Original Article

Antimalarial effect of synthetic endoperoxide on synchronized *Plasmodium chabaudi* infected mice

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Abstract

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The discovery of new antimalarial drugs can be developed using asynchronized *Plasmodium berghei* malaria parasites in vivo in mice. Studies on a particular stage are also required to assess the effectiveness and mode of action of drugs. In this report, we used endoperoxide 6-(1,2,6,7-tetraoxaspiro [7.11] nonadec-4-yl) hexan-1-ol (N-251) as a model antimalarial compound on *P. chabaudi* parasites. We examined the antimalarial effect of N-251 against ring-stage- and trophozoite-stage-rich *P. chabaudi* parasites and asynchronized *P. berghei* parasites using the 4-day suppressive test. The ED_{50} values were 27, 22, and 22 mg/kg, respectively, and the antimalarial activity of N-251 was verified in both rodent malaria parasites. To assess the stage-specific effect of N-251 in vivo, we evaluated the change of parasitemia and distribution of parasite stages using ring-stage- and trophozoite-stage-rich *P. chabaudi* parasites with one-day drug administration for one life cycle. We discovered that the parasitemias decreased after 13 and 9 hours post-treatment in the ring-stage- and trophozoite-stage-rich groups, respectively. Additionally, in the ring-stage-rich N-251 treated group, the ring-stage parasites hindered trophozoite parasite development. For the trophozoite-stage-rich N-251 treated group, the distribution of the trophozoite stage was maintained without a change in parasitemia until 9 hours. Because of these findings, it can be concluded that N-251 suppressed the trophozoite stage but not the ring stage. We report for the first time that N-251 specifically suppresses the trophozoite stage using *P. chabaudi* in mice. The results show that *P. chabaudi* is a reliable model for the characterization of stage-specific antimalarial effects.

Keywords: *Plasmodium chabaudi*, synchronization, stage-specific activity, antimalarial N-251

Introduction

A new antimalarial drug study usually begins with the use of *Plasmodium falciparum* in vitro and rodent malaria parasites such as *Plasmodium berghei* and *Plasmodium chabaudi* in vivo [1,2]. However, drug effectiveness for rodent malaria species does not always mimic those of the human malaria parasite, *P. falciparum* [3]. In mice *P. chabaudi* produces a high parasitemia and generates synchronous infections by circadian rhythm, thus enabling

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studies on parasite stage specificity [4,5]. The several *Plasmodium* spp. life cycle stages offer various patterns and targets for antimalarial chemotherapy [6].

The drug candidate 6-(1,2,6,7-tetraoxaspiro [7.11] nonadec-4-yl) hexan-1-ol endoperoxide (N-251) has high antimalarial activity against both *P. falciparum* in vitro and *P. berghei* in vivo, as described by our previous studies [7,8]. We also found that N-251 inhibited the trophozoite stage exclusively on synchronized *P. falciparum* with sorbitol treatment in vitro [9]. In this research, we used N-251 as a model antimalarial compound for analyzing stage-specific inhibition using synchronized *P. chabaudi* in vivo.

Melatonin is a natural hormone synthesized by the pineal gland and controlled by the circadian rhythm [10]. Some reports found that *Plasmodium* becomes more synchronized by melatonin [11,12]. We evaluated the ability of melatonin to obtain a high yield of synchronized *P. chabaudi* parasites. Our study aimed to examine the feasibility of an animal experimental model for stage-specific antimalarial effect using N-251 on the predominant ring and trophozoite stages of *P. chabaudi*.

Materials and Methods

Parasites and mice

P. chabaudi AS strain (*P. chabaudi*) and *P. berghei* NK65 strain (*P. berghei*) parasites were maintained in the laboratory. ICR male mice (The Jackson Laboratory, Kanagawa, Japan) were maintained in a 12 h light/dark cycle at room temperature ($23 \pm 2°C$) with free access to food (MF diet, Oriental Co., Tokyo, Japan) and water. The study was carried out in compliance with the Okayama University Guidelines for Animal Experimentation and was authorized by the Institutional Animal Care and Use Committee (permission number: OKU-2022815).

Comparison between parasite growth of *P. chabaudi* and *P. berghei*

To initiate growth rate assays, mice were intravenously (iv) inoculated with 1×10^6 *P. chabaudi* or *P. berghei* parasite-infected erythrocyte suspensions obtained from infected donor ICR mice. The parasitemia was assessed using a microscope after the preparation of a thin smear. Data were expressed as the mean ($n = 5$) with a bar showing the standard deviation (SD) value.

Effect of melatonin

Melatonin (Sigma, St. Louis, MO, USA) was dissolved in 10.0% ethanol, 10.0% Cremophore EL, and 80.0% saline (1:1:8 [v/v/v]). *P. chabaudi* and *P. berghei* parasites were evaluated by iv infusion of 3×10^5 infected erythrocytes in a healthy mouse. Mice received 4 days of melatonin iv administration at a rate of 50 mg/kg/day (days 0, 1, 2, and 3). Vehicles were the only treatment for the control groups. Parasitemia and distribution of parasite stages were checked on day 4. Data were expressed as the mean ($n = 5$) with a bar indicating the SD value.

Stage-specific effect of N-251

Ring-stage- and trophozoite-stage-rich parasite groups of *P. chabaudi* were examined fol-

lowing iv administration of 1×10^7 infected erythrocytes in mice. When the ring and trophozoite stages reached about 60.0% of the abundance of all stages, N-251 (Fig. 1) (dissolved in 10.0% ethanol, 10.0% Cremophore EL, and 80.0% saline (1:1:8 [v/v/v]) was given iv at a dose of 68 mg/kg every 8 h for 1 day. Control groups were treated with vehicles only. As shown in Fig. 2, blood smears were examined every 4 h for 25 h. Data were expressed as the mean ($n = 5$) with the bar indicating the SD value.

Four-day suppressive test of N-251

The antimalarial effect of N-251 was analyzed using the 4-day suppressive test in vivo [13]. Synchronized *P. chabaudi* and asynchronized *P. berghei* parasites were tested by iv infusion of 1×10^6 infected erythrocytes in a healthy mouse. We prepared 2 synchronized *P. chabaudi* groups: one in which the ring stage made up roughly 60% of the infected erythrocytes and the other in which the trophozoite stage made up more than 60% of the infected erythrocytes. Two hours post-infection, mice were iv administered N-251 at doses of 5, 20, and 50 mg/kg/day for 4 days. Control groups were treated with vehicles only. On day 4, thin blood films were prepared and the parasitemias were calculated. Data were expressed as the mean ($n = 5$) $\pm$ SD value.

Statistical analysis

Results were examined using Microsoft Excel 2016 and the data were expressed as the mean $\pm$ SD value. Percent parasitemia was computed by dividing the number of infected RBCs by the total number of RBCs indexed and multiplied by 100. ED_{50} and ED_{90} values were collected by sigmoidal dose-response curve analysis.
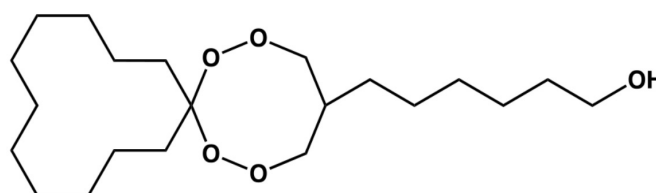
Fig. 1. Structure of N-251. The chemical structure of 6-(1,2,6,7-tetraoxaspiro [7.11] nonadec-4-yl) hexan-1-ol (N-251) [7].

Fig. 2. Scheme for analyzing the stage-specific effect of N-251. The drug was administered at a dose of 68 mg/kg every 8 h for 1 day. The control group was treated with vehicle only at the time indicated by the scheme, parasitemias, and parasite stage distributions were found. (A) Parasites have many ring stages. (B) Parasites abundant in the trophozoite stage.

Results

Comparison between parasite growth of *P. chabaudi* and *P. berghei*

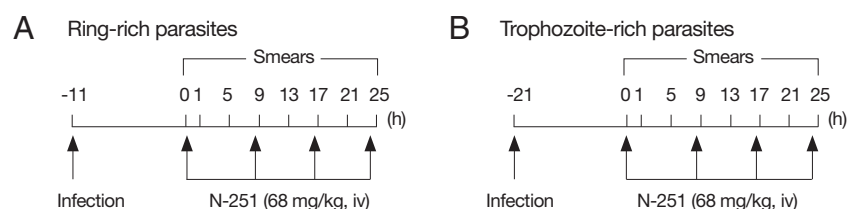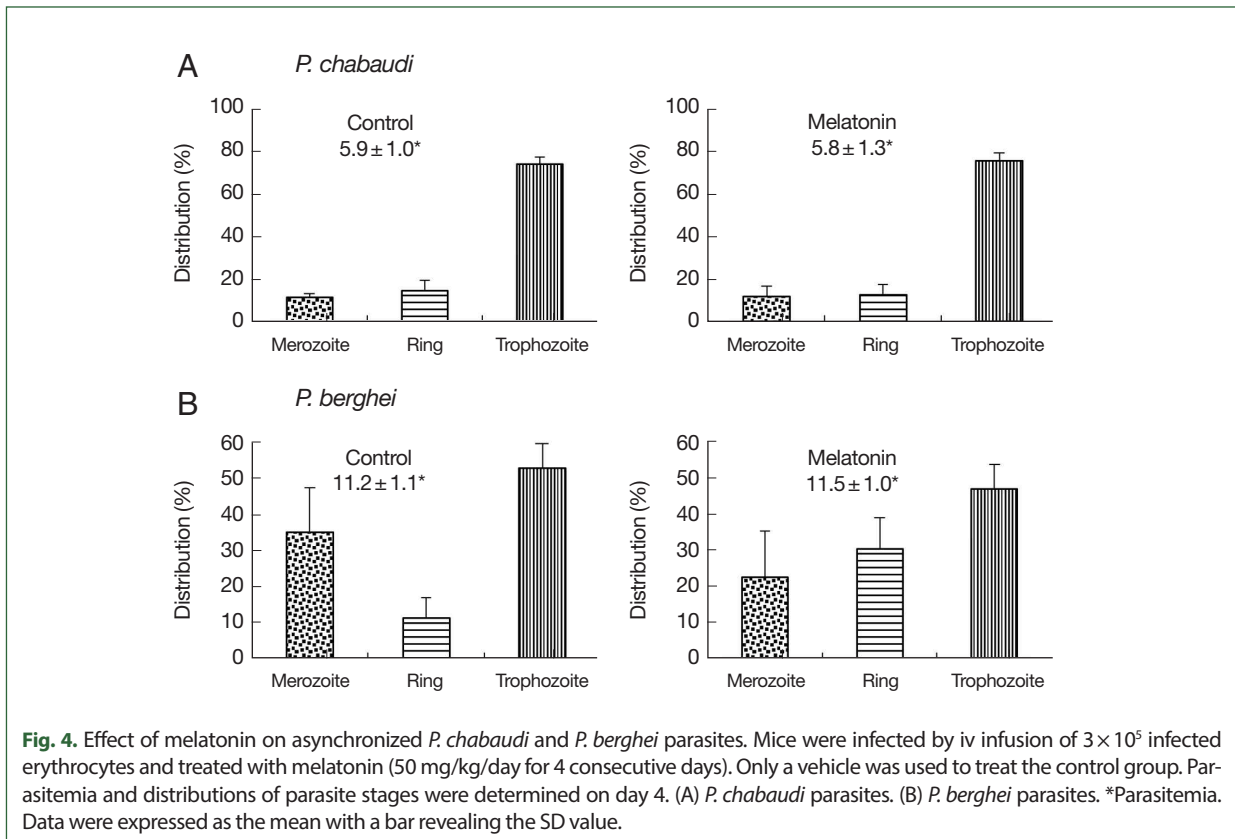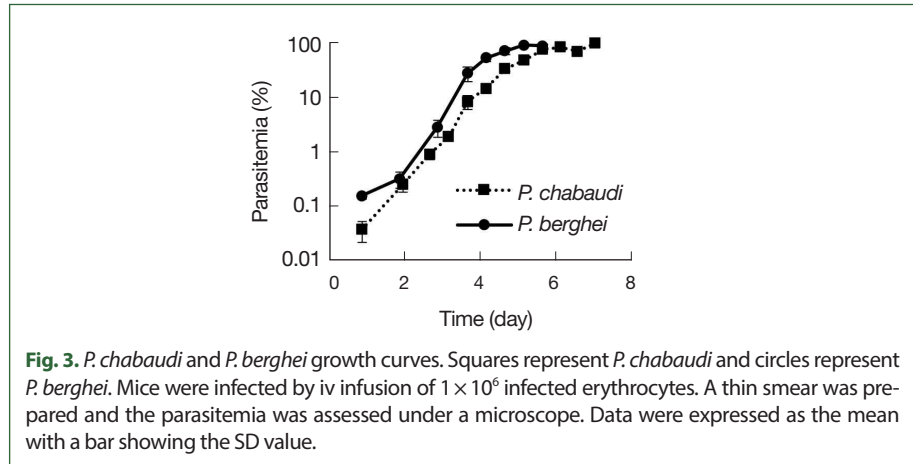The growth rates of *P. chabaudi* and *P. berghei* parasites in mice are displayed in Fig. 3. Parasite growth was more delayed in *P. chabaudi* than *P. berghei*, and the growth curves slope were otherwise similar for both parasite strains, and all infected mice died after day 7.

Fig. 3. *P. chabaudi* and *P. berghei* growth curves. Squares represent *P. chabaudi* and circles represent *P. berghei*. Mice were infected by iv infusion of 1×10^6 infected erythrocytes. A thin smear was prepared and the parasitemia was assessed under a microscope. Data were expressed as the mean with a bar showing the SD value.

Fig. 4. Effect of melatonin on asynchronized *P. chabaudi* and *P. berghei* parasites. Mice were infected by iv infusion of 3×10^5 infected erythrocytes and treated with melatonin (50 mg/kg/day for 4 consecutive days). Only a vehicle was used to treat the control group. Parasitemia and distributions of parasite stages were determined on day 4. (A) *P. chabaudi* parasites. (B) *P. berghei* parasites. *Parasitemia. Data were expressed as the mean with a bar revealing the SD value.

Effect of melatonin on parasite synchronization and growth

The effect of melatonin on *P. chabaudi* and *P. berghei* is shown in Fig. 4. In the *P. chabaudi* parasite groups, more than 70.0% of parasites were synchronized to the trophozoite stage in both the control and melatonin-treated groups. The parasitemias were 5.9% and 5.8%, respectively, in the control and melatonin-treated groups (Fig. 4A). This data demonstrated that melatonin did not affect the synchronization and growth of *P. chabaudi*. For *P. berghei* parasites, the distributions of merozoites, ring, and trophozoite stages in the control group were 35.3, 11.4, and 53.1%; while in the melatonin-treated group, the distributions were 22.6, 30.4, and 46.9%, respectively, without change in parasitemia (control: 11.2%, melatonin: 11.5%) (Fig. 4B). From these findings it was apparent that melatonin had a partial effect on the development of parasite stages in *P. berghei*. As a result, we used *P. chabaudi* without melatonin in the next experiment.

Stage-specific effect of N-251

To assess the stage-specific effect of N-251 in vivo, we examined the change of parasitemias and distributions of parasite stages using preparations of ring-stage- and trophozoite-stage-rich *P. chabaudi* parasites. The outcomes employing parasite preparations rich in ring stages are shown in Fig. 5A. In the control group, the parasitemia increased after 17 h, and ring-
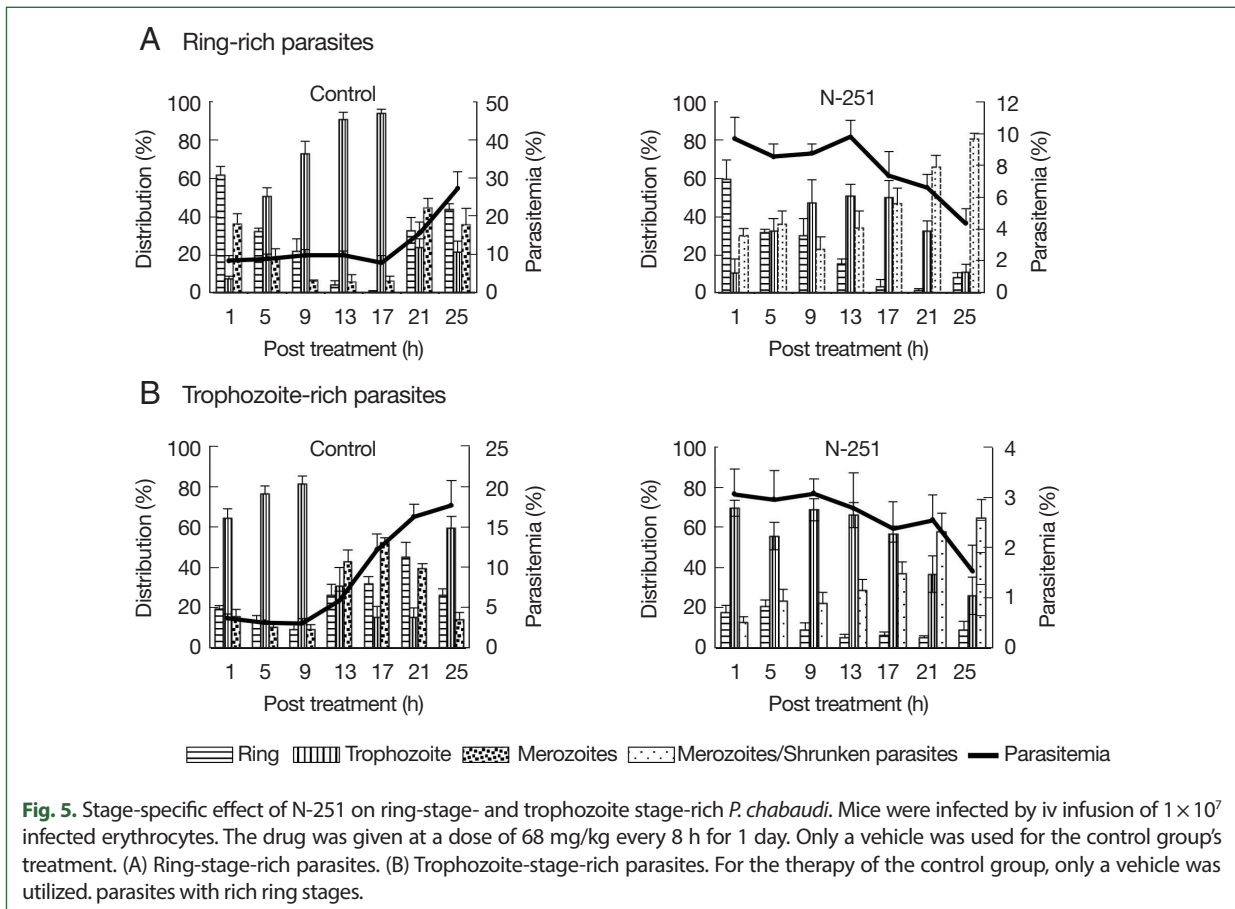

Fig. 5. Stage-specific effect of N-251 on ring-stage- and trophozoite stage-rich *P. chabaudi*. Mice were infected by iv infusion of 1×10^7 infected erythrocytes. The drug was given at a dose of 68 mg/kg every 8 h for 1 day. Only a vehicle was used for the control group's treatment. (A) Ring-stage-rich parasites. (B) Trophozoite-stage-rich parasites. For the therapy of the control group, only a vehicle was utilized. parasites with rich ring stages.

Table 1. Effect of N-251 on parasitic rodent malaria. Using a 4-day suppression test, the in vivo antimalarial activity of N-251 was evaluated against asynchronized *P. berghei* and ring-stage- and trophozoite-stage-rich *P. chabaudi*

N-251 (mg/kg)	*P. berghei*	*P. chabaudi*	
	Asynchronized	Ring-rich*	Trophozoite-rich*
ED$_{50}$	22.0 ± 2.0	27.0 ± 4.0	22.0 ± 3.0
ED$_{90}$	45.0 ± 1.0	48.0 ± 2.0	47.0 ± 2.0

*represents around 60% of the parasite life cycle. Data were presented as the mean and standard deviation.

stage parasites developed to the trophozoite stage until 17 h. The distribution of ring-stage parasites in the N-251 group reduced from 60.0 to 15.0% after 13 h, whereas trophozoite-stage parasites rose from 10.1 to 51.2% in the same period (Fig. 5A). The effect of N-251 on trophozoite-stage-rich parasites is depicted in Fig. 5B. The trophozoite-stage parasites made up around 81.0% of all parasites within the same parasitemia up to 9 h in the control group, after which the parasitemia dropped to 17.7% at 25 h. For the N-251 group, the parasitemias and the distribution ratio of the trophozoite stage were not changed until 9 h; and finally, the parasitemia decreased to 1.5% at 25 h. To compare ring-stage- and trophozoite-stage-rich N-251 groups, the time point of decreasing parasitemia was 4 h earlier in the trophozoite stage than in the ring-stage-rich group without marked changes in stages distributions. According to these findings, N-251 selectively suppressed the trophozoite stage and had no discernible impact on the ring stage.

Antimalarial effect of N-251 using a 4-day suppressive test

We evaluated the antimalarial effect of N-251 against ring-stage- and trophozoite-stage-rich *P. chabaudi* and asynchronized *P. berghei* parasites (Table 1). N-251 inhibited the growth of *P. chabaudi* and *P. berghei* with similar ED$_{50}$ and ED$_{90}$ values. There was no discernible difference in the ED value between *P. berghei* and the various *P. chabaudi* parasite stages.

Discussion

Evaluation of the stage-specific activities of antimalarial candidates in vivo is crucial for studying the effectiveness and mechanisms of action. In this study, we evaluated the stage-specific antimalarial activity of *P. chabaudi* using the predominant ring and trophozoite phases. It was reported that the *P. chabaudi* malaria parasite possesses an auto synchronization of intraerythrocytic developmental stages via a circadian rhythm [14]. Additionally, according to some scientists, host melatonin synchronizes the growth of the parasite stages [15,16]. In a healthy human body, melatonin is typically secreted at a rate of up to 0.04 mg/kg. Melatonin has a short half-life in the blood, of less than 30 min and it is relatively non-toxic [17-19]. As a result, in this study, melatonin was administrated at 50 mg/kg and the effect on parasites was examined. We discovered that melatonin had no antimalarial activity or improvement of synchronization of intraerythrocytic parasite developmental stages in *P. chabaudi* (Fig. 4A). In the *P. berghei* melatonin-treated group, the distribution of merozoites was decreased and ring-stage parasites increased without antimalarial activity (Fig. 4B). These results show that melatonin had no antimalarial activity in both species of rodent

malaria parasites and affects young parasite (merozoite) development of *P. berghei*. Further studies are required to assess melatonin function in *P. berghei*. The *P. berghei* strain is not appropriate for analyzing the stage specificity of the drug in our study, similar to the reported results [20]. With *P. chabaudi*, it is plausible that the mechanism of action can be examined because the drug can act at a specific stage.

In studies of the antimalarial activity of drug candidates, including the characterization of the stage specificity of action, the drug sensitivity of given rodent malaria species does not always mimic that of *P. falciparum* [3]. We have previously investigated the generation of novel antimalarial drugs in *P. falciparum* in vitro and *P. berghei* in vivo employing thousands of molecules, including natural products, marine sources, and synthesized compounds [21-24]. From these studies, N-251 was chosen as a promising antimalarial candidate. N-251 specifically suppressed the trophozoite stage of *P. falciparum* in vitro, possibly by targeting the *P. falciparum* endoplasmic reticulum-resident calcium-binding protein (*Pf*ERC) [NCBI accession no. 124803623] [9,25,26]. The *Pf*ERC level changes with parasite growth, increasing to a maximum level during the trophozoite stage and was inhibited by N-251 treatment. We examined in vivo antimalarial activity of N-251. N-251 revealed strong antimalarial activity with a cure effect in *P. berghei* in mice, but the stage specificity of its action was not investigated [8]. In the present study, the stage-specific antimalarial activity of N-251 as a model compound on *P. chabaudi* in mice was studied. If we discover that N-251 specifically inhibits the trophozoite stage in vivo, then *P. chabaudi* would be an experimental model for stage-specific antimalarial assessment in vivo. The parasites continued to proliferate to the trophozoite stage before being inhibited by N-251 in the group of parasites with a high concentration of ring-stage parasites (Fig. 5). In the trophozoite-stage-rich N-251 group, the ratio of trophozoite-stage parasites was similar while merozoites and shrunken parasites were increased with the same parasitemia up to 9 h. Because we were unable to distinguish between typical merozoites and parasites with morphologically altered merozoite-shaped bodies, the results in Fig. 5 reflected the total of merozoites and shrunken parasites. The results report for the first time that N-251 particularly inhibits the *P. chabaudi* trophozoite parasite in mice. Further study is required to clarify N-251 stage-specific action using additional synchronized trophozoite parasites.

We expected higher effectiveness of N-251 could be collected using trophozoite-stage-rich *P. chabaudi*, but we received similar ED_{50} values with ring-stage- and trophozoite-stage-rich *P. chabaudi* and asynchronous *P. berghei* using the 4-day suppressive test. The 4-day suppressive test only reveals a drug's overall impact after 4 days; it does not provide specific information on the various parasite stages. The parasite synchronization may be interrupted in the experiment since the life cycle of rodent malaria parasites is one day.

We conclude that melatonin has no antimalarial or synchronizing effect on *P. chabaudi* malaria parasites. We discovered for the first time that N-251 specifically inhibits the trophozoite stage of *P. chabaudi*, and this parasite is accordingly suggested as a model for the study of stage-specific antimalarial effects in mice.

Acknowledgments

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