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Short communication

Artesunate, a potential drug for treatment of *Babesia* infection

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ABSTRACT

The effects of artesunate, a water-soluble artemisinin derivative, against *Babesia* species, including *Babesia bovis*, *Babesia gibsoni* and *Babesia microti* were studied. Cultures of *B. bovis* and *B. gibsoni* were treated with 0.26, 2.6, 26 and 260 μM artesunate, showing inhibition of parasite growth at concentrations equal to and greater than 2.6 μM artesunate by days 3 post-treatment for *B. gibsoni* and *B. bovis* in a dose-dependent manner. Consistent with *in vitro* experiments, artesunate was effective in the treatment of mice infected with *B. microti* at doses equal to and greater than 10 mg/kg of body weight on days 8–10 post-infection. Taken together, these results suggest that artesunate could be a potential drug against *Babesia* infection.

Babesiosis is a parasitic disease caused by intraerythrocytic protozoa of the genus *Babesia* and transmitted by ticks to their vertebrate hosts. The disease is recognized to be of veterinary importance in cattle, horses, and dogs and is highlighted as an emerging zoonosis in humans. Symptoms can include a malaria-like syndrome, including fever, haemolytic anemia, and hemoglobinuria, and clinical cases appear suddenly and severe [1]. There are a number of babesiacides, but only a few drugs are currently available such as 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, clindamycin and atovaquone (Mepron®, Glaxo Welcome) for humans [2]. However, an increasing number of resistant parasites to commercial drugs are appearing, adverse effects of drugs are well documented, and the long term persistence of low level parasitemia after treatment still necessitate development of 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 leading to their quick elimination thereby rendering these derivatives effective against severe malaria. Furthermore, parasites are slow to develop resistance and these derivatives 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, has been the most commonly used derivative for more than 15 years; many clinicians feel that parenteral administration of artesunate is the most effective treatment for severe malaria [8,9]. Since *Babesia* species share a similar life cycle, as well as clinical symptoms, with *Plasmodium* species, coupled with the previously observed growth-inhibitory effect of artesunate on *Babesia* (*Theileria*) *equi* and *B. caballi in vitro* and on *B. microti* in hamster [10,11], we tested whether artesunate inhibited the growth of other *Babesia* species. In previous studies, the significant growth-inhibitory effects of atovaquone and diminazene aceturate were shown on *B. microti* and *B. divergens* and on *B. rodhani*, *B. divergens* and *B. bovis*, respectively [12–18]. Therefore, atovaquone and diminazene aceturate were used to compare an efficacy of artesunate against *Babesia* parasites. 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 humans *in vivo* and compared these growth-inhibitory effects with those of currently available drugs, such as atovaquone and diminazene aceturate.

For *in vitro* assays, solutions of 156 mM artesunate (Guangxi, China), 26 mM atovaquone (Toronto Research Chemical Inc., Canada) and 260 mM diminazene aceturate (Kanto Chemical Co., Inc., Japan) added to growth media were prepared. The Texas T2B strain of *B. bovis* and NRCPD strain of *B. gibsoni* were maintained in bovine and canine RBC as previously established methods [19,20]. The *in vitro* growth-inhibitory assay was carried out in 48-well tissue culture plates by modified methods described previously [10]. Initial *Babesia* parasite cultures containing 1% infected erythrocytes were prepared from cultures that had reached 3 to 5% parasitemia by mixing with

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uninfected bovine and canine RBC. To each well, 50 μl of infected erythrocytes were added to 450 μl of growth medium containing either 0.26, 2.6, 26 and 260 μM of one of the drugs (artesunate, atovaquone or diminazene aceturate). Evaluation of growth-inhibitory effects of each drug and concentration was performed for each parasite species and monitored in triplicate and in three independent 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 inhibitory effect was maintained after withdrawal of treatment, parasite cultures were subcultured with uninfected bovine and canine RBC as described above and parasite re-growth was monitored for another two days. Parasitemia in Giemsa-stained culture smears was calculated from eight to ten microscopic fields covering approximately 2000 cells.

B. bovis and *B. gibsoni* were grown *in vitro* beginning at 1% parasitemia in the presence of the aforementioned concentrations of artesunate, atovaquone and diminazene aceturate, and parasitemia was compared to a control. Statistical significance between the mean parasitemia of a control group and that of each group treated with drugs was determined using one-way ANOVA and Tukey's tests using the JMP Version 8 Program (SAS Institute Inc., USA). Beginning at day 2 post-treatment significant growth inhibition ($P<0.05$) of *B. bovis* was observed in groups treated with equal to and greater than 2.6 μM artesunate (Fig. 1A). Moreover, this growth-inhibitory effect was maintained in equal to and greater than 2.6 μM artesunate even after withdrawal of the treatment at day 4. Upon comparison of the growth-inhibitory effect against three drugs on *B. bovis*, it appears that artesunate may be moderately effective than atovaquone but less than diminazene aceturate at day 4 post-treatment (Fig. 1A versus 1B and C, respectively). These results could be explained by their mechanisms acting to parasites. Diminazene aceturate binds to the AT-rich domains of the DNA double helices, which leads to an interference with the activities of the eukaryotic type II topoisomerase enzyme and finally causes death of parasites [21,22]. On the other hand, atovaquone suppresses electron flow in mitochondrion of parasites by inhibition of binding between ubiquinone and cytochrome bc_1 [23]. In addition, artesunate inhibits sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) responsible for the maintenance of calcium ion concentrations, which is related to the generation of calcium-mediated signaling and the invasion of parasites to erythrocytes, thereby inhibiting the parasite growth [24].

Artesunate was also found to be effective against *B. gibsoni*, where significant growth inhibition ($P<0.05$) was observed at 26 and 260 μM from day 1 post-treatment. Moreover, this significant difference was observed in all test concentrations at day 3 post-treatment (Fig. 2A). Furthermore, upon withdrawal of the treatment, reemergence of the parasite failed to occur in concentrations equal to and greater than 2.6 μM artesunate. As with *B. bovis*, artesunate was found to be less effective than diminazene aceturate in suppressing the growth of *B. gibsoni* (Fig. 2). Regarding the efficacy of artesunate and atovaquone, while 2.6 μM artesunate could rather effectively inhibit parasite growth than 2.6 μM atovaquone, 26 μM atovaquone was more effective than 26 μM artesunate on *B. gibsoni*. Therefore, it is difficult to conclude which drug is more effective in the growth inhibition of *B. gibsoni*.

The growth inhibition of both *B. bovis* and *B. gibsoni* exhibited a dose dependence and therefore the half maximal inhibitory concentration (IC_{50}) for each parasite was calculated as the concentration required for a 50% reduction in the mean parasitemia of drug-treated groups by a comparison with that of control groups at day 4 post-treatment. The IC_{50} was calculated using non-linear curve-fitting of the percent inhibitions against various concentrations of three drugs by a calculation software (Sigma Plot, Japan). Although the IC_{50} values for diminazene aceturate (*B. bovis*, 24.82 ± 2.37 nM; *B. gibsoni*, 41.93 ± 2.32 nM) suggested this drug to be more effective than artesunate (*B. bovis*, 372.2 ± 24.32 nM; *B. gibsoni*, 924.0 ± 97.26 nM) in treatment of both *B. bovis* and *B. gibsoni*, previous attempts at using diminazene aceturate for treatment of babesiosis failed due to its toxicity to kidney, brain, and liver which can result in serious side-effects such as weakness, paralysis, lack of responsiveness to stimuli in the central nervous system especially in dogs as well as humans [25–27]. Moreover, due to these side-effects, the diminazene aceturate was recently withdrawn from the market in Japan, and this drug is not approved by the Food and Drug Administration (FDA) in the U.S.A. [28]. In contrast to diminazene aceturate, few significant side-effects of artesunate have been reported in more than two million patients treated with artesunate [3,6]. Therefore, artesunate could be a preferential choice for the treatment of *B. bovis* and *B. gibsoni* based on these results.

In order to determine anti-babesial effects of artesunate against *B. microti*, female 6-week-old BALB/c mice (Japan CLEA, Japan) were used in an experimental infection study. Moreover, atovaquone was tested in parallel on mice infected with *B. microti* as a currently available anti-babesial drug in order to compare its anti-babesial effects with artesunate. Infection was initiated by intraperitoneal (i.p.) injection of 1×10^7 *B. microti* Munich strain infected erythrocytes isolated from a mouse with 40.7% parasitemia. Infected mice were divided into 8 groups as follows: control groups were either administered 5% sodium bicarbonate (SB) for artesunate or phosphate buffered saline (PBS) for atovaquone intramuscularly. Experimental groups were divided as 1, 10 or 50 mg/kg of body weight of artesunate (AR1, AR10 and AR50, respectively) or atovaquone (AT1, AT10 and AT50, respectively) and subsequently administrated 0.2 ml of the indicated doses of artesunate or atovaquone dissolved in 5% sodium bicarbonate or PBS, respectively, by intramuscular route. The doses of atovaquone for this study were decided based on previous studies in which 50–100 mg/kg of body weight of atovaquone inhibited the growth of *B. microti* in hamster and Mongolian gerbil [13,14]. Parasitemia was monitored by examination of Giemsa-stained, thin blood smears using a light microscope. The body weight of mice was measured every 2 days and each mouse was given the indicated doses of the drug once per day for 6 consecutive days beginning 2 days post-infection. The infected erythrocytes appeared in peripheral blood of all mice on day 2 post-infection and thus treatment was started from day 2 post-infection. In Fig. 3A, peak parasitemia (35.5%) was observed in SB on day 10 post-infection. In contrast, lower parasitemia was observed in AR10 as well as AR50. Statistical significance of mean parasitemia between SB or PBS and each experimental group was determined using ANOVA and Tukey's tests using the JMP Version 8 program (SAS Institute Inc., USA). Significant differences ($P<0.05$) between SB and experimental AR50 and AR10 groups were observed on days 8–10 post-infection. Although artesunate 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 controlling *B. microti* infection. Moreover, artesunate was able to suppress more effectively the increase in parasitemia compared to atovaquone which showed significant inhibition ($P<0.05$) between PBS group and AT50 only at day 11 post-infection. Atovaquone did not effectively inhibit parasite growth in BALB/c mice, which is different from previous studies in gerbils and hamsters. Although mice, gerbils and hamsters are closely related in aspect of taxonomy, species differences in pharmacokinetics might affect absorption, distribution, metabolism and excretion of drugs [29,30]. The efficacy of artesunate could be improved by a combination with another effective babesiacide. Indeed, the combination of artesunate with other drugs has been advocated to malaria patients to prevent drug resistant parasites and to improve its efficacy by using drugs which have the different mode of action [31].