Discovery and evaluating new drugs for babesiosis treatment

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バベシア症に対する新薬候補の特定と評価

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Contents

Abbreviations

Abbreviations

A	ART	Artemisinin
	ATO	Atovaquone
	AZI	Azithromycin
C	Cmax	Maximum (peak) plasma concentration of drug
	CDC	Centers for Disease Control and Prevention
	Cytb	*Cytochrome b*
D	DMSO	Dimethyl sulfoxide
	DPI	Days post-infection
	DV	Digestive vacuole
E	ELQs	Endochin-like quinolones
F	FDA	Food and Drug Administration
G	G6PD	Glucose-6-phosphate dehydrogenase
H	HCT	Hematocrit
I	IC_{50}	Half maximal inhibitory concentration
	i.p.	Intraperitoneal injection
	iRBCs	Infected red blood cells
N	NQ	Naphthoquine
	NQP	Naphthoquine phosphate
P	PBS	Phosphate buffered saline

	PCR	Polymerase chain reaction
	PI4K	Phosphatidylinositol 4-kinase
	PI4P	Phosphatidylinositol 4-phosphate
	PIKs	Phosphatidylinositol kinases
R	RBC	Red blood cell
	RPL4	*ribosomal protein subunit L4*
	RPMI	Roswell Park Memorial Institute
S	SCID	Severe combined immunodeficiency
	SD	Standard deviation
	SG1	SYBR Green I
	SNP	Single nucleotide polymorphism
	SNV	Single nucleotide variant
	SO	Sesame oil
T	TAF	Tafenoquine
U	U.S.	United States
W	WT	Wild type

Unit abbreviations

bp	base pair
h	hour
mg	milligram
ml	milliliter
mM	millimolar

nM	nanomolar
nm	nanometer
min	minute
rpm	revolutions per minute
sec	second
µm	micrometer
µl	microliter
µg	microgram
%	percentage
°C	degree Celsius

General introduction

1. Babesiosis

Babesiosis is a global emerging tick-borne disease caused by infection with intra-erythrocytic parasites of the genus *Babesia.* It has a great economic, veterinary, and medical impact worldwide (Homer et al., 2000).

Since the first case report in cattle in 1888, over 100 known *Babesia* species have been identified which have a wide geographical range and are host-specific. *Babesia* species could infect many types of mammalian hosts, including domestic animals and humans (Fig. 1) (Babes, 1888; Homer et al., 2000; Bonnet and Nadal, 2021). The ixodid ticks are the main vector for babesiosis transmission. During blood meals, the mammalian host could transmit from the bite of infected ticks. Noted, human to human could be transmitted through the blood transfusion or from an infected pregnant woman to the fetus (Telford and Spielman, 1993; Kakoma and Mehlhorn, 1994; Gray and Weiss, 2008). The severity of *Babesia* infections is associated with immunity. The general symptoms of babesiosis include fever, anorexia, emaciation, hemoglobinuria, and even death in some severe cases (Homer et al., 2000).

2. *Babesia* species

The first case of human babesiosis was reported in 1957 and has gained attention in

the United States (U.S.) since 1969 (Skrabalo and Deanovic, 1957). Currently, six primary species have been described as agents for human babesiosis including *B. crassa*-like, *B. divergens*, *B. duncani, B. microti, B. motasi*, and *B. venatorum,* which have distinct geographic distributions (Krause, 2019; Kumar et al., 2021). Over the past few decades, human babesiosis has exponentially increased, particularly in northeastern and northern midwestern regions of the U.S., where babesiosis is caused by *B. microti* (Krause, 2019; Kumar, et al., 2021). *B. microti* infection shows a wide spectrum of symptoms, from asymptomatic to fatal disease. In. contrast, *B. divergens* infection is generally less severe (Vial and Gorenflot, 2006).

Bovine babesiosis has been considered the most important among arthropod-borne diseases in cattle (Bock et al., 2004). Most of the 1–2 billion cattle around the world are still threatened by babesiosis. *B. bovis, B. bigemina, B. orientalis,* and *B. divergens* are the pathogens closely related to the economy in cattle. The etiology of the disease varies according to the infecting species. In *B. bovis* infection, the accumulation of infected erythrocytes in lung and brain capillaries results in hypotension, respiratory stress syndrome, and neurological symptoms. Meanwhile, infections by *B. bigemina* and *B. divergens* show in high parasitemia resulting in severe anemia (Zintl et al., 2003; Bock et al., 2004).

Equine babesiosis is endemic in vast tropical and subtropical regions and *B. caballi* is the only recognized *Babesia* species that causes equine infection. It is a threat for the racehorse industry. In the chronic course, equines infected by *B. caballi* have a reduced capacity of erythrocytes to carry oxygen resulting in decreased performance. Hence, testing

for equine babesiosis is a mandatory requirement for the international movement of horses (Salim, et al., 2008; Onyiche et al., 2019).

Sheep and goat babesiosis usually have great economic importance in Europe, the Middle East, and some African and Asian countries (Yin and Luo, 2007; Ranjbar-Bahadori et al., 2012). The pathogens include highly pathogenic *B. ovis* and *Babesia* sp. Xinjiang, as well as moderately virulent *B. motasi* (Uilenberg, 2006; Xu, et al., 2021). *B. motasi* is often the cause of sheep babesiosis in northern Europe and mixed infections with *B. ovis* may occur in southern Europe and elsewhere (Christensson and Thunegard, 1981; Lewis, 1981).

Canine babesiosis is generally caused by *B. vogeli*, *B. canis, B. gibsoni, B. conradae,* and *B. rossi* (Zahler et al., 1998; Uilenberg, 2006). *B. rossi* infections provoke hemolytic anemia resulting in grave complications, such as neurological signs, acute renal failure, and pulmonary edema, with poor prognosis. *B. vogeli* infections can be fatal in puppies. The severity of *B. canis, B. gibsoni,* and *B. conradae* infections according to the individual (Irwin, 2009; Solano-Gallego and Baneth, 2011).

3. Conventional drugs for babesiosis treatment

Human chemotherapeutics

Most people with healthy immune systems do not require treatment. Immunocompromised patients are usually orally treated with atovaquone (ATO) plus azithromycin (AZI) for 7-10 days. This combination is recommended for all *B. microti*-infected patients (Krause et al., 2000) and shows lesser side effects than clindamycin plus

quinine, but in some severe cases, the treatment period will be prolonged due to mutations in the *cytochrome b* (*Cytb*) and *ribosomal protein subunit L4* (*RPL4*) which confer parasite resistance. Therefore, if initial antibabesial therapy failed, the patients will experience a prolonged relapse course of diseases. Although, clindamycin plus quinine is the standard of care for severely ill patients, this combination shows serious side effects in humans, such as headache, hearing loss, dizziness, and tinnitus. Furthermore, monotherapy of clindamycin or quinine has poor efficacy against *Babesia* (Ruebush et al., 1980; Rowin et al., 1982; AbouLaila et al., 2012). Hence, looking for new alternatives to treat babesiosis attracted the researcher's attention.

Animal chemotherapeutics

Imidocarb dipropionate and diminazene aceturate are commonly used drugs with anti-protozoan activity in animals. Imidocarb dipropionate is the first choice for treatment of bovine babesiosis caused by *B. bigemina*, *B. bovis*, *B. divergens*, and equine babesiosis caused by *B. caballi* (Kuttler, 1980). This compound is also used in ovine and canine babesiosis treatment (McHardy et al., 1986; Baneth, 2018). Imidocarb dipropionate treatment is given subcutaneously or intramuscularly at a recommended dose of 1-3 mg/kg (Kuttler, 1980), and could not be intravenously administered. It will provide protection from clinical diseases by allowing a sufficient level of infection for immunity to develop. However, long-term persistence of low-level parasitemia is considered a disadvantage, both for possible recrudescence and appearance of resistant parasites (Vial and Gorenflot, 2006). In addition, several studies have reported that imidocarb dipropionate treatment is associated with residue problems and remains detectable in edible ovine and bovine tissues

for long periods after dosing (Moore et al., 1996). Imidocarb dipropionate has a strong binding ability to nuclear components which causes accumulation of drug in the nucleus of the hepatocyte. Therefore, the use of imidocarb dipropionate in food-producing animals has caused concern.

Diminazene aceturate is an aromatic diamidine and was developed more than six decades ago. It has been proved by the U.S. Food and Drug Administration (FDA) as an anti-trypanosomal agent which is also used in the treatment or control of animal babesiosis (Vial and Gorenflot, 2006; Gohil, 2013). Diminazene aceturate treatment is given subcutaneously at a dose of 3-5 mg/kg. Diminazene aceturate shows efficacy against *B. bigemina*, but less efficacy against *B. bovis* and *B. divergens*. In addition, it has been used as a first-line agent for the treatment of *B. gibsoni* in Japan. However, diminazene aceturate often fails to eradicate *B. gibsoni* from dogs, and relapses may occur. Furthermore, diminazene aceturate can induce severe adverse effects in dogs, like cerebellar hemorrhage, hepatotoxicosis, and necrosis at the injection site (Sakuma, 2009). Hence, the discovery and evaluation of new drugs or drug targets for babesiosis treatment will contribute to preventing and controlling babesiosis.

4. Aim of the present study

Due to the *Plasmodium* and *Babesia* genera being closely related, some anti-*Babesia* drugs were initially developed as an anti-*Plasmodium* compound, e.g., ATO, clindamycin, quinine, and tafenoquine (TAF). Therefore, screening of drugs with known action modes

based on the efficacy against *Plasmodium* will reduce the cost and save time for anti-*Babesia* drug development. The objectives of the current study were as follows: (1) to screen potential anti-*Babesia* drugs from currently used or under development anti-*Plasmodium* compounds and evaluate their activity against *Babesia* species; (2) to identify and evaluate promising anti-*Babesia* druggable targets which can eradicate parasites.

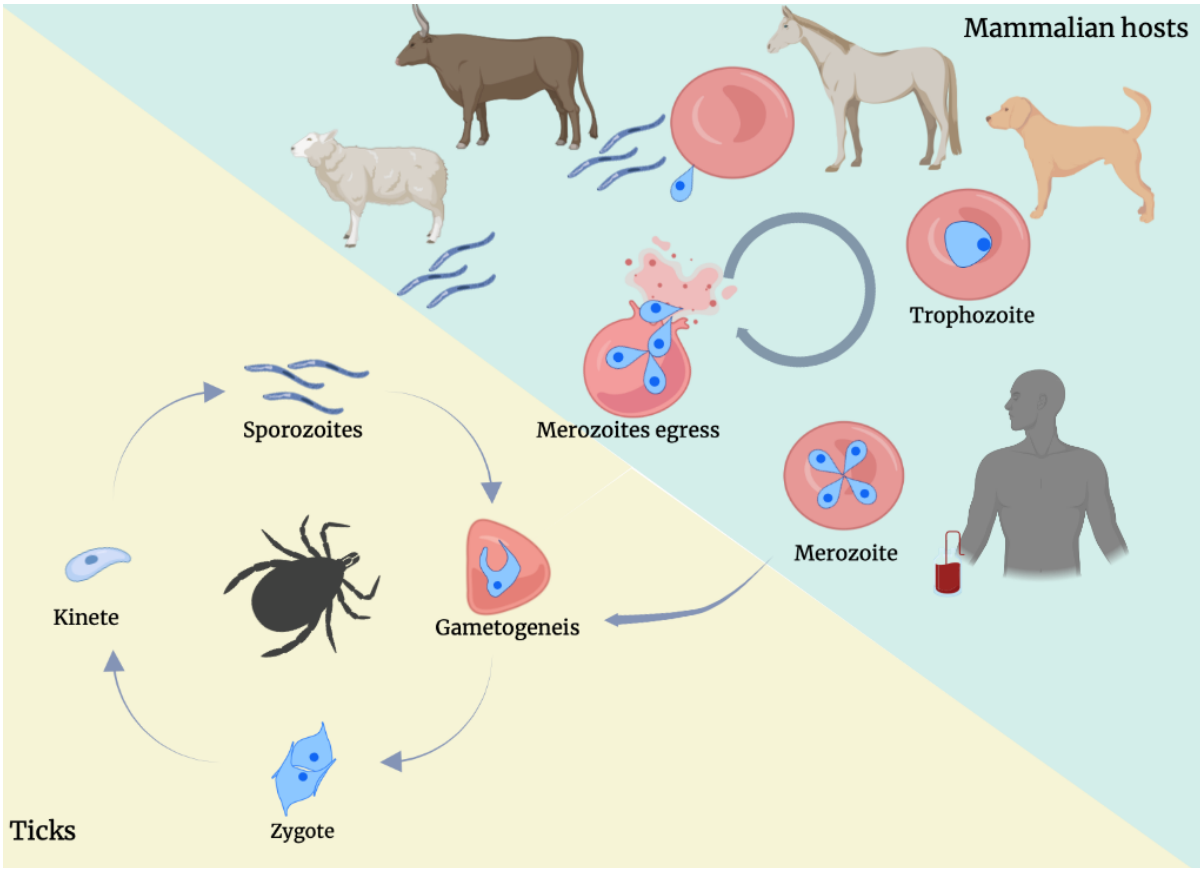

Fig. 1. Life cycle of *Babesia* spp.

Chapter 1

Inhibitory effect of naphthoquine phosphate on *Babesia gibsoni in vitro* and *Babesia rodhaini in vivo*

1.1. Introduction

Babesiosis is an infectious disease caused by intraerythrocytic parasites of the genus *Babesia*. The most notable species include *Babesia bigemina*, *B. divergens,* and *B. bovis* for bovine, *B. caballi* for equine, *B. canis* and *B. gibsoni* for canine, and *B. microti* and *B. rodhaini* for murine (Homer et al., 2000). Moreover, several *Babesia* spp. have been reported to infect humans. In the past five decades, the cases of human babesiosis have increased in the United States (U.S.) (Vannier et al., 2015). A review described that during 1982 to 1993, 139 hospitalizations occurred due to *Babesia* infection. Among the patients, 25% required intensive care stays and 9 patients died (White et al., 1998). Moreover, a recent surveillance in the U.S. found that a total of 7,612 cases of babesiosis were reported to the Centers for Disease Control and Prevention (CDC) from 2011 to 2015. Of 7,612 cases, 82.5% were classified by the reporting health jurisdiction confirmed as babesiosis and 17.5% as probable. Among these patients, 7 of 46 deaths were attributed to babesiosis and 4 of 46 deaths were not babesiosis-related, whereas whether the 35 patients' deaths were due to babesiosis were not verified (Gray and Herwaldt, 2019). Atovaquone (ATO) plus azithromycin (AZI) as the standard therapy for treating human babesiosis failed in some clinical cases caused by a single nucleotide polymorphism (SNP) in the *B. microti*

cytochrome b (*Cytb*) gene (Simon et al., 2017). In addition, although the ATO plus AZI is effective on *B. gibsoni* infections, a recent study found that 8.57% of *B. gibsoni* isolates obtained from Japanese dogs carry a SNP in the *Cytb* gene which was empirically proven to be associated with resistance to ATO (Iguchi et al., 2012; 2020). Therefore, new drugs are needed to combat the emergence of drug resistance and to develop effective therapies.

Naphthoquine (NQ) is an antimalarial drug that was first synthesized in China in 1986 and registered as naphthoquine phosphate (NQP) in 1993. Initial clinical trials showed that NQ monotherapy was highly efficacious without documented toxicity (Wang et al., 2004; Moore et al., 2016). Subsequently, a drug combination comprised of NQ and artemisinin (ART) at a fixed ratio of 1:2.5 was developed in order to retain the strongpoint of the two drugs and in prevention of the possible emergence of drug resistance. Safety data of NQ-containing therapies involving more than 4,000 patients was recorded without serious adverse reaction, hematology, and biochemistry changes. It has been considered as a promising antimalarial drug candidate and is marketed under the name of ARCO® in various tropical countries (Wang et al., 2004; Hombhanje and Huang, 2010; Moore et al., 2016). Due to the *Plasmodium* and *Babesia* are closely related, I investigated whether NQP has effects on the *in vitro* growth of *B. gibsoni*, a causative agent of canine babesiosis and *in vivo* propagated *B. rodhaini*, a highly pathogenic rodent *Babesia* species.

1.2. Materials and methods

Ethics statement

The protocols performed in the current study were carried out according to the ethical guidelines approved by the Obihiro University of Agriculture and Veterinary Medicine (permit numbers: animal experiment, 21-133; pathogen, 201712-5).

Chemicals

NQP was purchased from ChemScene (USA), and was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Japan) to prepare a 40 mg/ml stock solution. In parallel, tafenoquine (TAF, Sigma-Aldrich, Japan) was dissolved as mentioned above, and was used as a control treatment. Tafenoquine was previously reported as a potent antibabesial agent for treating *B. rodhaini* infection (Liu et al., 2021). SYBR Green I (SG1) nucleic acid stain was purchased from Lonza America (USA).

Maintenance of the parasites *in vitro* and *in vivo*

Babesia gibsoni Oita strain (Sunaga et al., 2002) was cultured and used for the *in vitro* growth inhibition assay. To maintain, the *B. gibsoni* was cultured in canine red blood cells (RBCs) and was suspended in a culture medium, RPMI-1640, supplemented with 20% canine serum. The culture was maintained in an atmosphere of 5% CO_2 and 5% O_2.

For the *in vivo* inhibition assay, *B. rodhaini* Australia strain (Terkawi et al., 2008) was recovered from the stock in our laboratory. For the maintenance of *B. rodhaini*, cryopreserved parasitized-RBCs were passaged by intraperitoneal (i.p.) injection of mice. Challenge infection was performed with i.p. inoculation of 10^7 fresh *B. rodhaini* infected RBCs (iRBCs). A total of 20 BALB/c mice (6-weeks old) were purchased from CLEA Japan and were used to maintain *B. rodhaini* for the *in vivo* study.

In vitro growth inhibition assay

To test the growth inhibitory effect of NQP on *B. gibsoni*, a SG1 fluorescence-based assay was performed as previously reported (Rizk et al., 2020). Briefly, the *in vitro* cultures of *B. gibsoni* were diluted to 1% parasitemia with fresh canine RBCs. The stock solution of NQP was diluted in medium to achieve final concentrations of 0.1, 0.5, 1.0, 2.5, and 5.0 μM, and incubated with iRBCs in triplicate in 96-well plates with 5% hematocrit (HCT) for 96 hours. After a lysis buffer containing a 2 × SG1 nucleic acid stain was added in each well, the fluorescence values were evaluated using the fluorescence spectrophotometer (Thermo Fisher Scientific, USA) and the inhibition activity and half inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism 8 (GraphPad Software Inc., USA).

In vivo growth inhibition assay

Fifteen BALB/c mice intraperitoneally challenged with 10^7 *B. rodhaini* (iRBCs) were randomly assigned to groups (n = 5). When parasitemia was about 3% to 5%, the drug treatment was initiated. The first group was treated orally with 40 mg/kg of NQP for 5 consecutive days as previously described (Tuvshintulga et al., 2016; Carvalho et al., 2020). The second group was treated orally with 20 mg/kg TAF as single-dose therapy, according to a previously regimen (Liu et al., 2021). The control group was treated orally with 5% DMSO in Milli-Q water. Parasitemia was calculated from Giemsa-stained blood smears by counting infected RBCs among 3,000 RBCs. HCT changes were monitored for the development of an index of anemia by using a hematology analyzer (Nihon Kohden Corporation, Japan) every other day until 42 days post-infection (DPI).

Statistical analysis

Data analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., USA). Differences in parasitemia between the control and treated groups were determined by one-way ANOVA analysis plus Tukey-Kramer post hoc analysis. Survival rates were calculated using the Kaplan–Meier method, with regard to the log-rank test. A P value < 0.05 was considered statistically significant.

1.3. Results

Effects of NQP on *B. gibsoni* growth *in vitro*

NQP significantly inhibited *B. gibsoni* growth at 2.5 μM and 5 μM (Fig. 2A). The IC_{50} value of NQP on *B. gibsoni* was 3.3 ± 0.5 μM (Fig. 2B). Meanwhile, the IC_{50} value of TAF was 20.0 ± 2.4 μM (Fig. 2C).

Effects of NQP on *B. rodhaini in vivo*

The highest parasitemia of *B. rodhaini* in DMSO treated group was 78.2% on day 8 DPI (Fig. 3A). In contrast, the parasitemia in NQP- and TAF-treated group were decreased after treatment and showed 95.1% (3.8% peak parasitemia) and 95.8% (3.3% peak parasitemia) inhibition compared to the highest parasitemia in the DMSO-treated group, respectively. A significant difference in parasitemia levels was calculated in NQP-treated group and TAF-treated group as compared with the DMSO-treated group at 6 and 8 DPI. Parasitemia was undetectable via Giemsa-staining method in mice treated with NQP and TAF at 10 DPI and 8 DPI, respectively. Afterwards, regrowth of parasites was observed in

both NQP- (n = 2/5) and TAF- (n = 1/5) treated group on 16 DPI and 22 DPI (Fig. 3A). Significant reductions in HCT values were observed in DMSO-treated mice on 6 DPI and 8 DPI as compared to the values recorded from the NQP- or TAF-treated group (Fig. 3B). All the mice in NQP- and TAF-treated group survived until the end of the experiment by 40 DPI, whereas none of DMSO-treated mice survived by 10 DPI (Fig. 3C). Compared with the DMSO-treated group, the TAF-treated parasites showed abnormal morphological changes such as faint chromatin staining and degenerative forms from 5 DPI onwards, while denigrative forms of NQP-treated parasites were observed at 8 DPI (Fig. 4).

1.4. Discussion

NQ is a 4-aminoquinoline antimalarial drug with a longer half-life but slower action than ART, and is currently combined with ART to treat malaria (Wang et al., 2004). This combination therapy is also effective on *Schistosoma mansoni* (El-Beshbishi et al., 2013). NQP combined with AZI for malaria treatment is available in the market (Bei et al., 2020). The toxic effects of NQP on mammalian hosts have been reported. Daily treatments in dogs for 14 days at a dose of 17.5 mg/kg/day and in rats for 70 mg/kg/day were safe. The safe doses in canine and rat models are equivalent to approximately 10 mg/kg/day in humans (Wang et al., 2003; Moore et al., 2016). A previous study reported that the concentration of NQP reached the peak level in plasma 2 hours post treatment with a dose of 10 mg/kg. The peak concentrations were 300.84 ng/mL and 273.29 ng/mL in plasma and erythrocytes, respectively, and the half-life of NQP was 198 hours (~8 days) in normal mice (Li et al., 2018). Interestingly, the concentrations were far greater in *Plasmodium berghei*-infected

mice (Li et al., 2018). The anti-parasitic activity of NQP, as shown by the inhibition of *B. gibsoni in vitro* (Fig. 2) prompted us to further explore its anti-*Babesia* activity *in vivo*. I used the lethal species *B. rodhaini* in the mouse model for evaluating NQP as a therapeutic. In the current *in vivo* trial, NQP exhibited excellent inhibitory efficacy as evidenced by reduced parasite growth (Fig. 3A) and degenerative morphological changes in the parasites (Fig. 4). Furthermore, the first 2 days of treatment with 40 mg/kg NQP prevented the rise of *B. rodhaini* parasitemia starting from day 6 post-infection compared with the typical rise of mean parasitemia in DMSO-treated mice. In addition, the accumulation of NQP in plasma (Li et al., 2018) by completion of the 4-day treatment resulted in morphological changes of parasites in all treated mice at day 8 post-infection. The TAF-treated group showed an aberrant parasite phenotype (Fig. 4) which has been associated with oxidative stress (Liu et al., 2021). *Babesia rodhaini*-infected mice in DMSO-treated group developed rapid anemia, whereas NQP and TAF prevented anemia development in infected mice (Fig. 3B).

Since TAF was approved by the U.S. Food and Drug Administration (FDA) as a single drug treatment for malaria, TAF studies have attracted much attention (Carvalho et al., 2020; Liu et al., 2021). The limitation of TAF is the risk of inducing severe hemolytic anemia in individuals with glucose-6-phosphate dehydrogenase deficiency (G6PD-deficiency) in humans and the relapse of parasites, which are well documented (Peters et al., 2009; Mordue and Wormser, 2019; Liu et al., 2021). A single treatment of TAF on immunocompromised hosts could not eliminate parasites (Mordue and Wormser, 2019; Liu et al., 2021). Recently, TAF showed strong and broad anti-parasitic activity against *Babesia*

spp., including *B. microti, B. gibsoni*, and *B. rodhaini* (Liu et al., 2021). Hence, TAF was selected as a reference drug in this study. In the present study, the relapse of parasites was observed both in NQP-treated group and TAF-treated group (Fig. 3A). Therefore, NQP may need to be accompanied by other anti-*Babesia* drugs to augment its effect and prevent the regrowth of parasites.

In addition, the mechanism of action of NQP has not been fully elucidated. The inhibition activity of NQP for *Plasmodium* was hypothesized to be through the inhibition of hemozoin bio-crystallization in the digestive vacuole (DV) of late-stage parasites and disruption of membrane system. Due to *Babesia* not producing hemozoin during parasite development, the inhibitory effect of NQP on the *Babesia* parasite is hypothesized to be related to targeting the parasite's membrane system (Rudzinska et al., 1976; Moore et al., 2016)

It should be noted that there are some limitations to the present study. Although NQP exhibited a potential antibabesial effect, it has a slower onset of action and a longer half-life, which may easily lead to drug build-up with increasing the probability of developing resistance. Therefore, future studies are warranted to analyze the possible synergistic effect of NQP when administrated in combination with other drugs which have a rapid onset of babesicidal action and a short half-life. Such analysis will help to determine the most effective composition ratio for treatment of *Babesia* in animals in clinical applications. Furthermore, the mode of action by which NQP inhibits the *in vitro* and *in vivo* growth of *Babesia* is still unknown. Subsequently, further studies are required to elucidate this point. Although the present study demonstrated the potential antibabesial efficacy of NQP in a

mouse model, additional *in vivo* experiments are required to confirm such inhibitory effect in *B. gibsoni*-infected dogs.

1.5. Summary

In summary, the present study demonstrated the growth inhibitory effect of NQP against *B. gibsoni in vitro* and *B. rodhaini in vivo*. These findings indicate that NQP is a potential candidate agent for the treatment of babesiosis and suggest further investigation on the possible use of this chemical for canine babesiosis and human babesiosis. Future work shall focus on the elucidation of the mechanism of action of NQP and the effect of its combination with other current promising drugs.

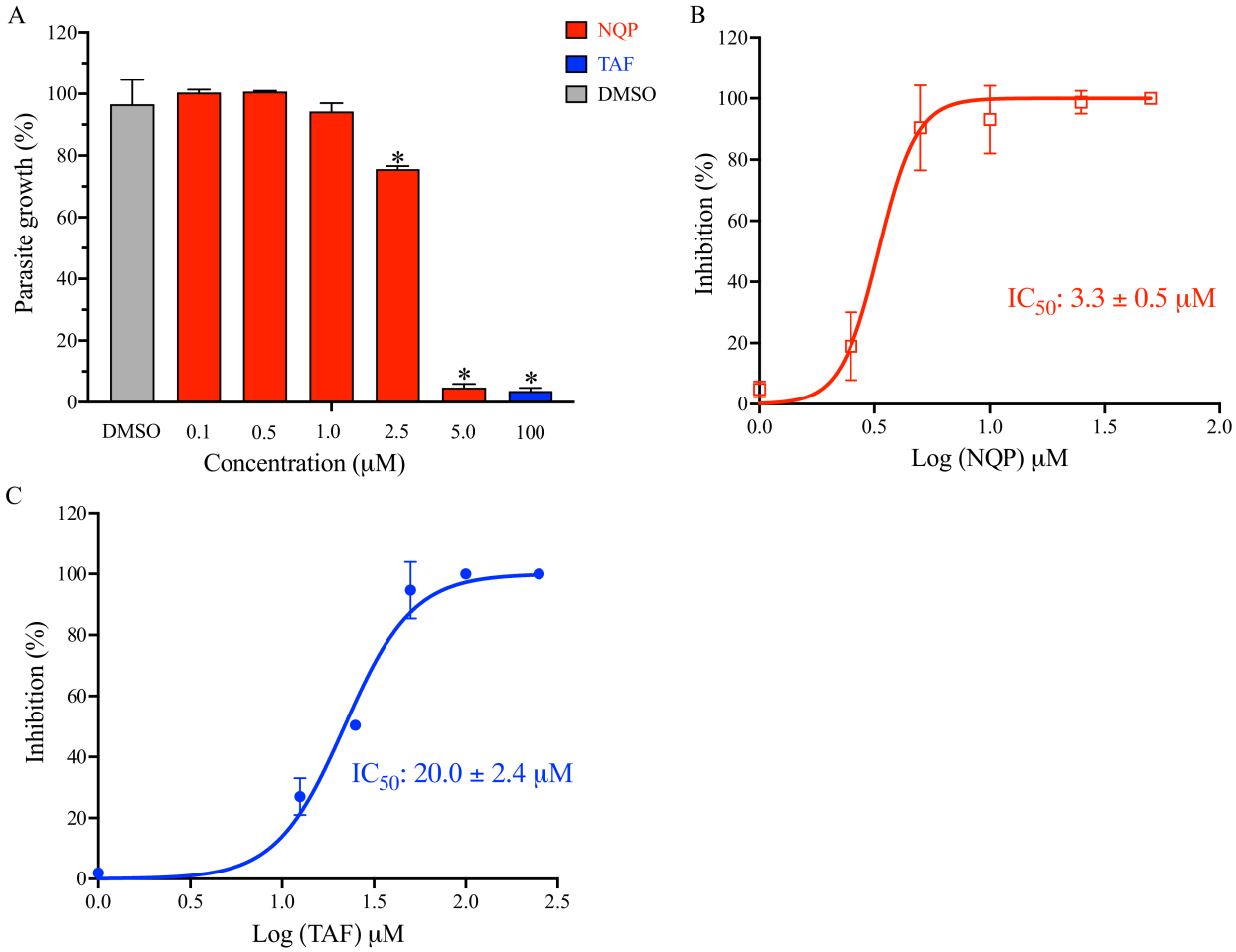

Fig. 2. The *in vitro* growth inhibitory effect of naphthoquine phosphate (NQP). (A) NQP inhibits growth of *B. gibsoni in vitro*. The grey column represents the DMSO-treated culture as a drug solvent control; the red columns represent the NQP-treated cultures; and the blue column represents 100 µM tafenoquine (TAF)-treated culture. (B) Dose-dependent inhibition curve of NQP on *B. gibsoni in vitro*. (C) Dose-dependent inhibition curve of TAF on *B. gibsoni in vitro*. Each value represents the mean ± standard deviation (SD) of three independent experiments carried out in a triplicate. The asterisks indicate a significant difference ($P < 0.05$) between the drug-treated cultures and the DMSO-treated culture.

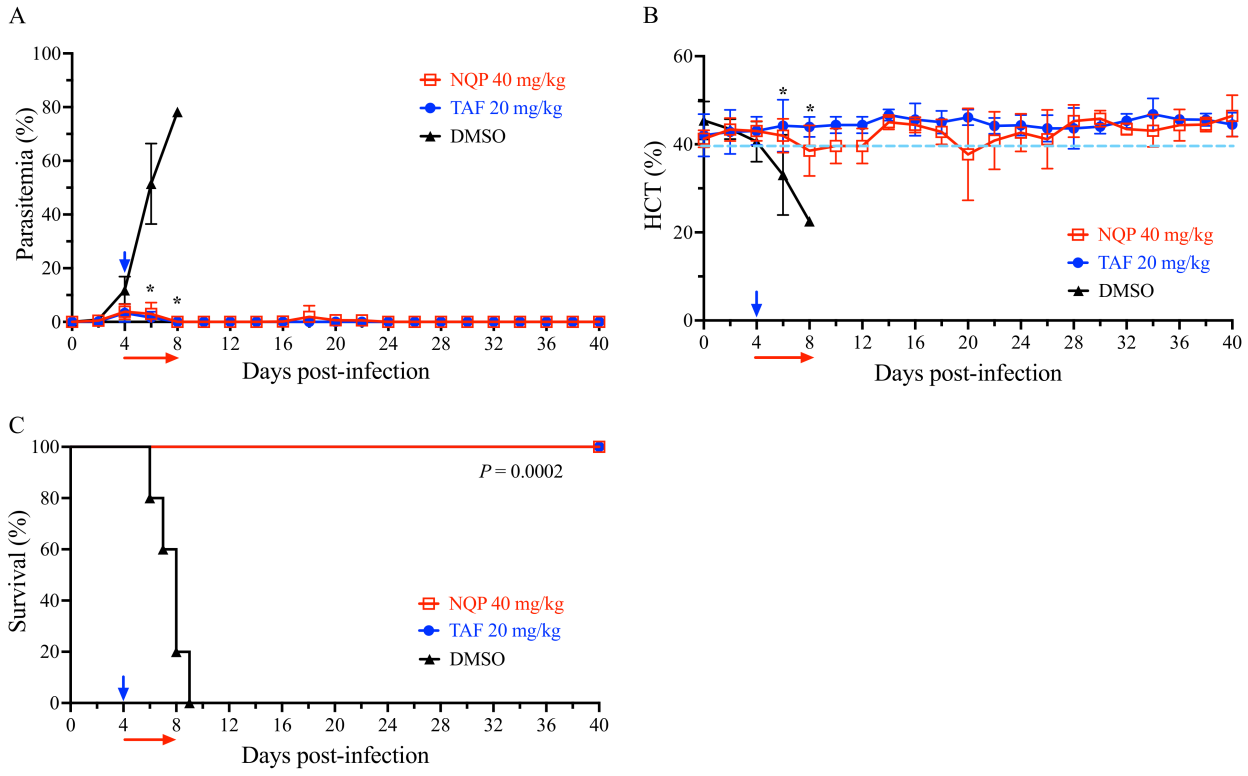

Fig. 3. The growth inhibitory effect of NQP on *B. rodhaini* in BALB/c mice. (A) NQP and TAF prevent the typical growth of *B. rodhaini* in mice as compared with that in DMSO-treated mice as a drug solvent control. (B) Changes of hematocrit (HCT) values in mice treated with NQP or TAF as compared with that in DMSO-treated mice. The asterisks indicate a significant difference ($P < 0.05$) between the NQP- or TAF-treated group and the DMSO-treated group. (C) Survival rates of NQP-, TAF-, and DMSO-treated mice. The arrows indicate time of treatment. Parasitemia was calculated by counting infected RBCs among 3,000 RBCs using Giemsa-stained blood smears. Dotted line indicates reference range.

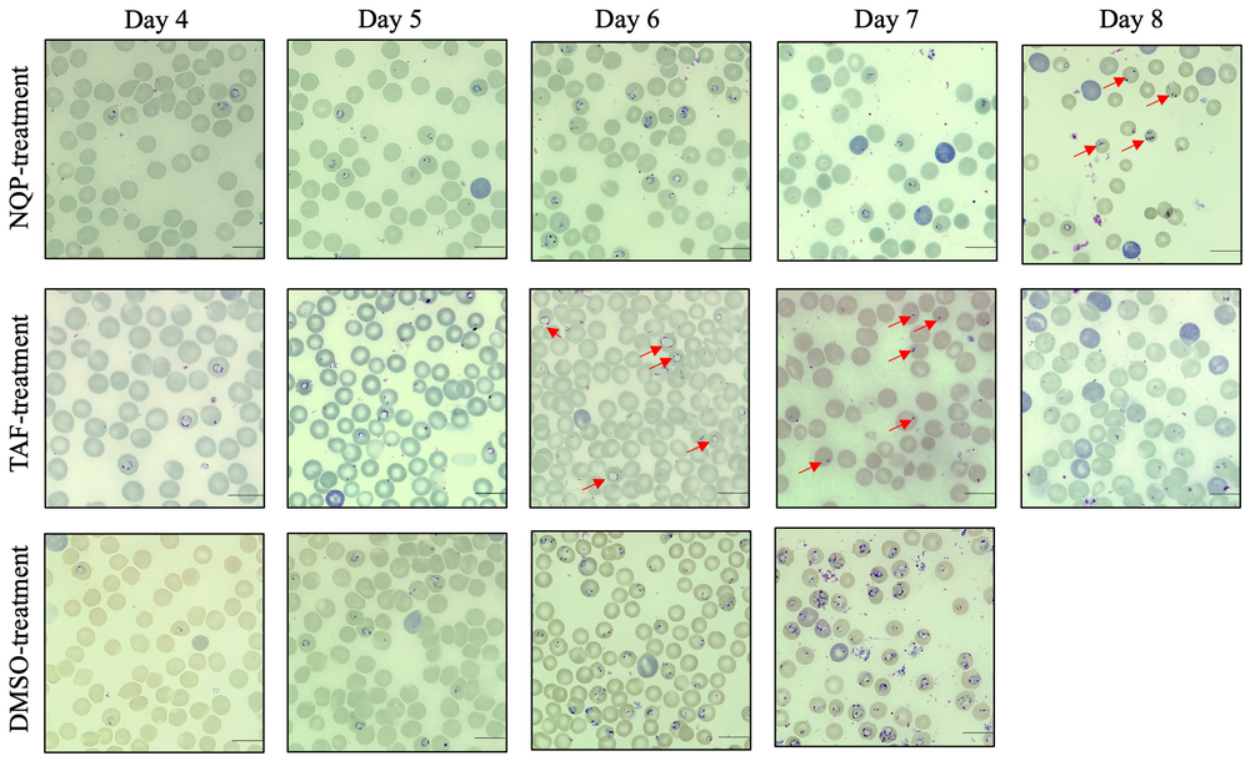

Fig. 4. Light micrographs of *B. rodhaini*-infected mice during NQP and TAF treatment (from 4 to 8 DPI) and of DMSO-treated mice (from 4 to 7 DPI). Compared with the DMSO-treated group, NQP treatment exhibits degenerated parasites at 8 DPI (red arrow), whereas parasites in the TAF-treated mice show a vacuole-like aberrant phenotype. Bars = 10 μm.

Chapter 2

Efficacy of the antimalarial MMV390048 against *Babesia* infection reveals phosphatidylinositol 4-kinase as a druggable target for babesiosis

2.1. Introduction

Babesiosis is a zoonotic disease caused by the tick-transmitted *Babesia* species, leading to huge economic losses and posing a serious health risk worldwide (Schnittger et al., 2013). Most species responsible for *Babesia* infections are host-specific, and over 100 *Babesia* species have the ability to infect many types of mammalian hosts, including domestic animals and human (Homer et al., 2000). With the rapid increase in the number of human babesiosis cases caused by *Babesia microti* reported over the past years, particularly in the northeastern and northern midwestern regions of the United States (U.S.) (Krause et al., 2019), babesiosis has been attracting interest as an emerging zoonosis (Homer et al., 2000).

Phosphatidylinositol 4-kinase (PI4K) is a ubiquitous eukaryotic lipid kinase and is involved in the synthesis of phosphatidylinositol 4-phosphate (PI4P), a member of the phosphoinositide family (Boura and Nencka, 2015). Phosphatidylinositol 4-phosphate is involved in the architecture of Golgi apparatus and trans-Golgi network, as well as regulates trafficking to and from the Golgi (McNamara et al., 2013). It plays a key role in

the synthesis of membrane polyphosphoinositides and regulation of multiple cellular functions (Godi et al., 2004; De Matteis et al., 2013). PI4Ks are classified as two classes based on enzymatic differences: type II (PI4KII) and III (PI4KIII). Each class contains α and β isoforms (Delang et al., 2012). Recently, *Plasmodium* PI4K (*Pf*PI4K) type III β was proven to be a promising druggable target for eliminating malaria (McNamara et al., 2013). So far, three novel anti-*Plasmodium* compound classes targeting *Pf*PI4K were reported to inhibit the multiple-stage development of *Plasmodium*, namely imidazopyrazine, quinoxaline, and 2-aminopyridine (McNamara et al., 2013; Paquet et al., 2017; Sternberg and Roepe, 2020). MMV390048 (Fig. 5A), also known as MMV0048, is a representative of 2-aminopyridine class and was developed based on the hits of a phenotypic high-throughput screen from the commercial BioFocus library (Younis et al., 2012). A previous report showed that MMV390048 could inhibit all *Plasmodium* life cycle stages except hypnozoites in the late liver stage (Paquet et al., 2017). Given the strong inhibitory effect and known action mode on *Plasmodium*, I investigated whether MMV390048 has the same inhibitory effect on *Babesia* species. Therefore, this study aimed to evaluate the efficacy of MMV390048 against *in vitro* growth of *B. gibsoni* and *in vivo* growth of the human babesiosis causative agent, *B. microti* Peabody mjr strain, and a highly pathogenic rodent species, *B. rodhaini*. Likewise, I elucidated the potential mechanisms of MMV390048 activity against *Babesia* parasites.

2.2. Materials and methods

Ethics statement

The protocols performed in the current study were carried out according to the ethical guidelines approved by the Obihiro University of Agriculture and Veterinary Medicine (permit numbers: animal experiment, 21-133; DNA experiment, 1723-5; pathogen, 201712-5).

Chemicals

MMV390048 was purchased from MedChem Express (USA). Atovaquone (ATO), azithromycin (AZI), and tafenoquine (TAF) were purchased from Sigma-Aldrich (Japan). The drugs were dissolved in vehicle (sesame oil, SO) (Sigma-Aldrich, Japan) to make a stock solution of 40 mg/ml and stored at 4°C until use.

Animal and parasites

Six-week-old female BALB/c mice and severe combined immunodeficiency (SCID) mice (CLEA, Japan) were used for the *in vivo* studies.

In vitro cultures of *B. gibsoni* (Oita strain) were used for the experiment. Parasites were grown in canine RBCs suspended with RPMI-1640 containing 20% normal canine serum. The culture was maintained in an atmosphere of 5% CO_2 and 5% O_2. For the *in vivo* study, the frozen stocks of *B. microti* Peabody mjr strain (ATCC® PRA-99™) and *B. rodhaini* Australia strain were thawed and intraperitoneally injected in BALB/c mice. When parasitemia levels were 10-20% in donor mice, the iRBCs were collected and diluted with phosphate-buffered saline (PBS). The *B. microti* challenge infection dose was 10^7 iRBCs in BALB/c mice and 2.0×10^7 in SCID mice, while the *B. rodhaini* dose was 10^7 in BALB/c mice.

In vitro anti-*Babesia* efficacy of MMV390048

The *in vitro* anti-*Babesia* activity was measured using a SYBR Green I (SG1) proliferation assay (Rizk et al., 2020). Briefly, 95 µl medium with various concentrations (100, 50, 25, 10, 5, and 1µM) of MMV390048 were added in triplicate in 96-well plates. Then, follow the procedure as described in Chapter 1 materials and methods.

In vivo anti-*Babesia* efficacy of MMV390048

The efficacy of MMV390048 on *Babesia* infection was evaluated in *B. microti*- and *B. rodhaini*-infected mice. For the treatment of *B. microti*-infected mice, 15 mice were divided in 3 groups. The dosages for treatment were determined based on *in vitro* results and previous reports. Group I (n = 5) was orally treated with 20 mg/kg MMV390048, while group II (n = 5) was orally treated with 20 mg/kg ATO plus 20 mg/kg AZI (Tuvshintulga et al., 2022). As the solvent control, group III (n = 5) was orally treated with 0.2 ml vehicle. All the treatments were given for 7 consecutive days starting at 4 DPI. For the treatment of *B. rodhaini*-infected mice, 15 mice were divided in 3 groups. Group I and III (n = 5) were treated as described above, whereas group II (n = 5) was treated with 20 mg/kg TAF once (Mordue and Wormser, 2019).

For the treatment of *B. microti*-infected SCID mice, 10 mice were divided in 2 groups. Group I (n = 5) was orally treated with 20 mg/kg MMV390048 for 7 consecutive days starting at 4 DPI, while group II (n = 5) was orally treated with 0.2 ml vehicle. Due to the relapse of parasites, the second 7-day treatment was given from day 28. The parasitemia in mice was monitored by examining 3,000 RBCs on Giemsa-stained thin smear slides.

Anemia development was monitored by the change of HCT values using an automated veterinary hematology analyzer (MEK-6550 Celltac α, Nihon Kohden, Japan).

Efficacy of MMV390048 on *Bm*PI4K mutant strain

To isolate the *Bm*PI4K L746S mutant strain, the parasites from the SCID mouse which relapsed after two regimens of MMV390048 was collected and passaged in a naïve SCID mouse. Then, to evaluate the efficacy of MMV390048, TAF, and ATO plus AZI on the *Bm*PI4K L746S mutant strain, twenty-six BALB/c mice were divided in 6 groups: group I and II (n = 3) were infected with *Bm*WT, while group III to VI (n = 5) were infected with *Bm*PI4K L746S mutation strain. Group II and III were orally treated with MMV390048, group I and group IV were orally treated with 0.2 ml vehicle, and group V and VI were treated with TAF and ATO plus AZI, respectively.

Detection of *B. microti* PI4K gene variants

At 60 DPI, the DNA of the relapsed parasites were extracted and sequencing was performed. Briefly, 10 uL blood was collected from the tail vein and diluted in 90 uL phosphate-buffered saline (PBS). Samples were incubated at 100°C for 5 min, then centrifuged at 10,000 rpm for 5 min. The supernatants were collected and used for the following PCR assay. The *B. microti* PI4K gene (3,212 bp) was amplified using KOD FX Neo DNA polymerase (Toyobo, Japan) using the primers listed in Table 1. The final reaction volume of 25 uL consisted of 4.5 µl of double-distilled water, 12.5 µl of 2 × PCR buffer, 5 µl of 2mM dNTP, 0.75 µl of 10 µM forward and reverse primers, 1 µl of DNA sample, and 0.5 µl of KOD FX Neo polymerase. The following thermocycling condition

was used: initial denaturation at 94°C for 2 min; 35 cycles of 98°C for 10 sec denaturation, 55°C (*Bm*PI4K-U-F and *Bm*PI4K-U-R2) and 58°C (*Bm*PI4K-D-F1 and *Bm*PI4K-D-R) for 30 sec annealing, 68°C for 1 min; and the final extension at 68°C for 7 min. The amplicons were purified using the QIAquick PCR purification kit (Qiagen, Germany) and were subjected to Sanger sequencing. The genetic variants of each amplicon were confirmed by alignment to the WT sequence as the reference. The obtained sequence was deposited in GenBank database with accession no. ON191810.

PI4K sequence alignment and homology modeling

B. microti RI strain PI4K (100% identity with Peabody mjr strain) (Genebank: XP_012649395), *B. gibsoni* PI4K (unpublished data), *B. bigemina* PI4K (Genebank: XP_012766582), *B. bovis* PI4K (Genebank: XP_001610874), and human PI4K (Genebank: NP_001185702) were obtained by homology search using *P. falciparum* PI4K (Genbank: XP_001351656). Sequence alignment was done using MUSCLE and analyzed using Jalview v2.8 software. The three-dimensional structures prediction of *Bm*PI4K was done using AlphaFold as described by Jumper et al., 2021. The docking poses were generated by Smina (Trott and Olson, 2010). The molecular docking results of MMV390048 with *Bm*PI4K and hydrogen bonds as well as van der Waals interaction were evaluated using Discovery Studio 3.5 Visualizer.

Statistical analysis

Data analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., USA). The differences in parasitemia and HCT values between the control and the treated group

were analyzed using one-way ANOVA test. Survival rates were calculated using the Kaplan–Meier method, with regard to the log-rank test. A P value < 0.05 was considered statistically significant.

2.3. Results

Inhibitory efficacy of MMV390048 on *B. gibsoni in vitro* and *B. microti in vivo*

The *in vitro* activity of MMV390048 against *B. gibsoni* showed a steep inhibition curve with a half inhibitory concentration (IC_{50}) value of 6.9 ± 0.9 μM (Fig. 5B). In *B. microti*-infected BALB/c mice, parasitemia significantly increased in vehicle-treated control group and reached the highest parasitemia (average 30.5%) at 8 days post-infection (DPI) (Fig. 5C). In contrast, the parasites were significantly inhibited by MMV390048 and ATO plus AZI (P < 0.05). The peak parasitemia of MMV390048-treated and ATO plus AZI-treated group were 3.4% and 4.0%, indicating growth inhibition of 88.9% and 86.9%, respectively, compared with the control group (peak parasitemia 30.5%) (Fig. 5C). However, ATO plus AZI-treated group eventually relapsed and maintained a low parasitemia (< 1%) until 24 DPI, whereas the blood smears of the control group mice were negative from 28 DPI. Hematocrit (HCT) changes were monitored as the index for anemia in *B. microti*-infected mice. There were no significant HCT reduction noted in MMV390048-treated and ATO plus AZI-treated groups. In contrast, significant HCT reductions were observed in vehicle-treated group from 8 DPI to 28 DPI (P < 0.05) (Fig. 5D), indicating that MMV390048 treatment could block the development of anemia on *B.*

microti-infected mice.

Inhibitory efficacy of MMV390048 on *B. rodhaini*-infected mice

To confirm if MMV390048 has the ability to inhibit the growth of other *Babesia* species, the lethal *B. rodhaini* was used for further study. In the vehicle-treated group, mice infected with *B. rodhaini* showed high parasitemia (82%) (Fig. 6A) and severe anemia. The *B. rodhaini*-infected mice died within 10 DPI (Fig. 6B), whereas administration of MMV390048 or TAF inhibited the rapid growth of parasite, which prevented anemia development and with no animals succumbing to lethal *B. rodhaini* infection. Moreover, relapse was observed in TAF-treated group at 16 DPI and parasitemia was eliminated eventually by immunity, similar with previous reports (Liu et al., 2021).

Identification of a *B. microti* PI4K mutation in a MMV390048-treated immunodeficient mouse with relapsed babesiosis

Following the promising results described above, MMV390048 was further evaluated in *B. microti*-infected severe combined immunodeficiency (SCID) mice with twofold higher inoculum (2×10^7 infected red blood cells, iRBCs) to test a more rapidly progressive babesiosis model. In the vehicle-treated mice, the highest parasitemia reached 40 to 50% at 10-14 DPI (Fig. 7A), whereas treatment with MMV390048 prevented the rise of parasitemia, with no parasites observed on blood smears starting 8 DPI (Fig. 7B). However, the first 7-day treatment of MMV390048 failed to prevent relapse. Notably, two (MMV390048 #1 and MMV390048 #4) of five MMV390048-treated mice relapsed at 26 DPI. Thus, a second 7-day treatment was given to all mice, but an unexpected relapse in

MMV390048 #2 mouse at 52 DPI, with increased parasitemia reaching 18.9%, was observed (Fig. 7B). As the PI4K mutation in *P. falciparum* confers resistance to MMV390048 (McNamara et al., 2013; Paquet et al., 2017), I confirmed if this was also present in the SCID mouse (MMV390048 #2) that had re-emergent parasitemia by sequencing the *B. microti* PI4K (*Bm*PI4K; Genebank: XP_012649395), a homologue of *Pf*PI4K obtained by BLASTP search. A single nucleotide variant (SNV) of *Bm*pi4k with a substitution at position 2,237 (from T to C) was found, which led to a non-synonymous coding change from leucine to serine (L746S) (Fig. 7C and D).

B. microti PI4K L746S as the resistance-conferring mutation against MMV390048 action

The *BmP*I4K L746S mutant strain was isolated and passaged to a donor SCID mouse subjected to uninterrupted monotherapy with MMV390048. Then, BALB/c mice were infected with either the purified *Bm*PI4K mutant isolate or the *B. microti* wild-type (*Bm*WT) to confirm the association of the L746S mutation with *B. microti* resistance to MMV390048. Consistent with the above results, *Bm*WT-infected mice treated with MMV390048 exhibited decreased parasitemia compared with the vehicle-treated group (Fig. 8A). On the other hand, the *Bm*PI4K L746S mutation resulted in ineffectiveness of MMV390048 against parasite growth (Fig. 8B). Meanwhile, TAF-treated and ATO plus AZI-treated groups showed significant inhibition on *Bm*PI4K L746S mutant strain (Fig. 8B). The morphological changes of parasites in all groups are shown in Fig. 8C. MMV390048-treated *Bm*WT showed severe degenerative changes at 6 DPI (Fig. 8C) compared with vehicle-treated *Bm*WT. At 6 DPI, TAF-treated *Bm*PI4K L746S mutant parasites showed

abnormal morphology, as described in previous reports (Liu et al., 2021), whereas ATO plus AZI treatment caused severe degeneration of *Bm*PI4K L746S mutant parasites. No significant morphological changes were observed in MMV390048-treated *Bm*PI4K L746S mutant parasites compared with vehicle-treated parasites.

Multiple sequence alignment of *Babesia* PI4K and molecular docking study

The full-length amino acid sequence of *Bm*PI4K shared identity values of 41.3%, 62.8%, 59.0%, 60.5%, 44.3% with human (Genebank: NP_001185702), *P. falciparum* (Genbank: XP_001351656), *B. bovis* (Genebank: XP_001610874), and *B. bigemina* (Genebank: XP_012766582), and *B. gibsoni* (unpublished data) PI4K, respectively. The results revealed that PI4K is evolutionarily conserved across *Babesia* species, especially the C-terminal of PI4K (Fig. 9A). The kinase domain of *Pf*PI4K (ranging from residues 1,261 to 1,559) was assigned in a previous report (McNamara et al., 2013). Herein, I predicted the structure of the catalytic domain of *Bm*PI4K and performed molecular docking with MMV390048 (Fig. 9B). The docking model showed that the MMV390048 is embedded in the kinase domain of *Bm*PI4K and positioned in the binding pocket (Fig. 9B). Likewise, the hydrogen bonds (H-bonds) between MMV390048 and *Bm*PI4K at residues K741, L790 and S795 were predicted, whereas, the mutation site found in this study (L746) showed van der Waals interaction with the MMV390048 (Fig. 9C).

2.4. Discussion

MMV390048 is a new compound that inhibits *Plasmodium* development by targeting

*Pf*PI4K. A previous clinical trial of MMV390048 demonstrated the promise of this compound as a single drug for *Plasmodium* treatment (McCarthy et al., 2020). Such results provide ideas for screening or developing new compounds for treating babesiosis. Indeed, this study confirmed that MMV390048 showed potent inhibition against *Babesia* species. The IC_{50} value of MMV390048 against *B. gibsoni in vitro* was 6.9 ± 0.9 μM (Fig. 5B), which is lower than TAF (IC_{50} = 20 ± 2.4 μM) (Result in Chapter 1), but higher than the IC_{50} value of ATO (IC_{50} = 89.0 ± 17.3 nM) (Matsuu et al., 2004). Likewise, the current MMV390048 IC_{50} value was higher compared with the IC_{50} value against the intraerythrocytic stage of *P. falciparum* NF54 drug-sensitive strain (28 nM) (Paquet et al., 2017). Although *Plasmodium* and *Babesia* are closely related, some variation in its features, such as solute permeability of infected erythrocytes, may have resulted in lower sensitivities of the *Babesia* species to drugs compared with the *Plasmodium* species (Alkhalil et al., 2007). A previous study showed that peak concentrations (Cmax) of MMV390048 reached 5.4 μg/ml (13.7 μM) in mouse plasma and 3.9 μg/ml (9.9 μM) in monkey plasma after a single oral dose administration of 20 mg/kg (Paquet et al., 2017). In another clinical trial, the peak Cmax of MMV390048 was 1.1 μg/ml (2.8 μM) in humans after a single oral dose administration of 120 mg (McCarthy et al., 2020, Sinxadi et al., 2020). Therefore, the recommended dose for treating human babesiosis should be higher than 120 mg to reach a high plasma concentration, which is expected to eventually eliminate parasites. Nonetheless, the ensuing safety issues should also be considered.

In the current study, the *in vivo* inhibitory effects of MMV390048 on *B. microti* were comparable with those of ATO plus AZI, the drug treatment recommended by the U.S. CDC

and anemia development was significantly prevented (Vial and Gorenflot, 2006). In subsequent experiments, mice infected with the lethal *B. rodhaini* showed a high parasitemia of 82% and died within 10 DPI, whereas MMV390048 effectively inhibited rapid growth of the parasite, thereby protecting the mice from death. Tafenoquine was more effective than MMV390048 as only one dose is required. Despite this, the limitation of TAF use is its risk of inducing severe hemolytic anemia in some glucose-6-phosphate dehydrogenase-deficient (G6PD-deficient) patients (Peters and Van Noorden, 2009), whereas MMV390048 is deemed safe as a treatment for G6PD-deficient patients (Paquet et al., 2017). The first-line drug for babesiosis treatment is ATO plus AZI. A recent report showed that *B. microti*-infected SCID mice consecutively treated with ATO plus AZI relapsed on 28 DPI with no response to the subsequent treatment (Tuvshintulga et al., 2022). Indeed, acquired drug resistance has been reported in immunocompromised patients, whom will experience a relapse of disease if initial antibabesial therapy failed (Krause et al., 2008). In the present study, I used *B. microti*-infected SCID mice to evaluate the effect of MMV390048 on immunocompromised host. *Babesia microti* was potently inhibited by MMV390048 after a 7-day treatment, but parasite recurrence was observed after two weeks, prompting us to give a second 7-day treatment. Although the twice-given treatment did not achieve radical cure of babesiosis, tolerance to MMV309948 was better than ATO plus AZI. Moreover, subsequent experiments proved that the potential drug target of MMV390048 was *Bm*PI4K.

In this study, a single nucleotide mutation was detected in relapsed *B. microti* infection by sequencing the *Bm*PI4K gene, and a non-synonymous coding change of *Bm*PI4K L746S

resulted in less sensitivity of parasites to MMV390048. In the case of *P. falciparum*, whole genome sequencing of the MMV390048-resistant strain revealed that the *Pf*PI4K S743T or A1319V mutation conferred the resistance to the parasites (Paquet et al., 2017). Moreover, the H1484Y and S1320L mutations in *Pf*PI4K also conferred some degree of cross-resistance to MMV390048. The *Bm*PI4K L746S mutation was characterized by parasite growth and morphology phenotypes similar to those of the wild-type parasites after MMV390048 treatment. On the other hand, the *Bm*PI4K L746S mutation did not affect the inhibitory effects of other antibabesial drugs such as TAF and ATO plus AZI on parasites, further validating that the mutation in the *Bm*PI4K renders MMV390048 inefficacious against *B. microti* infection. Altogether, similar as in *P. falciparum*, these data demonstrate that the PI4K is the target of MMV390048 action in *B. microti*.

PI4K is ubiquitous in eukaryotes and it regulates intracellular signaling and trafficking via phosphorylation of lipids (McNamara et al., 2013). Kinase domain alignment of PI4K orthologues showed high identity among the different *Babesia* species, and with its human orthologue. However, MMV390048 had been proven to have no affinity with human kinase, apart from human PIP4K2C (uniprot: Q8TBX8), ATM (uniprot: Q13315), and TNIK (uniprot: Q9UKE5). Human PIP4K2C is the main target of MMV390048 and its inhibition on the host is unknown (Paquet et al., 2017). A docking study explained that the most significant difference in the binding cavities between HuPI4K and *Pf*PI4K is the change of Q606 in HuPI4K to S1365 in *Pf*PI4K and that the sidechain of HuPI4K Q606 clashes with the CF3-pyridyl of MMV390048, resulting in low potency of MMV390048 to HuPI4K (Fienberg et al., 2020). However, this conflict does not appear to exist in *Babesia* species

as evidenced by the different residues and significant inhibition on parasites. In addition, I observed that the residue L746 was spatially close to the K741 which generates H-bonds and contributes to anchor the MMV390048 onto the binding pocket. Hence, I speculate that the *Bm*PI4K L746S mutation may affect the H-bonds between K741 and MMV390048 to prevent compound binding to the pocket. Obviously, *Bm*PI4K plays a key role for parasite survival and could develop resistance under drug pressure. This resistance confers some degree of fitness advantage for parasite survival but also may incur a fitness cost (Mackinnon and Read, 2004). However, I did not observe prominent difference on parasite growth or virulence between *Bm*PI4K L746S mutant strain and *Bm*WT strain. Therefore, this mutation may not be enough to markedly affect catalytic function.

2.5. Summary

Despite the excellent inhibition shown by MMV390048 on *Babesia* spp., there are some limitations in the present study. For instance, the twice 7-day regimen of MMV390048 did not eradicate *Babesia* infection in immunocompromised mice and did not halt the emergence of resistant parasites, suggesting that evaluating various reasonable modes of administration and combination with currently used drugs are necessary. Collectively, my results demonstrate that MMV390048 is a promising drug for babesiosis treatment and *Babesia* PI4K is a druggable target for babesiosis.

Table 1. List of primers used in this study

Primers	Sequence (5’ ⟶ 3’)
*Bm*PI4K-U-F	ATGACAAGCGGATTAGATGAAAAT
*Bm*PI4K-U-R1	CAAACGTACTGAAGAAGAGATCCAC
*Bm*PI4K-U-R2	GCGTAAATTACTGACAAGGAACTC
*Bm*PI4K-D-F1	AACGAGCATCTGAATCGTCTAAAAC
*Bm*PI4K-D-F2	GAATATGTTAATAGACACCAATGGG
*Bm*PI4K-D-R	GCGTATAACTAATGGTATACGTTGA

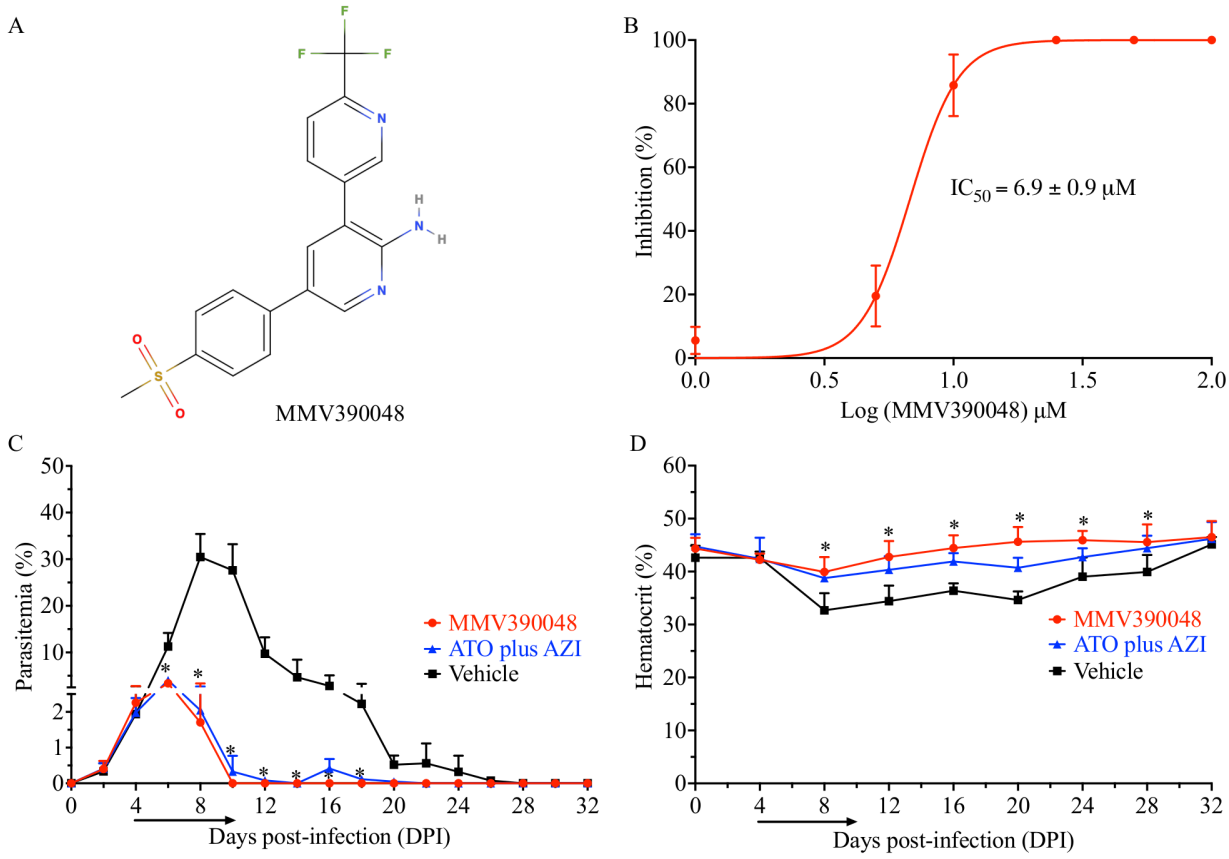

Fig. 5. MMV390048 demonstrates potent inhibition on *B. gibsoni in vitro* and *B. microti in vivo*. (A) Chemical structure of MMV390048. (B) Dose-dependent inhibition curve of MMV390048 on *B. gibsoni in vitro*. Each value represents the mean ± standard deviation (SD) of three independent experiments carried out in triplicate. (C) Inhibitory effects of MMV390048 and atovaquone (ATO) plus azithromycin (AZI) on the growth of *B. microti* in BALB/c mice. (D) Changes of hematocrit (HCT) values in mice treated with MMV390048 or ATO plus AZI compared with those of in vehicle-treated mice. The arrows indicate time of treatment. The asterisks indicate a significant difference ($P < 0.05$) between the drug-treated groups and vehicle-treated control group.

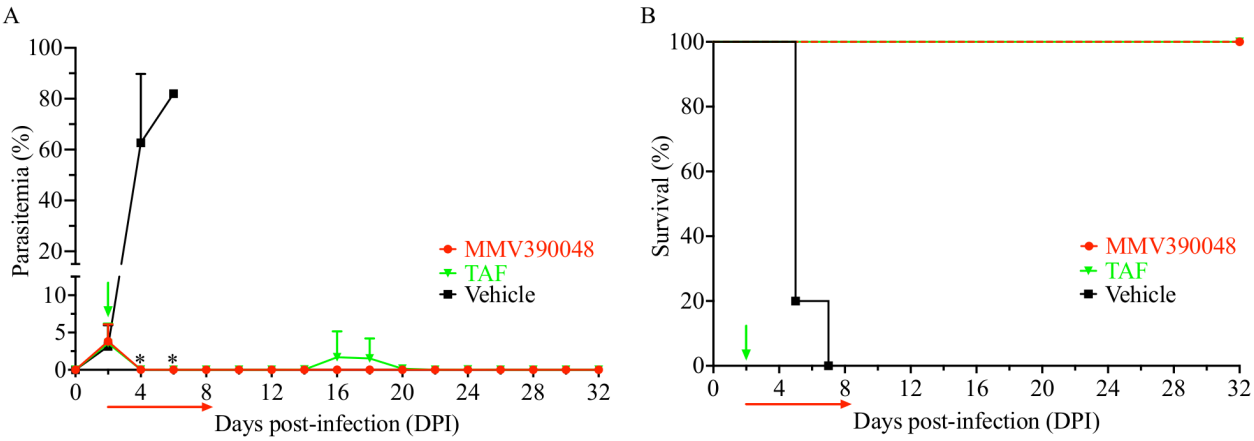

Fig. 6. Efficacy of MMV390048 against lethal *B. rodhaini* infection in BALB/c mice. (A) MMV390048 and tafenoquine prevented *B. rodhaini* growth in mice, compared with vehicle-treated mice. (B) Survival rates of MMV390048-, TAF-, and vehicle-treated mice. The asterisks indicate a significant difference ($P < 0.05$) between the drug treated groups and vehicle-treated group. The arrows indicate time of treatment.

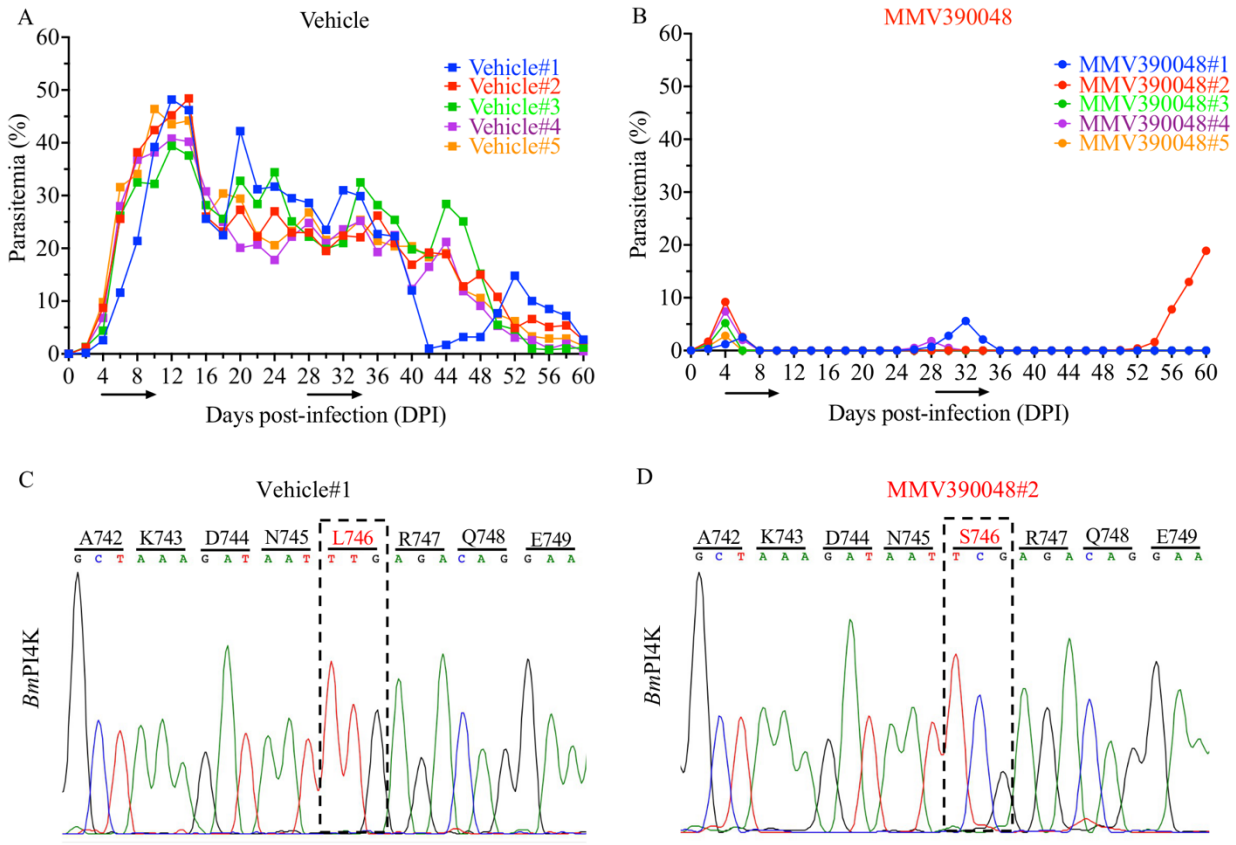

Fig. 7. Efficacy of MMV390048 against *B. microti* in SCID mice and *Bm*PI4K mutation as the target of MMV390048. (A and B) Parasitemia changes of vehicle- or MMV390048-treated *B. microti*-infected SCID mice. The arrows indicate time of treatment. (C and D) Representative sequencing chromatogram of recrudescent parasites from MMV390048-treated *B. microti*-infected mouse. The parasite DNA of MMV390048#2 was extracted from blood sample at 60 days post-infection. The DNA was used to amplify the *B. microti* PI4K gene and sequenced.

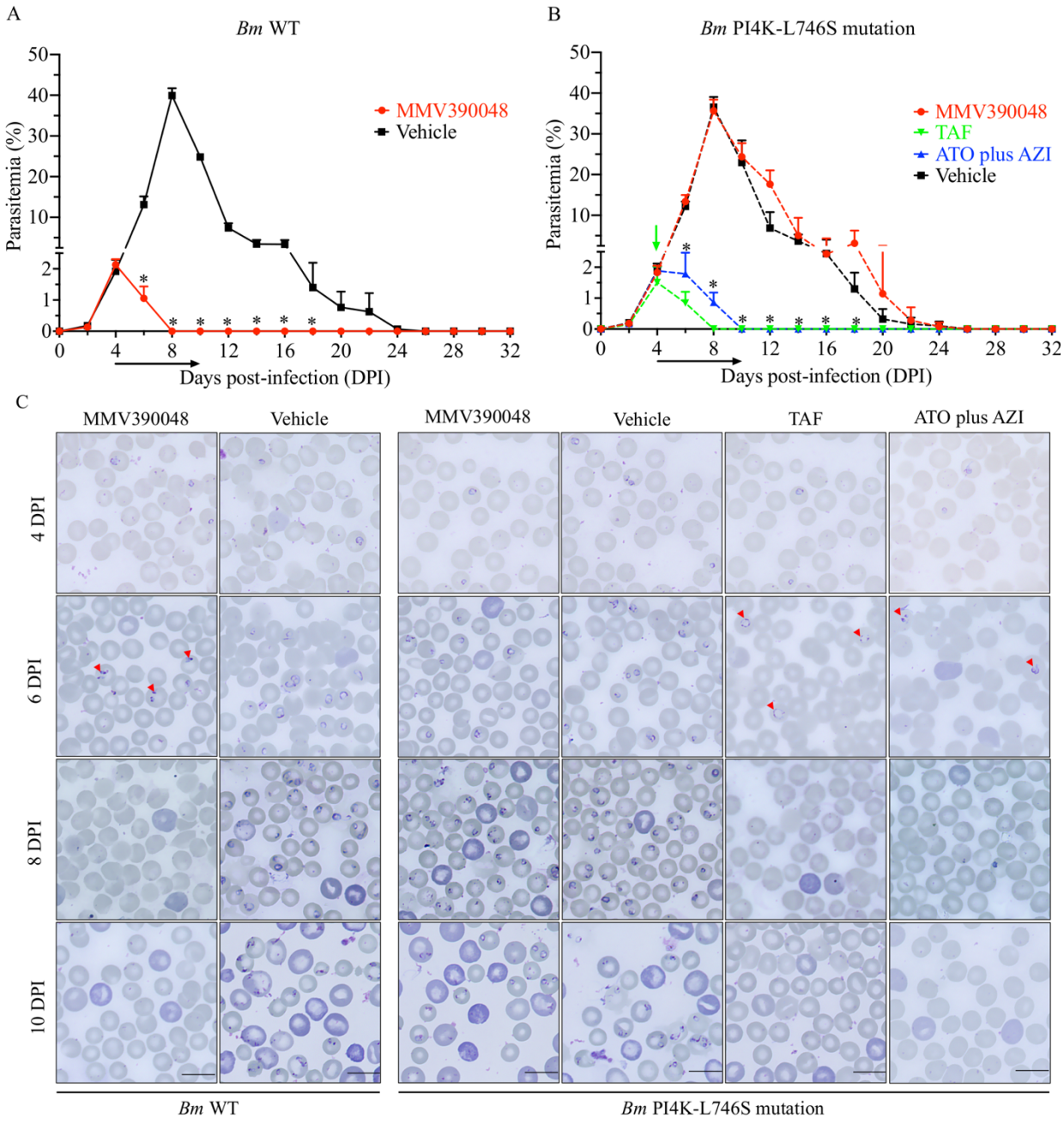

Fig. 8. Efficacy of MMV390048, TAF, and ATO plus AZI against *B. microti* PI4K L746S mutant strain. (A) Growth of *B. microti* wild-type (WT) parasites in vehicle- and MMV390048-treated group (as control groups; n = 3). (B) Growth of *B. microti* PI4K L746S mutant strain in vehicle-, TAF-, ATO plus AZI-, and MMV390048-treated BALB/c

mice (n = 5). The arrows indicate time of treatment. The asterisks indicate a significant difference ($P < 0.05$) between the drug-treated groups and vehicle-treated group. (C) Light micrographs of *B. microti*-infected mice during vehicle and MMV390048 treatment, as well as, light micrographs of *B. microti* PI4K mutant-infected mice during vehicle, TAF, ATO plus AZI, and MMV390048 treatment. Bar = 10 μm.

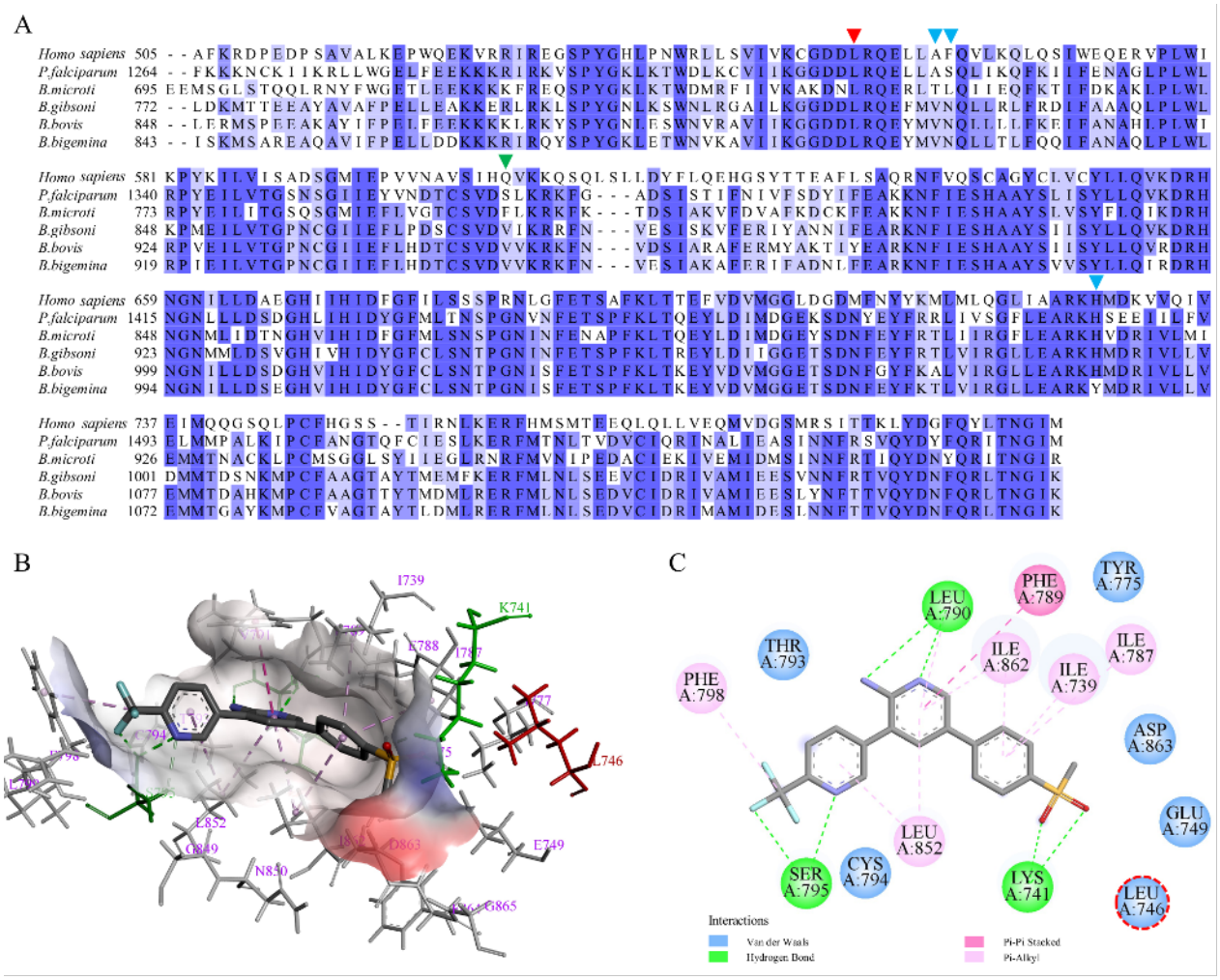

Fig. 9. Multiple sequence alignment of *Babesia* PI4K and molecular docking study. (A) Multiple sequence alignment of PI4K kinase domains. The mutation site of *Bm*PI4K found in this study is indicated by a red inverted triangle, while the sites related with *P. falciparum* drug resistance are marked with blue inverted triangles. The key site that blocks MMV390048 affinity with human PI4K (McCarthy et al., 2020) is labelled with a green inverted triangle. (B) Docking representation of binding modes of MMV390048 to the pocket of *Bm*PI4K. (C) 2D representation of *Bm*PI4K and MMV390048 interactions. The mutation site was labeled in red.

Chapter 3

Phosphatidylinositol 4-kinase is a viable target for radical cure *Babesia microti* infection in immunocompromised hosts

3.1. Introduction

For the time being, the treatment of human babesiosis, recommended by Centers for Disease Control and Prevention (CDC), usually involves the combination of atovaquone (ATO) plus azithromycin (AZI) for 1-2 weeks, which exhibits fewer side effects compared to an alternative combined therapy of clindamycin and quinine (Vannier and Krause, 2012). The treatment period for babesiosis may extend to six weeks or more in severely immunocompromised patients, and *cytochrome b* (*Cytb*) and *ribosomal protein subunit L4* (*RPL4*) mutations were associated with parasite resistance to ATO and AZI, respectively, resulting in treatment failure (Krause et al., 2008; Wormser et al., 2010; Vannier and Krause, 2012; Simon et al., 2017). Therefore, the buildout of new drug candidates or targets is urgently needed for the control and treatment of human babesiosis. Phosphatidylinositol 4-kinase (PI4K) is a ubiquitous eukaryotic lipid kinase involved in the production of phosphatidylinositol 4-phosphate (PI4P) (Li et al., 2021). PI4K has been reported to play a key role in the occurrence and development of cancer, viral infections, and malaria (Li et al., 2021). PI4K not only exhibits the potential for eliminating malaria (McNamara et al.,

2013; Paquet et al., 2017), but also possesses an inhibitory effect against *Babesia* species, as described in Chapter 2. The 2-aminopyridine MMV390048, a representative of a new chemical class of *Plasmodium* PI4K inhibitor (Paquet et al., 2017), showed potent inhibition against *B. gibsoni in vitro*, and against *B. rodhaini* and *B. microti in vivo*. However, twice-administered, short-time treatment did not eliminate the parasite from immunocompromised hosts. Hence, the purpose of this study was to test whether uninterrupted treatment targeting *B. microti* PI4K could eradicate *B. microti* infection in immunocompromised hosts.

3.2. Materials and methods

Ethics statement

The protocols performed in the current study were carried out according to the ethical guidelines approved by the Obihiro University of Agriculture and Veterinary Medicine (permit numbers: animal experiment, 21-133; DNA experiment, 1723-5; pathogen, 201712-5).

Chemicals

MMV390048, sesame oil (SO), atovaquone (ATO), and azithromycin (AZI) were purchased from Sigma-Aldrich (Japan). MMV390048, ATO, and AZI were dissolved in SO to prepare a stock solution with a concentration of 40 mg/ml and kept at 4°C before use. KOD FX Neo DNA polymerase was purchased from Toyobo (Japan). The Big Dye Terminator v3.1 cycle sequencing kit was purchased from Applied Biosystems (Japan).

Parasites and mice

Babesia microti Peabody mjr strain (ATCC® PRA-99™) was purchased from ATCC and stocked in our laboratory. For the maintenance of *B. microti*, cryopreserved parasitized RBCs were passaged by intraperitoneal (i.p.) injection in donor mice. Six-week-old female severe combined immunodeficiency (SCID) mice and BALB/c mice were purchased from CLEA (Japan) and used for *in vivo* studies.

Mouse infection and drug administration

To confirm the efficacy of uninterrupted treatment targeting PI4K, 15 SCID mice were randomly divided equally into 3 groups and intraperitoneally injected with 10^7 *B. microti*. Blood smears were prepared every other day, and the hematocrit (HCT) was measured every four days. Treatment was initiated at 4 days post-infection (DPI) when mouse parasitemia is ~1%. This timeline was followed since early clinical manifestations are observed when parasitemia exceeds 1% in babesiosis patients (Akel and Mobarakai, 2017). Daily treatment with 20 mg/kg MMV390048, 20 mg/kg ATO plus 20 mg/kg AZI, and 0.2 ml vehicle (sesame oil) was given orally to each group, respectively. These treatments were discontinued when mice tested negative by PCR detection of *B. microti* 18S ribosomal RNA (18S rRNA) gene. To isolate the *B. microti Cytb* mutant strain, parasites from the SCID mouse treated with ATO plus AZI were collected and passaged in a donor SCID mouse. To evaluate the efficacy of PI4K inhibitor on ATO-resistant parasites, 15 BALB/c mice were randomly divided equally into 3 groups and intraperitoneally injected with 10^7 *B. microti Cytb* mutant strain. A 7-day treatment was given to mouse groups as described above and the parasitemia and HCT levels were monitored.

Detection of *B. microti* 18S rRNA gene and surveillance of gene variants

Blood samples were collected from the tail vein and were diluted in PBS, followed by incubation at 100°C for 5 min. After incubation, the samples were centrifuged at 10,000 rpm for 5 min and the supernatants were collected and used for detection. To rule out false negative results, the samples were checked using Qubit™ 1 × dsDNA BR assay kit (Thermo Fisher Scientific, Japan) and Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Japan) before running the PCR assay to ensure that genomic DNA was present. Detection of the 18S rRNA gene started 16 DPI and was used to evaluate whether the parasites were cleared from the SCID mice. Gene amplification was performed following a previously described protocol (Persing et al., 1992). The *Cytb* and *RPL4* mutations were determined by Sanger sequencing (Tuvshintulga et al., 2022). The obtained sequence was aligned with the wild type sequence. A genetic variant was detected in *Cytb* gene and deposited in GenBank database with accession no. ON815034.

Statistical analysis

The parasitemia and HCT were performed using GraphPad Prism 8. The differences between control and treated groups were analyzed using one-way analysis of variance. A *P* value of < 0.05 was considered to be statistically significant.

3.3. Results

Radical cure of babesiosis by uninterrupted treatment targeting phosphatidylinositol 4-kinase

Treatment with MMV390048 showed potent efficacy against *B. microti*, evidenced by abated parasitemia from 5 DPI and undetectable parasites in blood smears from 8 DPI (Fig. 10A). Moreover, parasites were no longer detectable by PCR from 64 DPI to 92 DPI (Fig. 11). At 10 DPI, parasites in the vehicle-treated group reached the highest parasitemia (average 60.6%), with a transient and slight decline at 14 DPI, and maintained fluctuating parasitemia until the end of the trial. The mean parasitemia significantly differed between the vehicle-treated and MMV390048-treated groups from 6 DPI (Fig. 10A). In ATO plus AZI-treated group, parasites were initially inhibited until 22 DPI, but the parasitemia rapidly increased from 24 DPI and reached its peak at 32 DPI (average 40.1%) (Fig. 10A). From 30 DPI, the ATO plus AZI was ineffective on parasite growth as no significant difference in parasitemia was observed when compared to the vehicle-treated group. In addition, markedly lower HCT levels were recorded in vehicle-treated and ATO plus AZI-treated groups from 8 DPI and 32 DPI, respectively (Fig. 10B). The *Cytb* and *RPL4* genes were sequenced from relapsed parasites and a single nucleotide variant (SNV) of the *Cytb* gene was detected as a non-synonymous coding change at position 272 (Y272C) (Fig. 12). Meanwhile, there was no mutation detected in the *RPL4* gene.

Inhibitory efficacy of MMV390048 against ATO-resistant *B. microti* strain

The next step was to evaluate the sensitivity of *B. microti Cytb* mutant strain to MMV390048. In the vehicle-treated group, *B. microti Cytb* mutant strain rapidly increased in mice and reached peak parasitemia at 10 DPI (average 36.7%) and lower HCT level was observed from 12 DPI (Fig. 13). As expected, ATO plus AZI was ineffective against the *B. microti Cytb* mutant strain (Fig. 13A). No significant difference in the level of parasitemia

was observed between vehicle and ATO plus AZI-treated groups. In contrast, the growth of *B. microti Cytb* mutant strain and development of anemia was significantly inhibited upon treating mice with MMV390048 (Fig. 13B).

3.4. Discussion

To avoid developing drug resistance, the treatment for human babesiosis usually consists of a two-drug combination, such as ATO plus AZI (Krause et al., 2000). Despite this, acquired drug resistance is well documented in some severe cases in immunocompromised patients (Krause et al., 2008; Wormser et al., 2010; Vannier and Krause, 2012; Simon et al., 2017). Hence, the radical cure of babesiosis remains challenging in severely immunocompromised patients. In the recent past, a few compounds have been reported as promising drugs against human babesiosis, namely endochin-like quinolones (ELQs) (Lawres et al., 2016; Chiu et al., 2021), tafenoquine (Mordue and Wormser, 2019; Liu et al., 2021;), and clofazimine (Tuvshintulga et al., 2022). ELQs showed inhibitory effects against apicomplexan parasites by targeting *Cytb* (Doggett et al., 2012; Stickles et al., 2015). In babesiosis, a 7-day treatment of ELQ-334 plus ATO prevented the recrudescence in the SCID mouse model of *B. microti* infection (Lawres et al., 2016). Similarly, a 10-day treatment of ELQ-502 monotherapy or in combination with ATO resulted in the radical cure of babesiosis with no recrudescence in the mouse model (Chiu et al., 2021). Tafenoquine (TAF) was approved by U. S. Food and Drug Administration (FDA) in 2018 for the radical cure of *Plasmodium vivax* infection and chemoprophylaxis of malaria (Watson et al., 2021). In SCID mice, TAF showed strong

inhibition against *B. microti* infection, evident from the single dose requirement (Mordue and Wormser, 2019; Liu et al., 2021). Tafenoquine treatment in cases of relapsing babesiosis caused by drug-resistant *B. microti* is followed by resolution of parasitemia and symptoms in the patient, demonstrating TAF's excellent effectivity in clinical settings (Marcos et al., 2022; Rogers et al., 2022). However, the use of TAF entails the risk of inducing severe hemolytic anemia in glucose-6-phosphate dehydrogenase-deficient (G6PD-deficient) patients (Peters and Van Noorden, 2009). Clofazimine combined with ATO was also evaluated as a candidate for human babesiosis. Uninterrupted treatment of clofazimine with ATO resulted in the radical cure of *B. microti*-infected SCID mice in 44 days (Tuvshintulga et al., 2022). Phosphatidylinositol kinases (PIKs) are essential in the regulation of cell proliferation, survival, and membrane trafficking (Hassett and Roepe, 2018). Currently, six *P. falciparum* genes are hypothesized to encode PIKs, while in *Babesia* species, these genes are still unidentified (Hassett and Roepe, 2018). In *Plasmodium*, PI4K phosphorylates lipids to regulate intracellular signaling and trafficking, making it a druggable target to eliminate malaria (McNamara et al., 2013). In blood stages, the inhibitor prevents the parasite's development by disrupting plasma membrane ingression around the developing daughter merozoites. *B. microti* PI4K shares an identity value of 62.8% with *P. falciparum* and is highly conserved among *Babesia* species (Result in Chapter 2). Hence, I speculate that the mechanism in *Plasmodium* also applies to *Babesia* species. MMV390048 is an inhibitor of *Plasmodium* PI4K which was under evaluation in human clinical trials (Sinxadi et al., 2020). MMV390048 has potent inhibition against *Babesia* species by targeting PI4K, revealing a promising druggable target (Result in

Chapter 2). In light of this, I further examined if targeting PI4K by monotherapy is sufficient to achieve the radical cure of *B. microti* infection in SCID mice. In this study, I used MMV390048 as an inhibitor for *B. microti* PI4K (*Bm*PI4K) and a 64-day uninterrupted treatment with MMV390048 succeeded in curing babesiosis in *B. microti*-infected SCID mice. Although this therapy was longer than in ELQ-502 (10 days) and clofazimine with ATO (44 days), I have confirmed that PI4K is a promising target for the clearance of parasites. Meanwhile, in ATO plus AZI-treatment group, the parasite established resistance to ATO and AZI, in addition to the development of a single amino acid mutation (Y272C) in the *B. microti Cytb* gene. Y272 is highly conserved among apicomplexan parasites and the site mutation of Y272 will confer a high degree of resistance to the drug (Fisher and Meunier, 2008; Lemieux et al., 2016). Moreover, *B. microti* Y272C mutation has been described in a clinical case and caused treatment failure (Simon et al., 2017). MMV390048 also demonstrated potent inhibition of *B. microti Cytb* mutant strain. Despite MMV390048 exhibiting a good inhibitory effect, *Babesia* tends to be more tolerant than *Plasmodium* when treated with MMV390048. Hence, if MMV390048 were to be used for treating human babesiosis, the recommended dose should be higher than 120 mg (a safety dose test in human clinical trials) (Sinxadi et al., 2020). Moreover, the accompanying safety issues should be considered and future work can focus on developing and designing *Babesia* PI4K-specific inhibitors, as well as the development of MMV390048-combined therapeutics. This will accelerate the development of next-generation anti-babesiosis therapeutics to eliminate *Babesia* infection.

3.5. Summary

In summary, results from the present study revealed that targeting *Bm*PI4K not only suppresses parasite growth but also eradicates parasites in immunocompromised hosts, especially in relapsing infections caused by ATO-resistant *B. microti* strain. Based on the preceding findings, I can conclude that *Bm*PI4K is a feasible and promising drug target for the elimination of *B. microti* infection. This study opens new avenues to rationally design inhibitors with improved drug-like properties against *Babesia* species.

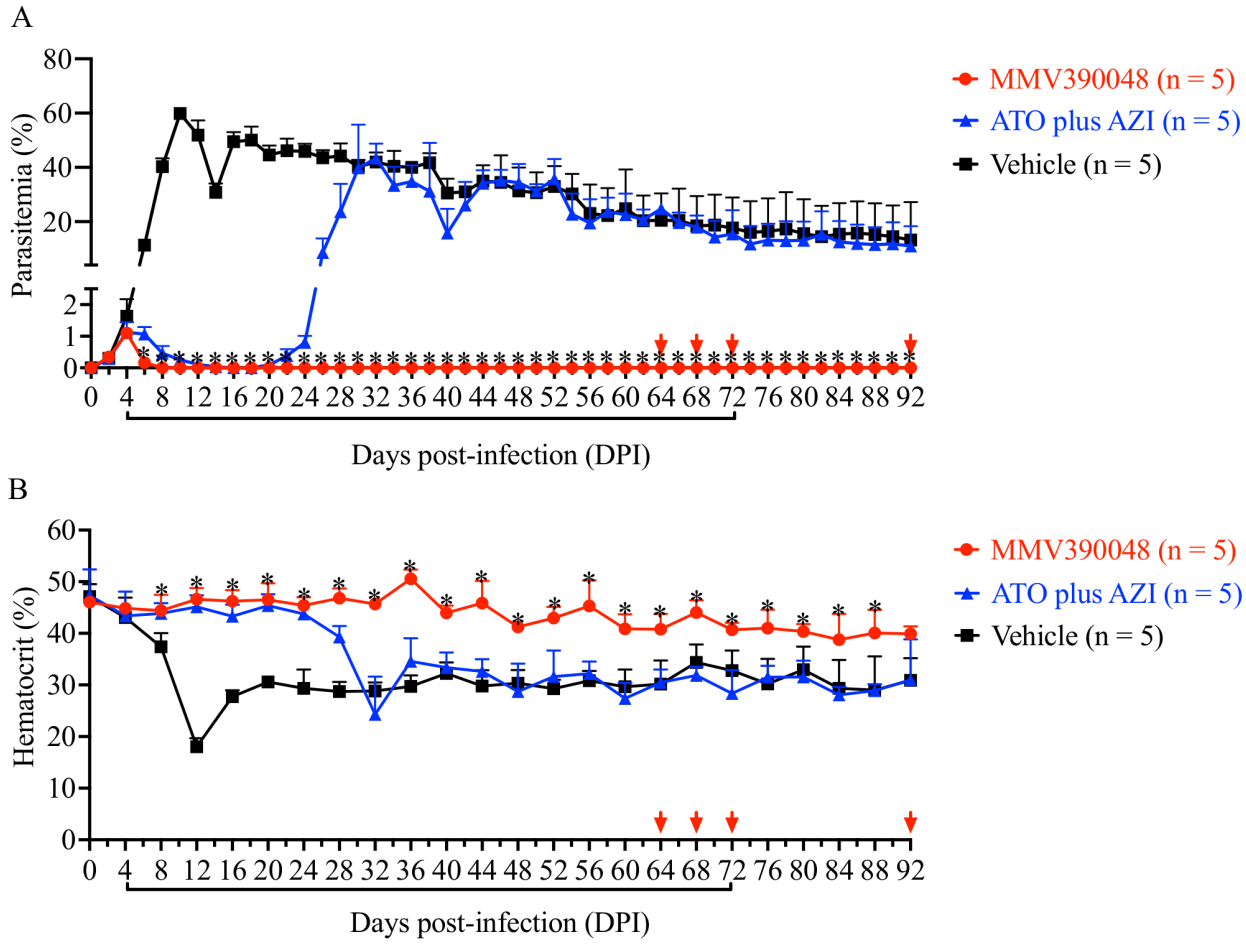

Fig. 10. The efficacy of consecutive treatment with MMV390048 in *Babesia microti*-infected severe combined immunodeficiency (SCID) mice. (A) Course of parasitemia in vehicle-, or atovaquone (ATO) plus azithromycin- (AZI), or MMV390048-treated groups. (B) Hematocrit changes in *B. microti*-infected SCID mice treated with vehicle, or ATO plus AZI, or MMV390048. Treatment was given orally starting from 4 days post-infection (DPI). The black arrows indicate time of treatment and the red arrows indicate testing negative in PCR assay. The asterisks indicate a significant difference ($P < 0.05$) between the drug-treated groups and vehicle-treated group. Parasitemia was calculated by counting infected RBCs among 3,000 RBCs using Giemsa-stained blood smears.

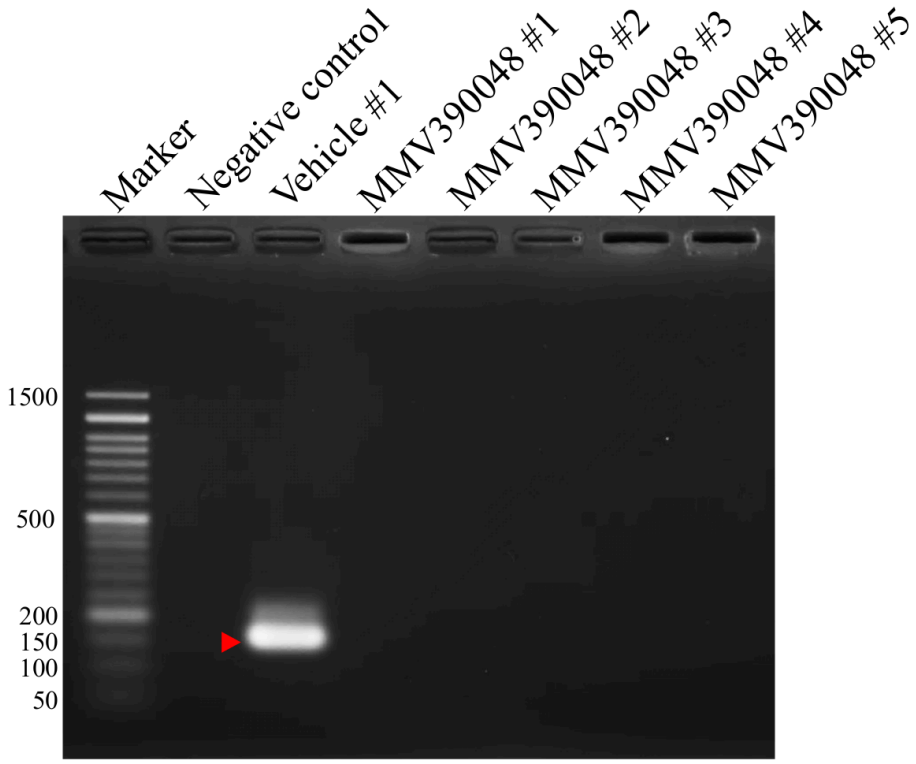

Fig. 11. Molecular detection of parasite DNA in the blood of MMV390048-treated group (#1-5) at 92 DPI. M indicates the DNA marker; NC indicates negative control from naïve SCID mouse; PC indicates positive control representing blood sample collected from vehicle-treated group. The arrow shows the expected band length of 154 bp for the *B. microti* 18S rRNA gene.

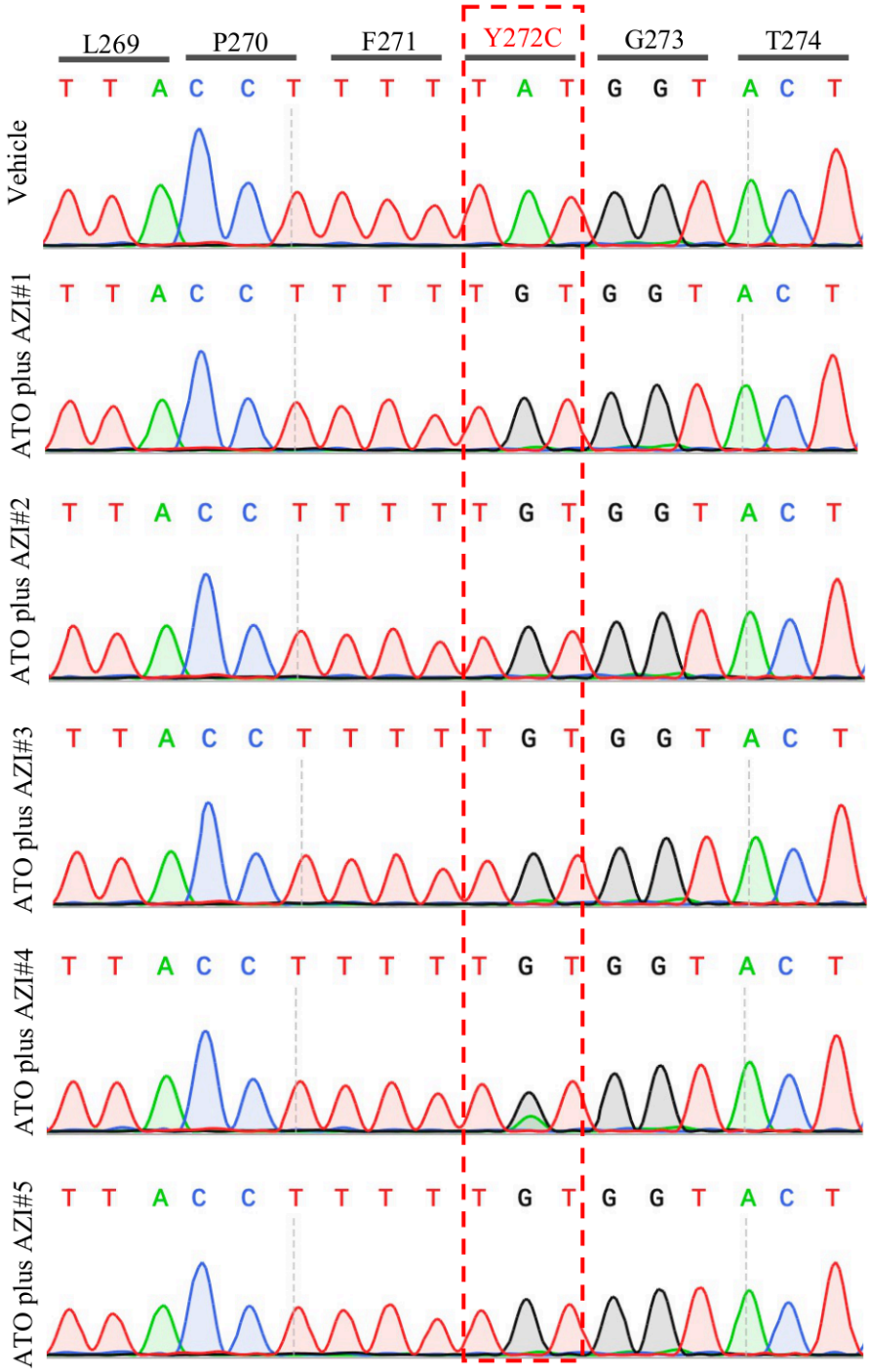

Fig. 12. Representative sequencing chromatogram of *cytochrome b* (*Cytb*) gene in recrudescent parasites. The parasite DNA of vehicle #1 and ATO plus AZI #1 were extracted from a blood sample at 92 days post-infection and was used to amplify and sequence the *B. microti Cytb* gene.

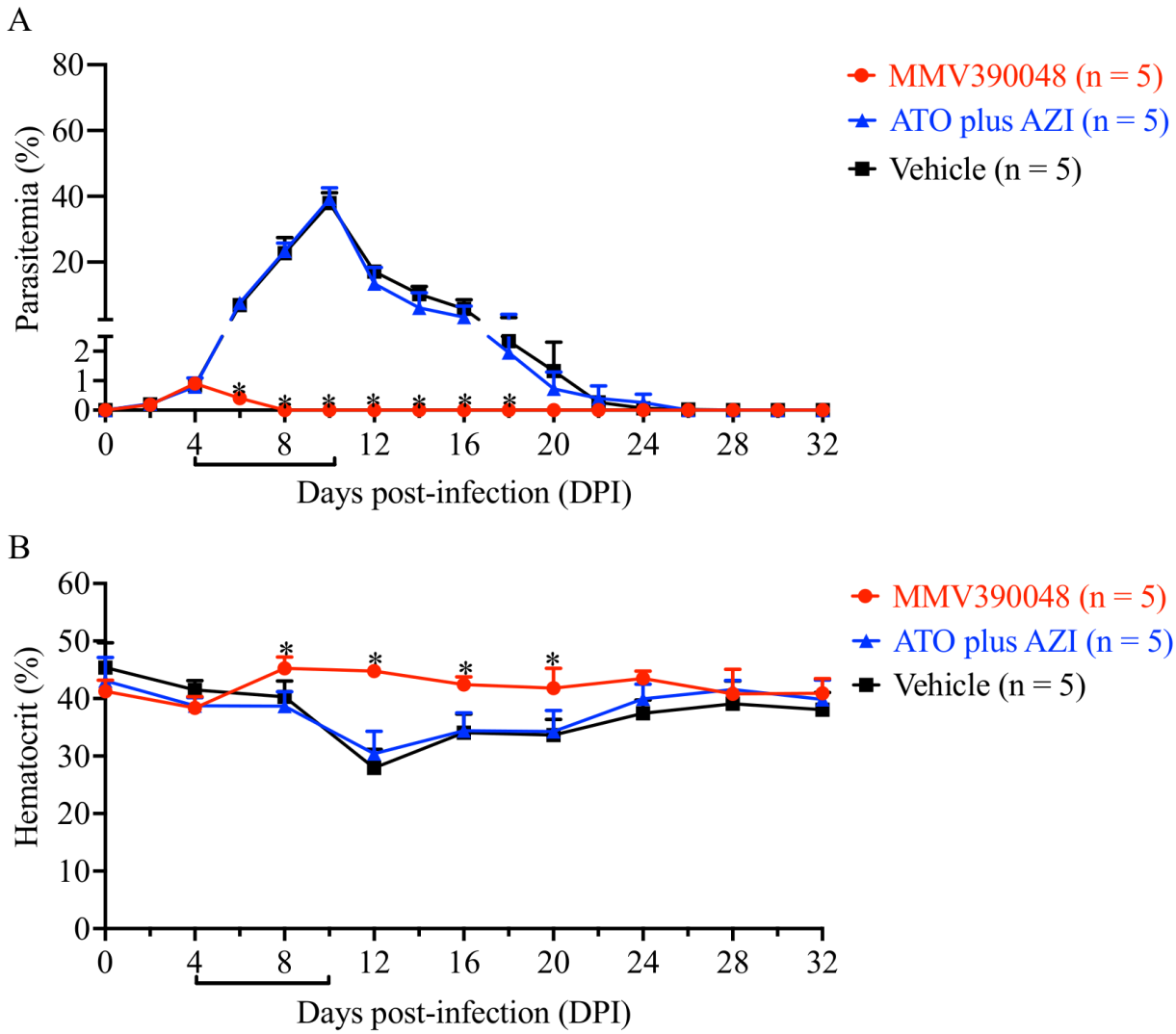

Fig. 13. The efficacy of MMV390048 against *B. microti Cytb* mutant strain in BALB/c mice. (A) Course of parasitemia of vehicle- or ATO plus AZI-, or MMV390048-treated BALB/c mouse groups. (B) Hematocrit changes of *B. microti Cytb* mutant strain in mice treated with vehicle, or ATO plus AZI, or MMV390048. The black arrow indicates the time of treatment. The asterisks indicate a significant difference ($P < 0.05$) between the drug-treated groups and vehicle-treated group. Parasitemia was calculated by counting infected RBCs among 3,000 RBCs using Giemsa-stained blood smears.

General discussion

Babesiosis is a zoonosis caused by genus *Babesia*, a member of the phylum Apicomplexa and causes a remarkable impact on host groups of domestic animals, humans, and wildlife species (Homer et al., 2000). The ixodid ticks are the main vector for babesiosis, which could transmit the parasites from the bite of infected ticks to hosts (Mordue and Wormser, 2019, Kakoma and Mehlhorn, 1994). The parasite infects the erythrocytes of mammals and causes malaria-like illness in the host. The control of babesiosis is usually by medicinal treatment. However, conventional drugs that have been used for years have proven increasingly ineffective due to their toxicity and drug resistance (Renard and Ben Mamoun, 2021). Therefore, the development of effective drugs and druggable targets for babesiosis is essential for the control of *Babesia* infection. Over the recent years, the development of new therapeutics for babesiosis has focused on repurposing known anti-parasite agents, such as anti-malaria compounds. Even this, a large number of drugs with anti-malaria activities, such as artesunate, artemether, dihydroartemisinin, chloroquine, lumefantrine, and so on, have been assessed against *Babesia*, but failed to demonstrate much efficacy against parasite infection at the selected dose (Renard and Ben Mamoun, 2021). In this study, I found two anti-malaria compounds with potent anti-*Babesia* activities, namely NQP and MMV390048. I have evaluated their inhibition effects on *Babesia* spp. and revealed the action mode of MMV390048.

In chapter 1, I used *in vitro* culture of *B. gibsoni* to confirm the inhibition of NQP on parasites. Then, I infected BALB/c mice with *B. rodhaini* and treated them with NQP to confirm its inhibition *in vivo*. NQP was developed by the Academy of Military Medical Sciences of China and is under the same family as chloroquine. Interestingly, the *Babesia* spp. did not respond to chloroquine, whereas NQP showed inhibitory effects on *in vitro* culture of *B. gibsoni* and *in vivo* growth of *B. rodhaini*. The differences in efficacy between chloroquine and NQP may cause by the different action modes. In malaria, the major action of chloroquine is thought to be preventing the polymerization of toxic heme released during proteolysis of hemoglobin (Hb) in the digestive vacuole (DV) (Rudzinska et al., 1976; Moore et al., 2016). However, *Babesia* does not produce hemozoin during parasite development, which may explain the reason why chloroquine did not show inhibition against *Babesia*. Meanwhile, the activity of NQP on malaria was hypothesized in two ways: first was through the inhibition of hemozoin bio-crystallization in the DV of late-stage parasites; and second was through the disruption of the membrane system. Apparently, the inhibitory effect of NQP on *Babesia* is more inclined to the latter.

In chapter 2, I evaluated the inhibition of MMV390048 on *in vitro* culture of *B. gibsoni*, as well as on *in vivo* growth of *B. rodhaini* and *B. microti*. Likewise, the mechanism of MMV390048 on parasites was elucidated by a molecule docking study. MMV390048 is a new anti-malaria compound that inhibit *Plasmodium* by targeting *Pf*PI4K. The genetic analysis of PI4K genes reveal that PI4K are highly conserved among the *Babesia* spp. Hence, I think MMV390048 also has inhibitory effects on other *Babesia* parasites, such as *B. bovis* and *B. bigemina*. Additionally, MMV390048 has been evaluated in clinical trials

and a single dose of 120 mg was safe to human. In my opinion, the dosage of MMV390048 on babesiosis treatment should be higher than 120 mg and more than one dose. Hence, before the apply MMV390048 for babesiosis treatment, the tolerability of MMV390048 on the host should be evaluated to ensure that the dose of MMV390048 is within a safe range.

Furthermore, the relapse of *Babesia* spp. have been well documented in immunocompromised host, including humans. I have confirmed the target of MMV390048 on *Babesia* spp. in chapter 2, hence, I want to test if targeting PI4K consecutive treatment could eliminate parasites from the host. Subsequently, I uninterruptedly treated *B. microti*-infected SCID mice with MMV390048, meanwhile, I used ATO plus AZI as a positive control. As expected, in ATO plus AZI treated group, a mutation was found in Y272C in *Cytb* gene which has been reported to be closely related with the development of drug resistance, whereas in MMV390048 treated group, after 64 days treatment, the parasites could not be detected by a nested PCR assay targeting the *B. microti* 18S-RNA. This result indicates that therapies targeting PI4K have the potential to eradicate parasites from immunocompromised hosts. I strongly believe that the results presented here will contribute to the design of new inhibitors, specifically those targeting *Babesia* PI4K, and not only limited to MMV390048.

Although, I have evaluated the anti-*Babesia* activity of NQP and MMV390048 in this study. There are still has some unsolved queries: (1) the mechanism of NQP is not elucidate in this study; (2) there is a missed opportunity for the development of a combination therapy, for instance, NQP combine with MMV390048. Hence, future research is needed to explain these scientific problems and develop new combinations to eradicate the babesiosis.

General summary

Babesiosis has a wide geographical distribution and is a host-specific zoonotic disease. Essentially, the disease causes economic losses in livestock industry and notably threatens human health. To date the lack of effective vaccine, leaves the use of chemical treatment as the most commonly used control strategy. However, there are concerns about the side effects and drug resistance of currently used anti-*Babesia* drugs. Hence, discovery and development of more effective therapeutic drugs or druggable targets are urgent needed. In this research project, I screened two potential anti-*Babesia* drugs from currently used and under development anti-*Plasmodium* compounds, as well as evaluate their activity against *Babesia* species. Likewise, I identified and evaluated promising anti-*Babesia* druggable targets, with a specific direction toward eradicating parasites.

In chapter 1, I found NQP, which currently used for malaria treatment, showed anti-*Babesia* activity. I evaluated the efficacy of NQP on *B. gibsoni in vitro* and *B. rodhaini in vivo*. The IC_{50} of NQP against *B. gibsoni* was 3.3 ± 0.5 μM and five days treatment with NQP at a dose of 40 mg/kg significantly inhibited *B. rodhaini* growth in a mouse model. These results demonstrated that NQP can be a candidate for babesiosis treatment. Future study can focus on elucidating the mechanism of NQP to provide a new druggable target for the development of more drugs.

In chapter 2, I evaluated the inhibitory effects of MMV390048 on *in vitro* cultured *B.*

gibsoni, as well as, *B. rodhaini* and *B. microti in vivo*. MMV390048 is an anti-malaria compound that is under evaluation in clinical trials. In this study, MMV390048 against *B. gibsoni* with a IC_{50} value of 6.9 ± 0.9 μM. Moreover, MMV390048 showed comparable anti-*Babesia* activity with ATO plus AZI and TAF *in vivo* study of *B. microti* and *B. rodhaini*, respectively. Furthermore, I isolated a MMV390048-resistant strain and revealed the target of MMV390048 as well as its mechanism. Overall, this finding provided an alternative drug for babesiosis treatment and a new druggable target for babesiosis treatment.

In chapter 3, I evaluated the efficacy of uninterrupted targeting *Babesia* PI4K treatment. After 64 days consecutively treating *B. microti*-infected SCID mice with MMV390048, the PCR detection targeting *B. microti* 18S-RNA was negative until the end of the trial. Furthermore, I isolated a *B. microti* ATO-resistant strain from the control group and a genetic mutation in Y272C in *Cytb* gene was detected by sequencing. This mutation has been reported in clinical case of human babesiosis. I further confirmed that MMV390048 also showed potent inhibition against the ATO-resistant strain. These results demonstrated that PI4K is a promising target for eliminating parasites from immunocompromised hosts, especially in relapsing infections caused by ATO-resistant *B. microti*.

In summary, I have found and evaluated two drugs that showed inhibition against *Babesia* spp. which can be candidates for babesiosis treatment. Moreover, the new drug target identified in this study will provide insights to accelerate the development of next-generation anti-babesiosis therapeutics.

和文要約

バベシア症は、地理的に広く分布しており、宿主特異的な人獣共通感染症である。この疾病は畜産業に経済的損失をもたらし、さらに人の健康も脅かす。今日まで有効なワクチンがなく、最も一般的に使用されている制御戦略として化学療法が挙げられる。しかし、現在使用されている抗バベジア薬には副作用や薬剤耐性が懸念されている。したがって、より効果的な新規治療薬開発が急務である。本研究では、抗マラリア原虫作用を示す化合物について、抗バベシア効果を評価した。

第 1 章では、現在マラリア治療に使用されている NQP が抗バベシア活性を示すことを見出した。*B. gibsoni* に対する *in vitro* 試験および *B. rodhaini* に対する *in vivo* 試験において NQP の有効性を評価した。*B. gibsoni* に対する NQP の IC_{50} は 3.3 ± 0.5 μM であり、40 mg/kg の用量の NQP を 5 日間投与すると、マウスモデルで *B. rodhaini* の増殖が有意に阻害された。これらの結果は、NQP がバベシア症治療薬の候補となりうることを示している。今後の研究では、NQP の作用機序を解明して、より多くの新規治療薬候補を特定することに焦点を当てることが重要である。

第 2 章では、抗マラリア臨床治験中の化合物である MMV390048 の *in vitro* 培養 *B. gibsoni*、および *in vivo* 試験での *B. rodhaini* と *B. microti* に対する阻害効果を評価した。*B. gibsoni* に対する MMV390048 の IC_{50} 値は 6.9 ± 0.9 μM であった。一方、MMV390048 は、*B. microti* と *B. rodhaini* の *in vivo* 試験において、それぞれ標準陽性化合物である ATO と AZI、および TAF と同等の抗バベシア活性を示し

た。さらに、MMV390048 耐性株を単離し、MMV390048 の分子標的とそのメカニズムを明らかにした。これらの結果は、バベシア症に対する新規治療法開発の可能性を示した。

第 3 章では、バベシア虫体の PI4K を分子標的とした治療法の有効性を評価した。*B. microti* 感染した SCID マウスに MMV390048 で 64 日間連続して投与した後、*B. microti* 18S rRNA をマーカーとする PCR 検出では、試験終了まで陰性であった。さらに、対照群からは *B. microti* ATO 耐性株を分離し、シーケンスにより *Cytb* 遺伝子の Y272C に遺伝子変異を検出した。この変異は、ヒトのバベシア症の臨床例でも報告されている。MMV390048 は、ATO 耐性株に対しても強力な阻害作用を示すことを確認した。これらの結果は、バベシア虫体の PI4K が免疫不全状態の宿主から寄生虫を排除するための有望な分子標的であることを示唆し、特に ATO 耐性 *B. microti* によって引き起こされる感染の再発において効果が顕著であることを示した。

以上のことから、本研究ではバベシア種に対して増殖阻害効果を示す 2 つの薬剤候補を特定し、評価した。ここで得られた知見は、新規抗バベシア症治療法の開発に寄与できるものと考えられる。

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