhibiting *P. yoelii* growth where this strain is less susceptible to chloroquine than other rodent species such as *P. berghei*, *P. chabaudi* and *P. vinckei* (Beauté-Lafitte *et al*., 1994). It has been proven in this study where treatment of chloroquine at 1 mg/kg BW suppressed *P. berghei* growth 6.7% higher than *P. yoelii* growth *in vivo* (Table 1).

This study showed that the efficacy of goniothalamin as an antimalarial compound was potentiated by combination treatment with chloroquine. This phenomena is related to the study performed by Perez *et al.* (1994), where the combination of other compound, Ajoene (50 mg/kg), a product initially isolated from extracts of garlic (*Allium sativum*), with chloroquine (4.5 mg/kg), completely prevented the subsequent development of parasitemia in treated mice which led to 100% survival rate.

The study findings concluded that a combination of drug treatment has synergistic effect where the response is accelerated. Not only the combination treatment improved the parasitemia reduction but also prolong the survival of the treated mice.

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