Title

Artesunate, a potential drug for *Babesia* infection

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ABSTRACT

The effect of artesunate, a water-soluble artemisinin derivative, against *Babesia* species, such as *Babesia bovis*, *Babesia gibsoni*, and *Babesia microti* was studied. Cultures of *B. bovis* and *B. gibsoni* were treated with 0.26 μM, 2.6 μM, 26 μM, and 260 μM artesunate, and the growth-inhibitory effect was shown in over 2.6 μM artesunate in day 4 and day 3 post-subculture for *B. bovis* and *B. gibsoni*, respectively, in dose-dependent manner. In vivo experiment for *B. microti*, strong inhibition effects were observed in mouse groups treated with over 1.0 mg/kg body weight of artesunate on day 9 and 10 post-infection. These results suggest that artesunate could be a potential drug for *Babesia* infection.

Key words: Artesunate, *Babesia bovis*, *Babesia gibsoni*, *Babesia microti*

Babesiosis is a parasitic infection caused by intraerythrocytic protozoa of the genus *Babesia* and transmitted by tick to its vertebrate hosts. *Babesia* infection is a well-recognized disease of veterinary importance in cattle, horses, and dogs and it is highlighted as an emerging zoonosis in human. This disease can cause a malaria-like syndrome, including fever, haemolytic anemia, and hemoglobinuria, and clinical cases appear sudden and severe [1]. There are a number of babesiacides, but only a few drugs are currently available, for instance, imidocarb dipropionate (Imizol®, Schering-Plough Animal Health) and diminazene aceturate (Berenil®, Intervet India Pvt Ltd) for animals, such as cattle, horses, and dogs, and quinine and clindamycin for human [2]. However, an increasing of resistant parasites to commercial drugs, adverse effects of drugs, and the long term persistence of low level parasitemia after treatment still necessitate developing an effective treatment.

Artemisinin and its derivatives, such as artesunate, artemether, arteether, and dihydroartemisinin, are the most potent antimalarial drugs available throughout the world [3]. The artemisinin derivatives act rapidly on the parasites and are quickly eliminated, which renders these derivatives effective against severe malaria and slow to develop resistance, and those exhibit high efficacy against all asexual stages of *Plasmodium falciparum* with rare adverse effects [4-7]. Among artemisinin

derivatives, artesunate, a water soluble half-ester succinate derivative, is the most commonly used derivative and has been for more than 15 years. Moreover, many clinicians feel that artesunate administration in parenteral is the most effective treatment for severe malaria [8,9]. Since *Babesia* species share similar life cycle as well as clinical symptoms with *Plasmodium* species, and the growth-inhibitory effect of artesunate in *Babesia* (*Theileria*) *equi* and *B. caballi* was observed [10], it led us to test whether artesunate inhibits the growth of other *Babesia* species. With this in mind, we evaluated the efficacy of artesunate against *B. bovis* for cattle and *B. gibsoni* for dogs in vitro, and *B. microti* for mice and human in vivo.

Solution of 156 mM artesunate (Guanaxi, China) in growth medium for in vitro assay and in 5% sodium bicarbonate for in vivo assay was prepared before using. Texas T2B strain of *B. bovis* and NRCPD strain of *B. gibsoni* were grown in bovine and canine RBC using a method previously established [11,12]. The in vitro growth-inhibitory assay was carried out in 48-well tissue culture plate by modified methods previously described [10]. Initial *Babesia* parasite cultures containing 1% infected erythrocytes were prepared from cultures that had reached 3 to 5% parasitemia by mixing with normal bovine and canine RBC. To each well, 50 µl of the infected erythrocytes was added into 450 µl of the growth medium with 0.26 µM, 2.6 µM, 26 µM, and 260 µM artesunate. The evaluation of the growth-inhibitory effect per drug concentration per each parasite species was monitored in triplicate and in three separate trials. Culture plates were kept in a humidified 5% CO_2 incubator at 37˚C. Per well, 250 µl of the culture medium with the indicated concentration of drug was replaced daily for 4 days. Thereafter, to demonstrate whether the inhibition effect is maintained after a withdrawal of the treatment, the treated parasite culture was subcultured with healthy bovine and canine RBC as described above and parasite re-growth was monitored for another three days. Parasitemia in Giemsa-stained culture smears was calculated based on eight to ten microscopic fields covering approximately 2,000 cells.

B. bovis and *B. gibsoni* were grown in vitro culture from 1% parasitemia with the above-indicated concentration of artesunate, and parasitemia was compared with the control. Statistical significance of

the differences was analyzed by One-way ANOVA and student's *t* test using JMP Version 8 Program (SAS Institute Inc., USA). Significant growth inhibition (P <0.05) of *B. bovis* was observed in groups treated with more than 2.6 μM artesunate from day 3 post-subculture and in all test groups, even in the group treated with 0.26 μM artesunate, at day 5 post-subculture (Fig. 1). Moreover, this growth-inhibitory effect was maintained in over 2.6 μM artesunate even after withdrawal of the treatment. In growth inhibition test of *B. gibsoni*, significant difference (P <0.05) between control and test groups was shown in 26 μM and 260 μM artesunate from day 2 post-subculture and in all test groups at day 4 post-subculture (Fig. 2). After the withdrawal of the treatment, reemergence of the parasite did not occur in 26 μM and 260 μM artesunate and modest inhibition effect was observed in 2.6 μM and 0.26 μM artesunate. These growth inhibition results of *B. bovis* and *B. gibsoni* showed in dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) for each parasite was interpreted as the concentration required for 50% reduction in the mean parasitemia of groups treated with artesunate compared to that of control at day 4 post-subculture. The IC_{50} was calculated using non-linear curve-fitting of the percent inhibitions against various concentrations of artesunate by a calculation software (Sigma Plot, Japan). The values of IC_{50} for *B. bovis* and *B. gibsoni* were 372.2 ± 24.32 nM and 924.0 ± 97.26 nM, respectively, which is higher than that for *P. falciparum* [13]. According to a pharmacokinetic report, when patients with uncomplicated malaria were administrated 120 mg of artesunate by either i.v. or i.m route, the maximum concentration in plasma (C_{max}) was 42 μM for i.v. and 2.3 μM for i.m. [14] which is the actual concentration of artesunate to act on the parasites. In this growth inhibition assay, the growth of *B. bovis* and *B. gibsoni* was inhibited in 2.6 μM and 26 μM artesunate, respectively, which is less than above-mentioned C_{max} of artesunate for i.v., suggesting that artesunate could be used for *B. bovis* and *B. gibsoni* infection.

In order to determine anti-babesial effects of artesunate against *B. microti*, female 6-week-old BALB/c mice (Japan CLEA, Japan) were used. Infection was initiated by intraperitoneal (i.p.) injection of 1×10^7 *B. microti* Munich strain infected erythrocytes. Infected mice were divided into 5 groups as follows: control and a group for 5% sodium bicarbonate were administered with 0.2 ml

saline and 5% sodium bicarbonate intramuscularly. Groups for 1 mg/kg, 10 mg/kg, and 50 mg/kg body weight of artesunate (AR1, AR10, and AR50, respectively) were treated with 0.2 ml of the indicated doses of artesunate dissolved in 5% sodium bicarbonate intramuscularly and parasitemia was monitored by an examination of Giemsa-stained, thin blood smear using a light microscope. The body weight of mouse was measured every 2 days and each mouse was given the above doses of the drug once per day for 6 consecutive days from day 2 post-infection in which infected erythrocytes were observed in peripheral blood. As shown in Fig. 3, the infected erythrocytes appeared in peripheral blood of all mice on day 2 post-infection and treatment was started from day 2 post-infection. A peak parasitemia (42.5%) was observed in the control on day 10 post-infection, in contrast, low parasitemia was observed in AR10 as well as AR50. Significant difference (P <0.05) between the control and test groups, AR50, AR10, and AR1, was observed on day 9 and 10 post-infection. Although it failed to eradicate parasites and parasitemia increased up to 19.6% and 24.7% (standard deviation; ±6.32 and ±9.34) for AR10 and AR50 after the cessation of the treatment, respectively, artesunate not only inhibited the growth of the parasites but also delayed the increase of parasitemia, indicating that artesunate could be used for *B. microti* infection. However, in order to improve the efficacy of the parasite elimination, further studies about the combination of artesunate with another effective babesiacide are needed.

In this study, i.m. artesunate regimen was selected to treat *B. microti* infection, since a prompt treatment is necessary so as to treat *B. microti* infection that occurs sudden and severe in clinics. While i.v. administration is also recommended for patients in severe condition, particularly those in coma, venous access may not be possible where only basic health care facilities exist. In addition, even when the drugs can be administered in i.v., patients discomfort and inconvenience, as well as risks such as overhydration and thrombophlebitis may make i.v. less attractive than i.m. [14]. Although i.m. administration of the oil-soluble antimalarial artemisinins could damage brain stem centers mainly involved in auditory processing and vestibular reflexes [15-17], artesunate, a water-soluble artemisinin derivative, has shown less neurotoxic effects [18]. Mice treated with artesunate

here showed neither any decreases of body weight on day 12 post-infection compared to that on day 1 post-infection (data not shown) nor clinical abnormalities such as gait and equilibrium disturbances, suggesting that the doses of 1-50 mg/kg body weight of artesunate were not responsible for neurotoxicity.

In conclusion, we have demonstrated that artesunate inhibits the growth of *B. bovis* and *B. gibsoni* in vitro and i.m. administration of artesunate suppressed a growth of *B. microti* in vivo without side effects, suggesting that artesunate could be a potential drug for *Babesia* infection. However, in vivo experiment for *B. microti* showed a possibility of a recrudescence of parasite growth after a cessation of artesunate treatment. Further studies would be needed to evaluate a combination of other drugs with artesunate against *Babesia* species after a prudent screening to select drug candidates to be combined with artesunate.

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Figure legends

Fig. 1. Growth curves of *B. bovis* in vitro culture treated with 0.26 μM, 2.6 μM, 26 μM, and 260 μM artesunate. Cultures were started at 1% parasitemia and Giemsa-stained thin blood smears were prepared to determine daily parasitemia. ∗, the significant difference (P <0.05) between the control group and the groups treated with 2.6 μM, 26 μM, and 260 μM artesunate; ∗∗, the significant difference (P <0.05) between the control group and all the groups tested with artesunate.

Fig. 2. Growth curves of *B. gibsoni* in vitro culture treated with 0.26 μM, 2.6 μM, 26 μM, and 260 μM artesunate. Cultures were started at 1% parasitemia and Giemsa-stained thin blood smears were prepared to determine daily parasitemia. ∗, the significant difference (P <0.05) between the control group and the groups treated with 26 μM and 260 μM artesunate; ∗∗, the significant difference (P <0.05) between the control group and the groups tested with 2.6 μM, 26 μM, and 260 μM artesunate;

1 ***, the significant difference (P <0.05) between the control group and all the groups tested with
2 artesunate.
3
4 Fig. 3. Course of parasitemia in artesunate treatment against *B. microti* infection. *B. microti* infected
5 mice were treated with artesunate for 6 days, from day 2 to day 7 post-infection, and Giemsa-stained
6 thin blood smears were prepared to determine daily parasitemia. CON, control group; SB, group for
7 5% sodium bicarbonate; AR50, group for 50 mg/kg body weight of artesunate; AR10, group for 10
8 mg/kg body weight of artesunate; AR1, group for 1 mg/kg body weight of artesunate. *, the
9 significant difference (P <0.05) between the control group and the groups for AR50 and AR10; **, the
10 significant difference (P <0.05) between the control group and the groups for all groups treated with
11 artesunate.

Figure

Fig. 1

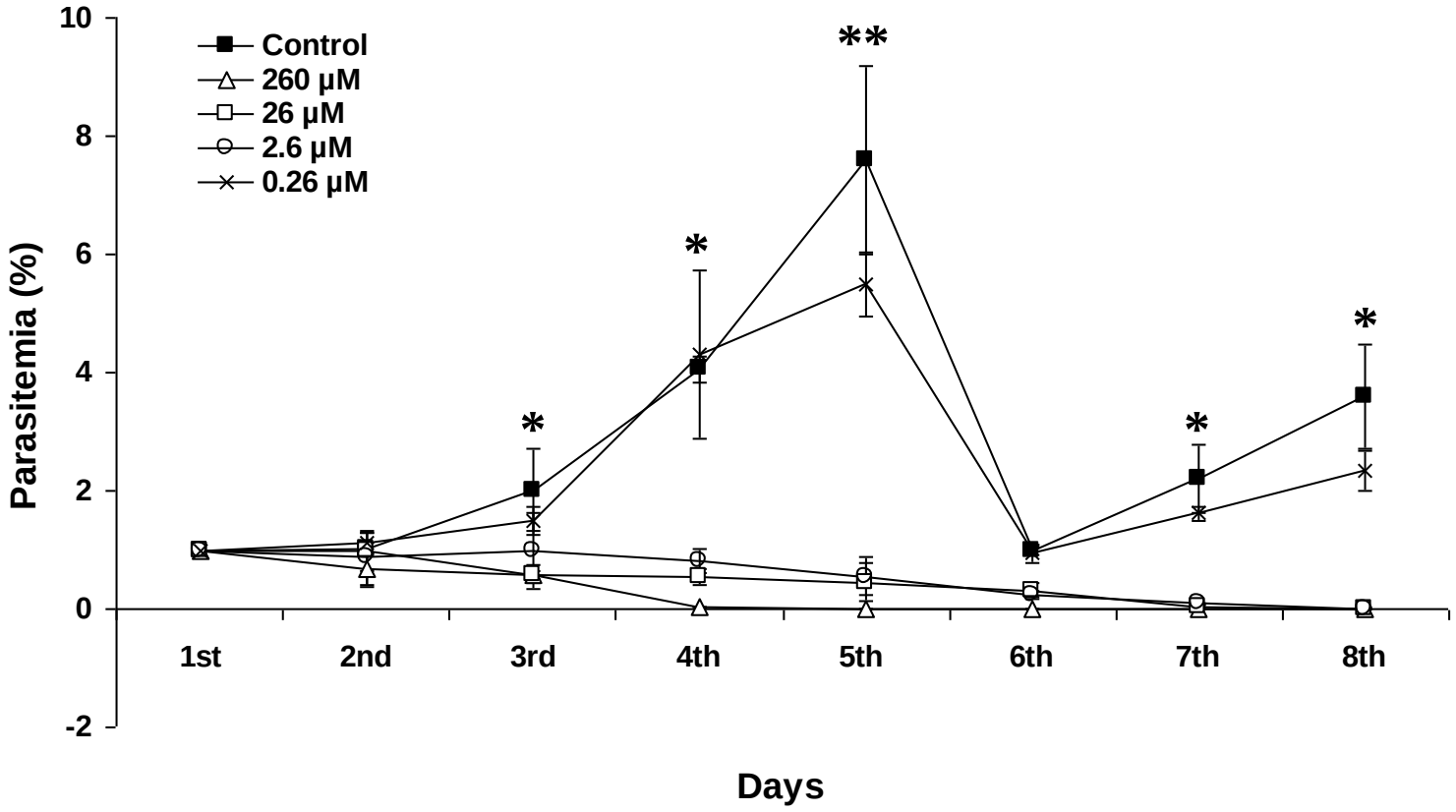

Control
260 μM
26 μM
2.6 μM
0.26 μM
Parasitemia (%)
10
8
6
4
2
0
-2
1st
2nd
3rd
4th
5th
6th
7th
8th
Days
*
*
**
*
*

Fig. 2

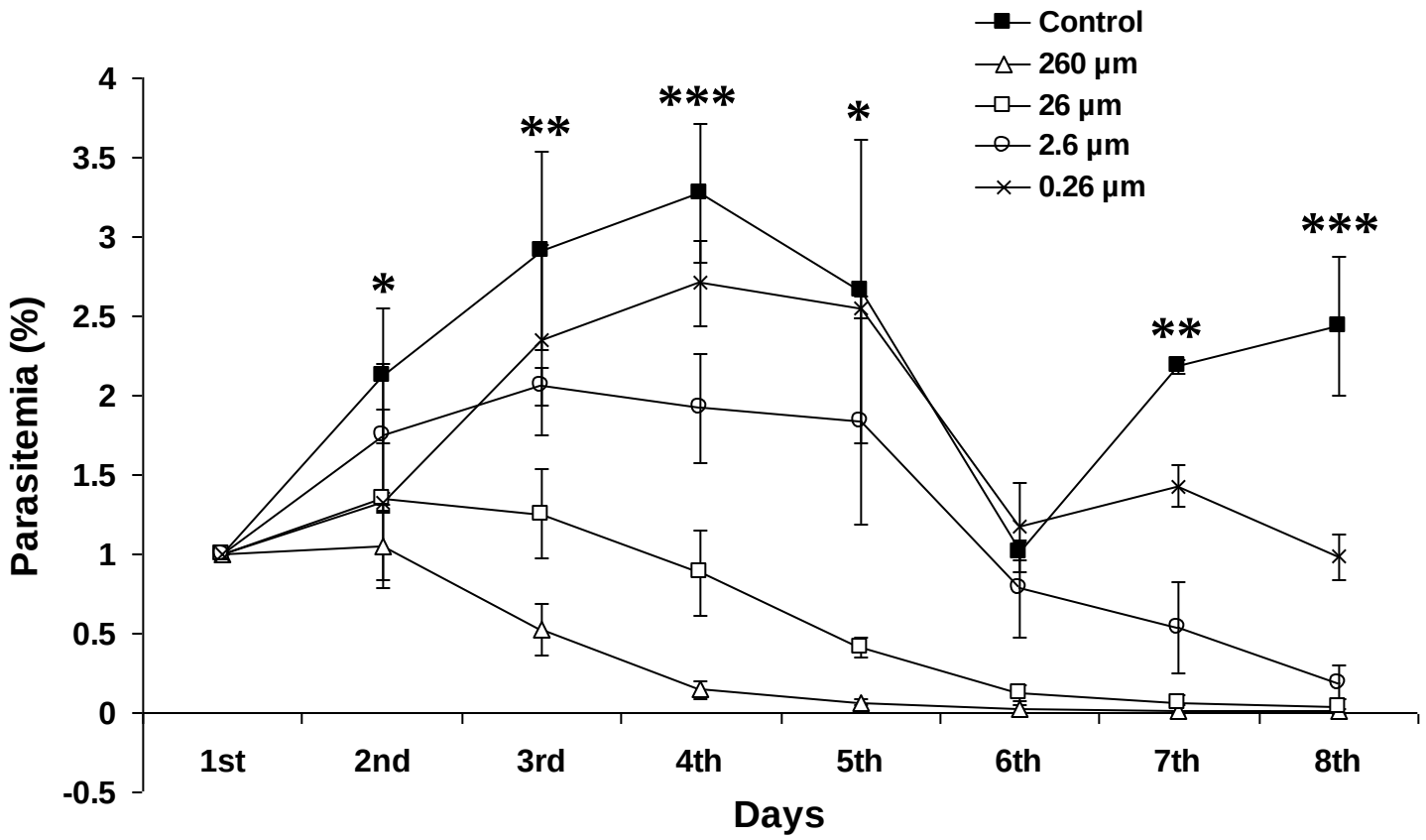
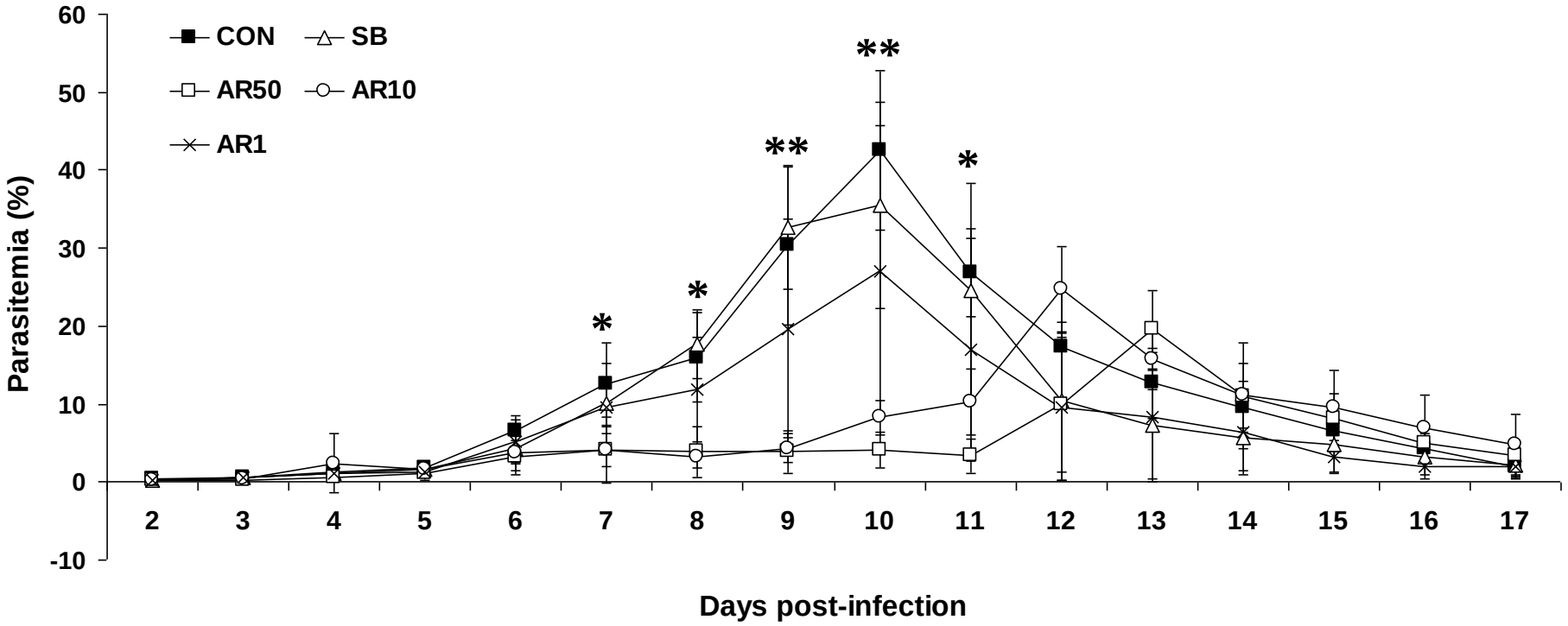

Control
260 μm
26 μm
2.6 μm
0.26 μm
Parasitemia (%)
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
1st
2nd
3rd
4th
5th
6th
7th
8th
Days
*
**
***
*
**
***

Fig. 3


CON
SB
AR50
AR10
AR1
Parasitemia (%)
60
50
40
30
20
10
0
-10
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Days post-infection
*
*
**
**
*