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In vitro efficacy of 5-aminolevulic acid against *Babesia gibsoni* by solo-use and combined use with atovaquone

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Abstract

***Babesia gibsoni* is a tick-borne parasite of dogs. Atovaquone (ATV) is used to treat *B. gibsoni* infections, but recurrent with a single-nucleotide polymorphism (M121I) is a serious concern. The present study aimed to evaluate the effect of 5-aminolevulinic acid (5-ALA) on inhibiting *in vitro* growth of *B. gibsoni* and to evaluate the effect of combined use of ATV and 5-ALA on suppressing *in vitro* growth and regrowth. The growth of *B. gibsoni* was suppressed by nearly 85% with 1250 μM 5-ALA. Although the growth of *B. gibsoni* was suppressed after the combined exposure, the regrowth was observed. M121I was not detected in the regrowth population. 5-ALA might be useful in preventing the development of refractory canine babesiosis due to ATV.**

Key Words: atovaquone, *Babesia gibsoni*, 5-amnolevulic acid

Babesiosis is a parasitic disease transmitted by ticks and caused by intraerythrocytic protozoa of the genus *Babesia*. *Babesia gibsoni* infection is frequently identified in dogs and recognized as a severe clinical problem worldwide. The clinical signs are hemolytic anemia, fever, jaundice, hemoglobinuria, and splenomegaly[3)]. Several treatment strategies have been used against this infection, including diminazene aceturate, a combination of antibiotics, and atovaquone (ATV)[2,11,14)]. Among these treatment options, ATV has been the preferred choice due to its early onset of symptom improvement and reduced adverse effects. However, the use of ATV has resulted in relapses and the proliferation of ATV-resistant parasites with a single nucleotide polymorphism at nt363 in cytochrome *b*, which has led to the replacement of methionine with isoleucine (M121I)[4)]. The parasites with M121I have been increasingly detected in Japan[6,7)]. It is required to search for alternative drugs for ATV in order to suppress the emergence of ATV-resistant *B. gibsoni*. However, the immediate effect and safety of ATV are very useful advantages in clinical practice. In previous study, the combined use of ATV and other drugs has been investigated for the suppression of *B. gibsoni* with M121I. The combination of ATV and proguanil has been reported and this combination showed a rapid therapeutic effect on infections of *B. gibsoni* with

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M121I[5,10], but was unable to suppress *B. gibsoni* with M121I. New combination drugs with ATV are required to prevent the appearance of *B. gibsoni* with M121I after using ATV.

5-aminolevulinic acid (5-ALA) is a type of natural amino acid that acts as a porphyrin precursor throughout the body. The combination of ALA and ferrous ion (Fe^{2+}) has been reported to inhibit the *in vitro* growth of *Plasmodium falciparum*[8]. Sodium ferrous citrate (SFC) plus 5-ALA has also been reported to have an antimalarial effect *in vitro* and experimental animal model and might facilitate the development of a new class of antimalarial drug[13]. We posited that 5-ALA has a growth-suppressing effect on other apicomplexan protozoa. A drug combining 5-ALA phosphate (5-ALA P) and Fe^{2+} is already available commercially (EneALA®, Neopharma Japan Co., Ltd, Tokyo, Japan). EneALA® has been shown to be safe for dogs and cats and is used to treat hyperlipidemia and chronic kidney disease. 5-ALA hydrochloride (5-ALA HCl) has been used in the clinical application of photodynamic diagnosis (PDD) (Aragurio®, SBI Pharmaceuticals Co., Ltd., Tokyo, Japan). 5-ALA HCl is currently a diagnostic agent, but this may be useful as a therapeutic agent. The purpose of this study was to evaluate the inhibitory effect of each product of 5-ALA on *B. gibsoni* growth. In addition, in order to investigate whether 5-ALA is useful as a combination drug with ATV, the growth inhibitory effect of *B. gibsoni* and the regrowth inhibitory effect of *B. gibsoni* with M121I were investigated by the combined use of ATV and 5-ALA.

Babesia gibsoni was isolated from a naturally infected Tosa dog in the Aomori Prefecture of Japan in 2004 and was maintained in an *in vitro* culture within the laboratory as wild-type (WT) *B. gibsoni*[11]. The parasite was placed in a 10% packed cell volume in each well of a 12-well plate and incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide[11].

We examined the sensitivity of WT *B. gibsoni* against 5-ALA P and 5-ALA HCl with SFC. Those 5-ALA products were obtained from Neopharma Japan Co., Ltd., and were mixed with diluted water for preparing stock solutions (2500 μM). The sensitivity tests were conducted according to previous report[12]. Both 5-ALA stock solutions were diluted with a culture medium to yield final concentrations of 78 to 1250 μM. Sodium ferrous citrate (SFC; 10 μM) was added to culture medium. The culture medium containing only SFC was used as controls. We dispensed 200 μL of WT *B. gibsoni* culture suspension per well in a 96-well plate in triplicate for each drug concentration. The infection rate of day 0 was diluted to 0.1 % with the culture medium. Every day, we replaced 80 μL of the culture medium with fresh medium. We stopped the culture on day 6. The infection rate on day 6 was calculated by counting the number of parasitized erythrocytes per 2000 erythrocytes in each of the wells containing the drug and that in the control wells without the drug. The growth inhibition rate was calculated by the formula = 100 - ((infection rate of each well at day 6 - 0.1)*100/(infection rate of control well at day 6 - 0.1))

The IC_{50} for each drug was defined as the concentration required for 50% reduction in the mean number of parasitized erythrocytes from that of the control culture[12]. The experiments were repeated three separate times, and the results are reported as mean ± standard deviation. Statistical analysis was performed using Student's *t*-test.

The growth of WT *B. gibsoni* was inhibited by 5-ALA P and 5-ALA HCl. Their IC_{50} values on day 6 were 246.1 ± 6.0 and 284.7 ± 22.3 μM, respectively (Table 1), with no significant difference (P=0.06).

We evaluated the growth-inhibition effect of the combined use of each 5-ALA and ATV (obtained from Wako Chemical, Osaka, Japan). The stock solutions of ATV were prepared in a solution of dimethyl sulfoxide (DMSO), which were diluted with a culture medium to yield final concentrations of 100 to 1600 nM. The final concentration of DMSO was adjusted to 0.1%. We added each concentration of ATV to culture medium with 5-ALA P or 5-ALA HCl. The concentration of each

Table 1. Inhibition rates of *Babesia gibsoni in vitro* growth with different concentrations of 5-ALA products for 6 days and IC_{50}'s of each 5-ALA product.

	Inhibitory rate (%)	
Concentration (μM)	5-ALA P	5-ALA HCl
78	26.5	13.2
156	37.7	32.1
312	56.3	53.1
625	70.9	77.9
1250	84.5	85.8
IC_{50}	246.1 ± 6.0	284.7 ± 22.3

*IC_{50}; the half maximal inhibitory concentration
5-ALA P: 5-aminolevulinic acid phosphate
5-ALA HCl: 5-aminolevulinic acid hydrochloride

5-ALA product used in this study was either 50 μM or IC_{50} equivalent (250 μM). Using the culture medium containing ATV and 5-ALA product at different concentrations, we performed culture of WT *B. gibsoni* for 6 days by the same method as the sensitivity test and determined the IC_{50} of ATV when ATV was used with 50 or 250 μM of each 5-ALA product with SFC. The experiments were repeated three separate times, and the results are reported as mean ± standard deviation.

The IC_{50} of ATV on day 6 after *B. gibsoni* exposed to 50 and 250 μM 5-ALA P with ATV was 148.5 ± 43.3 and 134.6 ± 1.3 nM, respectively. That exposed to 50 and 250 μM 5-ALA HCl with ATV was 155.2 ± 40.1 and 122.8 ± 14.0 nM, respectively.

Following the exposure to the combination of ATV and 5-ALA P for 6 days, we determined whether WT *B. gibsoni* showed regrowth under different conditions. We used 5 groups. After the exposure to 0-1600 nM ATV for 6 days, parasites were maintained in a culture medium which did not contain ATV (Group A). Group A was used as a control which parasites were not exposed to 5-ALA P. For group B and C, after initial exposure to 0-1600 nM ATV and 50 μM 5-ALA P for 6 days, the parasites were incubated with culture medium with different contents. For group B, the exposed parasites were incubated with culture medium which did not contain either ATV or 5-ALA P. For group C, the exposed parasites were incubated with culture medium which containing 50 μM 5-ALA P. For group D and E, after initial exposure to 0-1600 nM ATV and 250 μM 5-ALA P for 6 days, the parasites were incubated with culture medium with different contents. For group D, the exposed parasites were incubated with culture medium which did not contain either ATV or 5-ALA P. For group E, the exposed parasites were incubated with culture medium which containing 250 μM 5-ALA P. Experiments for group B-E were performed three times independently and the experiment for group A was performed only once. Every day, 400 μL of the medium was replaced with a fresh medium. Parasite growth was observed by counting the parasitized erythrocytes every 7 days using blood smears. When parasitemia reached 1.5% in three experiments, we judged that regrowth of the parasites were observed and the culture was stopped. The observation period lasted until day 56.

The results of group A showed that regrowth was observed on day 13 after exposure to ATV at 100, and 200 nM (data not shown). Parasites exposed to 400 nM showed regrowth on day 20, and those exposed to 800 and 1600 nM showed regrowth on day 34 (Fig. 1(a)).

The results of group B-E showed that regrowth was observed on day 13 after exposure to ATV at 0, 100, and 200 nM (data not shown). In group B, the parasites exposed to ATV of 400 nM, 800 nM, and 1600nM with 50 μM 5-ALA P for first 6 days showed regrowth on day 20, day 27, and day 41 respectively (Fig. 1(b)). In group C, the parasites exposed to ATV of 400 nM, 800 nM, and 1,600 nM with 50 μM 5-ALA P for first 6 days showed regrowth on day 20, day 27, and day 62 respectively (Fig. 1(c)). In group D, the parasites exposed to ATV of 400 nM, 800 nM, and 1,600 nM with 250 μM 5-ALA P for first 6 days showed regrowth on day 34, day 34, and day 48 respectively (Fig. 1(d)). In group E, the parasites exposed to ATV of 400 nM and 800 nM with 250 μM 5-ALA P for first 6 days showed regrowth on day 27 and day 55, respectively. Parasites exposed to ATV 1600 nM with 250 μM 5-ALA P for first 6

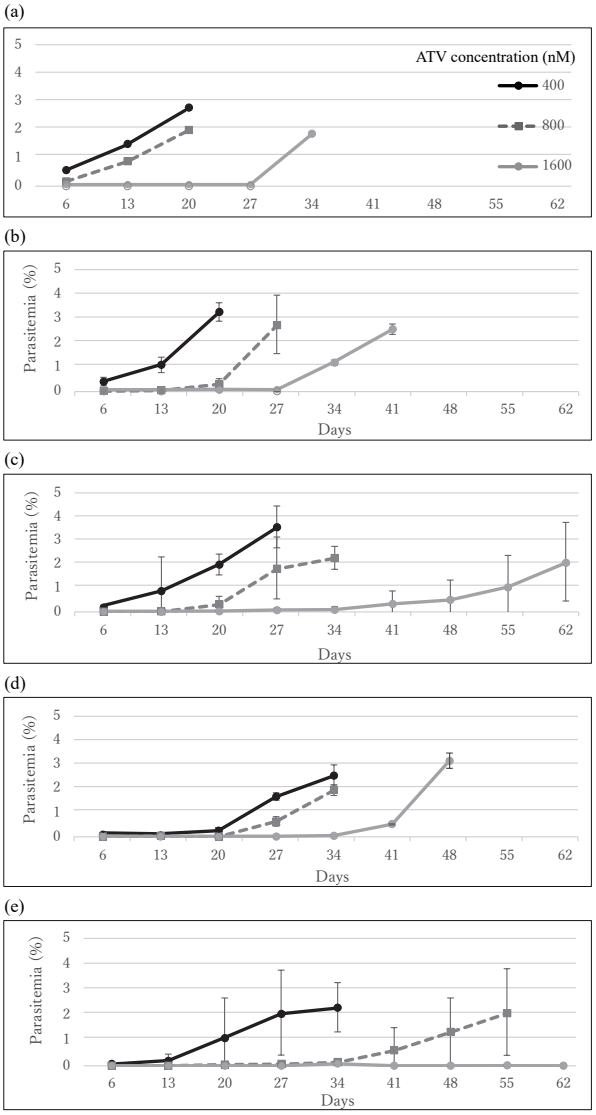

Fig. 1.
Change in parasitemia of regrowth population of *Babesia gibsoni* in various conditions (Group A to E). (a) Group A: after exposure to ATV for 6 days, the parasites were maintained in a culture medium without ATV. (b) and (c) Group B and C: After exposure to the combination of ATV and 50 μM 5-ALA P for 6 days, the parasites were maintained in a culture medium without both ATV and 5-ALA P (Group B), or in a culture medium with 50 μM 5-ALA P (Group C). (d) and (e) Group D and E: After exposure to the combination of ATV and the equivalent IC_{50} of 5-ALA P for 6 days, the parasites were maintained in a culture medium without both ATV and 5-ALA P (Group D), or in a culture medium with the equivalent IC_{50} of 5-ALA P (Group E).

days in group E showed no regrowth during the observation period (Fig. 1(e)).

In the experiments of group A, C and E, *B. gibsoni* DNA was collected once from the culture at 7 days from the day when the culture was stopped. *B. gibsoni* DNA was isolated using a genomic DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. An allele-specific SYBR green real-time polymerase chain reaction assay was used to quantify the M121I variant population[4]. To evaluate the M121I variant population, copy numbers were calculated for the wild-type allele (363G) and mutated allele (363T) in the cytochrome *b* (cyt *b*). Allele-specific forward primers were designed to specifically detect and quantify cyt b 363G and 363T alleles. A common reverse primer was used. Plasmid clones containing the target region, either 363G or 363T, were prepared and serially diluted tenfold to obtain 10^8 to 10^0 molecules/μL. A standard curve was created by plotting the log of the initial copy number of input plasmid DNA against the threshold cycle value[4]. gDNAs from WT and ATV-resistant *B. gibsoni in vitro* cultures were used as negative and positive controls, respectively. We calculated the proportion of the M121I variant population in the regrowth population using the copy number of 363G and 363T.

The proportions of M121I variant population in the regrowth population of group C and E with different initial exposure conditions of ATV concentration with 50 or 250 μM 5-ALA P were less than 1%. In contrast, the proportion of the variant population in group A with initial exposure to 1600 nM ATV was 44.59% (Table 2).

A previous study indicated that the growth inhibition effect was increased when the infected cells of *P. falciparum* were treated with 10 μM of SFC and 200 μM of 5-ALA compared with using 5-ALA alone[8]. After confirming that 10 μM of SFC did not affect the growth of *B. gibsoni* (data not shown), we used 5-ALA with 10 μM of SFC. In this study, both products of 5-ALA inhibited the growth of *B. gibsoni*. In the overview of nonclinical studies of 5-ALA HCl, it was reported that the Cmax when 5-ALA HCl was orally administered to dogs at 20 mg/kg was equivalent to 76 μM. It was also reported that 5-ALA HCl and 5-ALA P

Table 2. The M121I-variant populations of *Babesia gibsoni* in group A, C and E

ATV concentration (nM)	Group A (Without 5-ALA)	5-ALA P	
		Group C (50 μM)	Group E (250 μM)
0	0.03	0.05	0.01
100	0.05	0.03	0.04
200	0.03	0.05	0.03
400	0.06	0.04	0.02
800	0.19	0.01	0.13
1600	44.59	0.02	ND

*ND; not determined

showed comparable pharmacokinetics. However, when using 5-ALA P in clinical practice, it is expected that the dose will be reduced, and it is predicted that the blood concentration will also decrease. So, the target blood concentration was set as 50 μM in this study. The IC_{50} values in this study were much higher than the predicted blood concentration (50 μM). The growth inhibition rates at 78 μM in this study were 26.5% for 5-ALA P and 13.2% for 5-ALA HCl. These results indicate that the growth-inhibitory effect of 50 μM of 5-ALA was low. Since it is presumed that a sufficient growth inhibitory effect cannot be expected at the blood concentration, we considered that either type of 5-ALA used with SFC could not be expected to have a sufficient inhibitory effect on *B. gibsoni* in vivo.

In a previous study, when ATV was used alone to treat canine babesiosis, it became less effective in controlling recurrent *B. gibsoni* with M121I[11]. To solve this problem, the efficacy of ATV combined with other drugs was examined. The IC_{50}'s of proguanil and doxycycline against WT *B. gibsoni* were 42.3 ± 18.6 μM and 9.1 ± 5.5 μM, respectively[4]. Although the IC_{50} of these drugs was not lower than that of ATV, the combination of a medical compound (ATV and proguanil; Malarone®, GlaxoSmithKline, London, U.K.) with doxycycline was able to prevent recurrence[5]. Some pharmacologic agents could be useful in combination with ATV, even though the agent itself has a low growth inhibiting effect. Therefore, we investigated whether 5-ALA could be used concomitantly with ATV. We evaluated the effect of suppressing *B. gibsoni* growth by combining ATV and 5-ALA at a concentration equivalent to IC_{50}, and ATV and 5-ALA at 50 μM.

In this study, the IC_{50} values for ATV of WT *B. gibsoni* was indicated after exposure ATV with 5-ALA. The previous report showed that an IC_{50} value for ATV of WT *B. gibsoni* was 164 ± 42 nM[4]. The results in this report tended to be slightly lower than previous study. Although ATV has few adverse effects and a high therapeutic effect, prescription drugs containing ATV is very expensive. When the amount of ATV can be reduced by combining it with 5-ALA, the combination therapy can be useful as a treatment for canine babesiosis. Further in vivo studies are needed to assess the efficacy of combining ATV with each type of 5-ALA for *B. gibsoni* infection.

The parasites exposed to ATV 800 nM in group E showed the regrowth on day 55 and that exposed to 1600 nM in group C showed the regrowth on day 62. In addition, the parasites exposed to ATV 1600 nM in group E did not show the regrowth. On the other hand, the parasites exposed to ATV 800 nM and 1600 nM in group A showed regrowth on day 34. These results indicated that continuous use of 5-ALA might extend the period to regrowth.

In this study, the M121I-variant populations of parasites in group C and E were < 1% after exposed to ATV and 5-ALA. In the previous report, the presence of *B. gibsoni* with M121I after exposure to ATV was thought to be due to selective proliferation[4], which suggests that 5-ALA suppresses the emergence of *B. gibsoni* with M121I. The mechanism of action of ATV and 5-ALA against *B. gibsoni* has not been elucidated. In malaria, the binding site of ATV in mitochondria has been reported. In addition, the change of structure of the binding site caused a reduction of sensitivity against ATV[9]. It has also been reported that the abnormal accumulation of heme intermediate metabolites in intracellular organelles, including apicoplast and mitochondria, after using 5-ALA suppressed the growth of *P.*

falciparum[8]. Both of ATV and 5-ALA may also be targeting mitochondria in *B. gibsoni*. The drug that targets the same organelles may enhance their action and suppress the growth of *B. gibsoni* with M121I. Further research is needed to determine whether the action of ATV and 5-ALA suppressed the emergence of *B. gibsoni* with M121I, or whether 5-ALA suppressed the growth of *B. gibsoni* with M121I.

When ATV is used to treat *B. gibsoni* infections, the growth of *B. gibsoni* with M121I was observed with or without the combined use of other drugs. Therefore, decreased sensitivity to ATV has become a major problem at the time of recurrence. If the continued use of 5-ALA can suppress the emergence or growth of *B. gibsoni* with M121I, 5-ALA could be a new combination drugs with ATV.

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