氏　　名	DINH THI QUYEN
授与した学位	博　士
専攻分野の名称	薬　学
学位授与番号	博甲第 7373 号
学位授与の日付	令和 7 年 9 月 25 日
学位授与の要件	医歯薬学総合研究科病態制御科学専攻 （学位規則第５条第１項該当）
学位論文の題目	Investigating the effect of N-89 as a transdermal antimalarial drug on gametocytes of *Plasmodium berghei*-infected mice（マラリア病態モデルを用いた環状過酸化物・N-89 のガメトサイト原虫に対する解析）

学位論文要約

【背景・目的】

We previously reported that 1,2,6,7-tetraoxaspiro [7.11] nonadecane (N-89) is a novel oral antimalarial candidate against the asexual stage of parasites *in vitro* and *in vivo*. However, since most malaria deaths occur in children under the age of five, it was determined that multiple oral administrations would be difficult to implement in this population. To address this issue, we developed a transdermal formulation of N-89 (td N-89). Notably, td N-89 was administered at 60 mg/kg twice daily for four days at parasitemia of 0.2% in *P. berghei*-infected mice, targeting asexual trophozoites, achieved complete parasite clearance without recurrence. These results support the potential of N-89 as a transdermal antimalarial drug candidate. Recent climate change and expanded vector environmental conditions would require new drug development for transmission inhibitors. In this study, we aimed to investigate the effect of td N-89 on gametocytes, the sexual stage responsible for malaria transmission. To evaluate its gametocytocidal and transmission-blocking activities, we conducted the following experiments. We used *P. berghei* NK65 and ANKA strains to establish a suitable rodent malaria model for assessing the effect of td N-89 on the gametocyte stage. In addition, we evaluated the effect of td N-89 monotherapy on gametocyte development in *P. berghei*-infected mice.

【実験方法】

We used 5-8 weeks old ICR mice for all experiments. An ointment formulation containing either N-89 or ART (approximately 70 mg/application) was topically applied to a shaved area of around 4cm^2. The control group received only the vehicle (polyethylene glycol-based formulation). To monitor the expression of gametocyte-related genes, we used a standard curve-based method for relative RT-qPCR to assess changes in gene expression level during treatment under different regimens.

1. To establish a suitable rodent malaria model for evaluating the effect of td N-89, we carried out the following experiments with *P. berghei* NK65 and ANKA strains on ICR mice: analysis of parasitemia curves, changes in gametocyte-related gene expression levels during the post-infection period, and evaluation of responses to a 4-day suppressive treatment with either td N-89 or ART.

2. Three monotherapy regimens of td N-89 (at doses of ED_{90} values, curative dose, or insufficient therapy) were assessed. Artemisinin (ART)-an antimalarial compound is used as the first line for uncomplicated malaria in human patients. In my study, td ART was used as the reference compound.

【結果】

1. Comparison between *P. berghei* NK65 and ANKA strains in infected-ICR mice

The comparison involved assessments of parasitemia curves, expression profiles of gametocyte-related genes (Pbs21 as female and GCS as male gametocyte markers) during the post-infection period, and responses to a 4-day suppressive treatment with either td N-89 or ART. The results show that the antimalarial activities of td N-89 and td artemisinin, as assessed by ED_{50} and ED_{90} values, did not differ significantly between *P. berghei* NK65 and ANKA strains in mice (**Table 1**), Additionally, similar morphology of gametocytes and a few numbers of gametocytes revealed under light microscopy in both strains (**Fig. 1**). However, analysis of the parasitemia curves (**Fig. 2**) and gametocyte-related gene expression profiles (**Fig. 3**) demonstrated that the NK65 strain in ICR mice exhibited lower inter-individual variability and higher experimental reproducibility compared to the ANKA strain.

Based on these findings, we selected the NK65 strain-infected ICR mouse model for subsequent analyses of gametocyte-related gene expression profiles and to evaluate expression changes following treatment with td N-89 or artemisinin.

Table 1. The antimalarial activity of td N-89 and td ART
Data are expressed as mean ± SD (n=5)

Compounds	ED_{50} (mg/kg)		ED_{90} (mg/kg)	
	NK65 strain	ANKA strain	NK65 strain	ANKA strain
td N-89	18 ± 3.2	21 ± 2.7	46 ± 4.0	50 ± 1.5
td Artemisinin	9 ± 2.1	10 ± 2.0	22 ± 3.9	19 ± 3.2

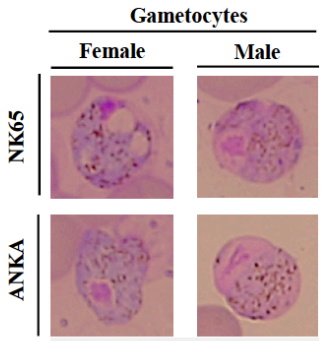

Figure 1. Morphology of gametocytes

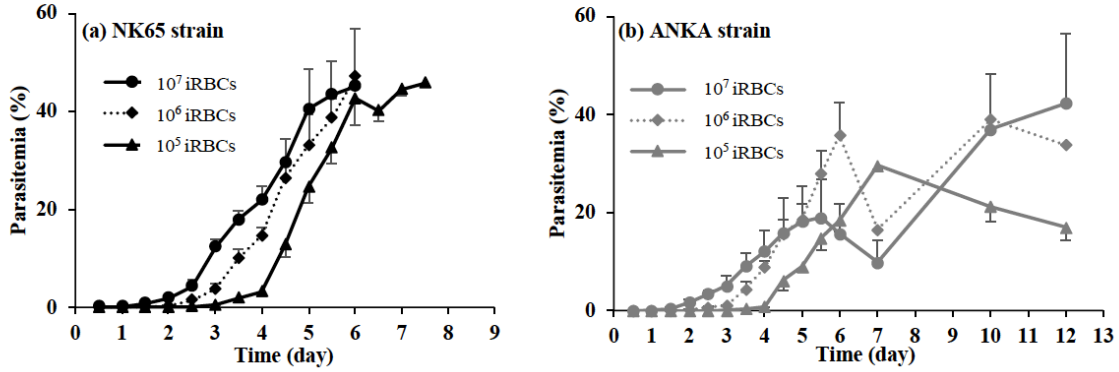

Figure 2. The growth curve of *P. berghei* NK65 and ANKA strains. Healthy 5-week mice were infused with i.v. of 1 × 10^7, 1 × 10^6, or 1 × 10^5 iRBCs. The parasitemia was determined using thin blood smears stained with Diff-Quik. The parasitemia is expressed as mean (n=4) ± SD values.

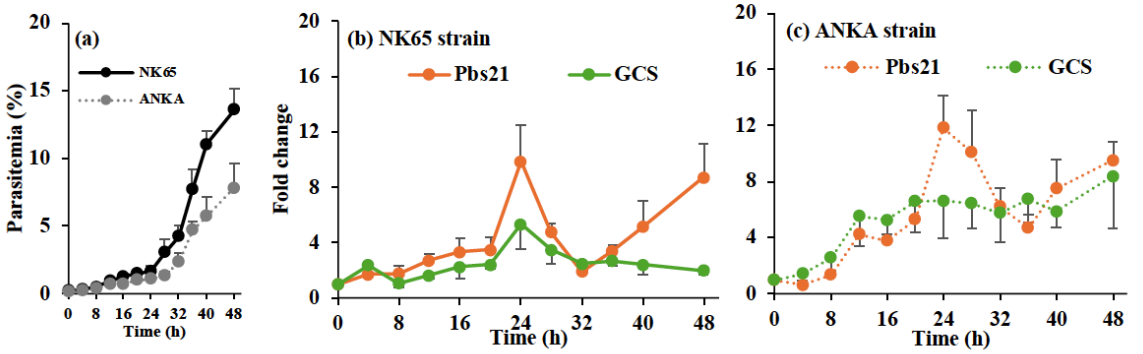

Figure 3. Parasitemia and gametocyte-related gene expression profiles of *P. berghei* NK65 and ANKA strains. **(a)** *P. berghei* NK65 and ANKA strains' parasitemia. Mice were inoculated with 1 x 10^7 iRBCs. **(b-c)** The expression levels of Pbs21 (female gametocyte gene marker) and GCS (male gametocyte gene marker) were built using the relative standard curve-based RT-qPCR method, with GAPDH as a reference gene. Other time points were normalized with 0 h (set 0h at 0.21 %) parasitemia. Data was shown as Mean ± SD (n=5).

2. Comparison analysis of parasitemia and gametocyte-related gene expressions following td N-89 or ART treatment at the ED_{90} values

In mice treated with td N-89 and td ART at ED_{90} doses, rapid parasitemia decreased in the td ART group than in the td N-89 group. The gametocyte-related gene expression levels were elevated in both groups after 24 hours of treatment (**Fig. 4**). However, expression was lower in the td N-89 compared to td ART.

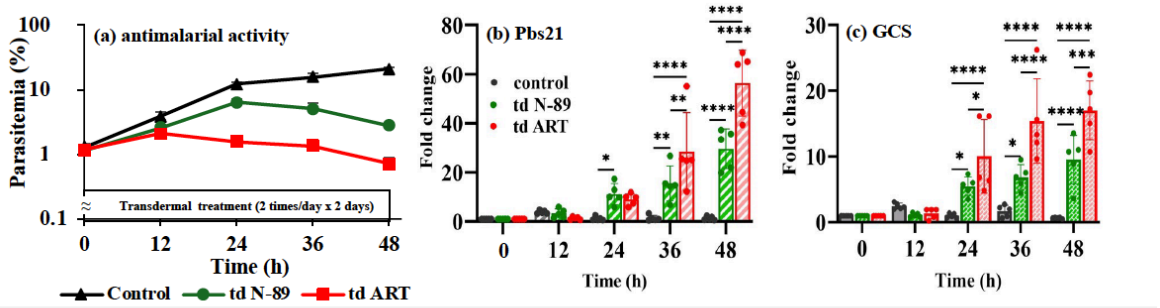

Figure 4. Comparison of the parasitemia and gametocyte-related gene expression levels following td N-89 or ART treatment at the ED_{90} values in *P. berghei*-infected mice. **(a)** Antimalarial activity of td N-89 and td ART against *P. berghei*-infected mice at ED_{90} values (ED_{90} value: td N-89; 46.0 mg/kg, and td ART; 21.8 mg/kg). Treatment was initiated at 1.2% parasitemia (set as sample 0h). **(b)** Gene expression of Pbs21. **(c)** Gene expression of GCS. The data are presented in mean ± SD (n=5). The two-way ANOVA with Turkey's multiple comparison tests determined statistical significance among control, N-89, and Artemisinin treatment groups at the same time points *(0.01<P<0.05); **(0.001<p<0.01); ****(p<0.0001).

3. Analysis of parasitemia and gene expression level of gametocytes for td N-89 at cure dose with different regimens

In the cure dose of td N-89, the parasitemia reached 2.7% at 12 hours and decreased over 108h. Finally, the parasite was not observed at 120h in the td N-89 group (**Fig. 5a**). As a result of monitoring the mice's parasitemia and survival until 30 days, two of six mice (33.3%) in the td N-89 group were cured without recrudescence of parasites. The expression level of the Pbs21 and GCS genes significantly increased from 24 hours. They remained elevated from 36 to 60/72 hours and then continued to decline, decreasing to 96/84 hours but were no longer detected at 108/96 hours **(Fig. 5b and c)**. Moreover, in the event of the inadequate regimen of the td N-89 group (60 mg/kg, twice daily for 48 hours), both gametocyte gene expression increased in a time-dependent manner, ranging from 0 to 48 hours. Conversely, the expression of the genes from 144 to 168 hours was found to be considerably lower than that observed in the td N-89 treatment and analogous to that of the control group (**Fig. 6**).

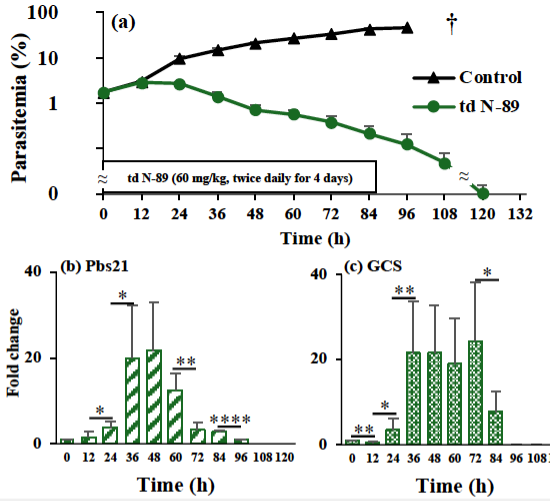

Figure 5. Evaluation of td N-89 at cure dose on parasitemia and gametocyte-related gene profiles. **(a)** Mice were transdermally administered N-89 at doses of 60 mg/kg twice daily for 96 hours when parasitemia reached 1.7%. **(b-c)** The levels of Pbs21 and GCS gene expression during N-89 exposure. All gene expression levels were normalized to the time point before the first dose (set as 0h). Data are shown as mean ± SD (n=6). Statistical significance between consecutive time points was determined using two-tailed Student's *t*-tests. Significance levels: *(0.01 < P < 0.05); **(0.001 < P < 0.01); ****(P < 0.0001). Mouse death: †

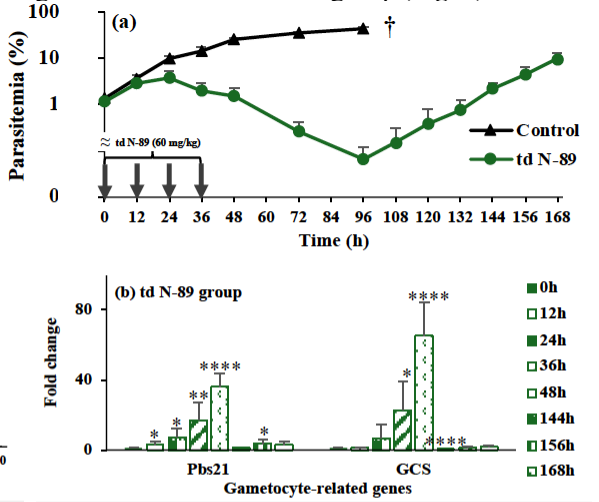

Figure 6. The impact of an insufficient td N-89 regimen on parasitemia and gametocyte-related gene expressions. **(a)** Parasitemia for untreated mice and those receiving N-89 at 60 mg/kg, twice daily for 48 hours, at 1.2% parasitemia (set as 0h). **(b)** Expression levels of Pbs21 and GCS. Relative expression levels were normalized to GAPDH. All time points were referenced to 0h. Data are expressed as mean ± SD (n=6). Statistical significance between different time points and the 0-hour baseline was determined using two-tailed Student's *t*-tests. Significance levels: *(0.01 < P < 0.05); **(0.001 < P < 0.01); ****(P < 0.0001). Mouse death: †

【考察】

In this study, we tried to investigate the potential of td N-89 for gametocytes using *P. berghei* (NK65)-infected mice. At low parasitemia or when assessing antimalarial effects, detecting gametocytes by light microscopy is often challenging due to the detection limit. To overcome this issue, we assessed the effect of td N-89 on gametocytes by monitoring the expression levels of gametocyte-related genes (Pbs21 as female and GCS as male gametocyte markers) in various treatment regimens.

In mice treated with td N-89 and td ART at ED_{90} doses, gametocyte-related gene expression levels were elevated in both groups after 24 hours of treatment. However, expression was lower in the td N-89 group compared to td ART (**Fig. 4**). This phenomenon would be explained by the stress response hypothesis which posits that parasites subjected to drug-induced stress may shift towards sexual differentiation as an adaptive survival strategy of parasites (Azimi, W.A. *et al*., *Malar. J*., 2024). Our study demonstrates that td N-89 maintained efficacy even at 1.7% parasitemia, although with a lower cure rate (33.3%). Notably, the transmission-blocking potential of td N-89 appears to be associated with sustained trophozoite clearance rather than direct gametocytocidal activity.

【結論】

My study demonstrates that td N-89 maintained efficacy even at 1.7% parasitemia, although with a lower cure rate (33.3%). Notably, the transmission-blocking potential of td N-89 appears to be associated with sustained trophozoite clearance rather than direct gametocidal activity. I reported for the first time that gametocyte-related gene expression progressively changes in the td N-89 group and can be detected for a long period. Conversely, td ART which exhibited stronger anti-asexual activity than td N-89 but failed to prevent recrudescence. Moreover, in mice with td artemisinin as a reference, Pbs21 and GCS expression levels were higher than those in the td N-89 group, indicating that td N-89 may exhibit a less side effect compared to td artemisinin.

【参考論文】

1. Thi Quyen Dinh, Hiroaki Matsumori, Mamoru Niikura, Shin-Ichi Miyoshi, Hye-Sook Kim. Evaluating the effect of new antimalarial N-89 for gametocytes in *P. berghei*-infected mice. *Parasitology International*, 109 (2025) 103093. (IF: 1.9)

【関連文献】

なし