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In vitro inhibitory effect of gedunin on *Babesia* and *Theileria* parasites

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ABSTRACT

Evaluation of novel and effective chemotherapeutic agents for bovine and equine piroplasmosis is urgently needed because of the toxic side effects of currently available drugs. Heat shock protein 90 (Hsp90) becomes a promising drug target due to its important roles in protecting the cells. In this study, we report the *in vitro* inhibitory effect of gedunin, an Hsp90 inhibitor, on the growth of *Babesia bovis*, *B. bigemina*, *B. caballi*, and *Theileria equi*. The IC_{50} values of gedunin for four parasites were 21.72 µM, 15.25 µM, 22.1 µM, and 33.21 µM, respectively. Severe morphological changes, such as pyknotic and degenerative change, were also observed in the parasites treated with gedunin. Although further experiments are required to evaluate the *in vivo* activity of gedunin against *Babesia* and *Theileria* parasites, the present study indicated that Hsp90 could be a drug target in bovine and equine piroplasms.

Keywords: *Babesia;* gedunin; Hsp90; *in vitro*; *Theileria*

INTRODUCTION

Babesia bovis and *Babesia bigemina* are two major species that cause clinically and economically significant bovine babesiosis (Bock *et al*., 2004). On the other hand, *Babesia caballi* and *Theileria equi* induce severe piroplasmosis in horses (Brüning, 1996). The infections with these parasites are commonly observed among cattle and horses reared not only in tropical but also in sub-temperate areas of the world (Uilenberg, 1995). The economical losses as a result of piroplasmosis are due to production losses, treatment costs, and the death of infected animals (Schnittger *et al*., 2012). *Babesia* and *Theileria* are transmitted by ticks of the Ixodidae family to the hosts, and the differences were observed in the life stages of the host animals. When sporozoites are injected into the bloodstream of the host, *Babesia* directly infects the erythrocytes, while *Theileria* infects lymphocytes (Brayton *et al*., 2007; Mehlhorn and Schein, 1998; Pain *et al*., 2005). Although numbers of chemotherapeutic agents are available to treat the infected animals, toxic side effects and possible development of drug resistance indicate the importance of introducing alternative treatment options (Gohil *et al*., 2013).

Heat shock proteins (Hsps) are found in almost all living organisms, and the Hsps play important roles in protecting cells from harmful conditions (De Maio, 1999; Santoro, 2000). Hsps are divided into several classes, such as Hsp33, Hsp60, Hsp70, Hsp90, and Hsp100, depending on their molecular mass and chaperone activity. Hsp90, which contains two binding regions, the amino and carboxyl terminals are highly conserved among classes of Hsps (Csermely *et al*., 1998). In cancer research, Hsp90 has been extensively studied as a drug target, and several drugs that target the N-terminal (novobiocin), C-terminal (geldanamycin), and co-chaperone (celastrol) of Hsp90 has been studied as specific inhibitors (Matts *et al*., 2011). In addition, Hsp90 has been identified as a drug target in protozoan parasites. A geldanamycin derivative (17AAG) inhibited the Hsp90-ATPase activity in *Plasmodium falciparum* and *Trypanosoma evansi* (Pallavi *et al*., 2010).

Gedunin, a natural product extracted from the Indian neem tree (*Azadirachta indica*), has a similar activity to that of celastrol (Matts *et al*., 2011). Gedunin directly binds to p23, the Hsp90 co-chaperone, and causes apoptotic cell death (Patwardhan *et al*., 2013). The gedunin showed antimalarial, insecticidal, and anticancer effects (Brandt *et al*., 2008; Khalid *et al*., 1989). In the present study, the effect of gedunin against *Babesia* and *Theileria* parasites are evaluated using an *in vitro* model.

MATERIALS AND METHODS

In vitro cultivation of *Babesia* parasites and *Theileria equi*

B. bovis (Texas strain), *B. bigemina* (Argentina strain), *B. caballi* (USDA strain), and *T. equi* (USDA strain) were maintained using a microaerophilic stationary-phase culture system (Igarashi *et al*., 1994). Briefly, a culture medium (Medium 199 for *B. bovis*, *B. bigemina*, and *T. equi*; RPMI 1640 for *B. caballi*) supplemented with 40% bovine or horse serum, 60 U/ml of penicillin G, 60 µg/ml of streptomycin, and 0.15 µg/ml of amphotericin B was used for the *in vitro* cultivation of the parasites. For cultivation of *T. equi*, 13.6 µg/ml of hypoxanthine was also added to the medium (Bork *et al*., 2004; Zweygarth *et al*., 1995). In culture plates containing the medium, parasitized red blood cells (RBCs) and normal bovine or equine RBCs were incubated at 37^{o}C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

In vitro growth inhibition assay

The experiment was conducted as previously described, using 96-well culture plates (Bork *et al*., 2003). Briefly, six different concentrations of gedunin, 1, 5, 10, 25, 50, and 100 µM, diluted from a working solution of 50 mM gedunin (Tocris Bioscience, UK) in dimethyl sulfoxide (DMSO) were tested for growth inhibition of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*. In addition, a medium without gedunin and a medium with DMSO were used as controls. Two hundred microliters of the medium, DMSO control, or medium with indicated drug concentrations and 20 µl of 1% parasitized RBCs were loaded into each well and incubated under appropriate conditions as described for the *in vitro* cultivation of parasites. Each experiment was performed in triplicate and repeated three times. Thin blood smears were prepared every 24 hours after initiation of the experiments until 96 hours. In addition, the medium, DMSO control, and medium with gedunin were replaced every 24 hours with fresh ones. Thin blood smears were stained with Giemsa solution and observed under a light microscope to calculate the parasitemia.

Viability test

After 96 hours, subcultures were prepared from each well and maintained using media without gedunin or DMSO for five days, and the growth of the parasites was monitored every 24 hours (Bork *et al*., 2003).

Statistical analysis

Independent Student's *t*-tests and one-way analysis of variance (ANOVA) were analyzed by using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA) to generate the differences of parasitemia percentage. *P*-values less than 0.05 were considered significant.

RESULTS

Inhibitory effect of gedunin on *Babesia* and *Theileria* parasites

The *in vitro* studies showed the ability of gedunin to inhibit the growth of *Babesia* and *Theileria* parasites. Treatment with 10 µM of gedunin significantly inhibited ($P < 0.05$) the growth of *B. bovis*, *B. caballi*, and *T. equi*, while *B. bigemina* growth was significantly suppressed in the presence of 5 µM of gedunin (Fig. 1). Complete eliminations of *B. bigemina* and *B. caballi* were observed when treated with 50 µM of gedunin, while *B. bovis* and *T. equi* required two times higher concentration. The 50% inhibitory concentration (IC_{50}) was calculated by a curve fitting technique on the day during which the parasitemia reached the maximum in DMSO-control wells (Bork *et al*., 2004). The IC_{50} values on the growth inhibition of *B. bovis* and *B. bigemina*, calculated on the third day of culture, were determined to be 21.7 µM and 15.3

μM, respectively, while the IC_{50} values for *B. caballi* and *T. equi* were 22.1 μM and 33.2 μM, respectively.

Assessment of parasite regrowth after treatment

Subsequent viability tests showed that there were no regrowth of *B. bovis*, *B. caballi*, or *T. equi* that had been treated with 50 μM of gedunin. In contrast, treatment with 25 μM of gedunin resulted in the loss of viability of *B. bigemina*. Blood-smear examinations showed severe morphological changes in *Babesia* and *Theileria* parasites after the gedunin treatment (Fig. 2). These abnormal morphologies of parasites included pyknotic and degenerative changes.

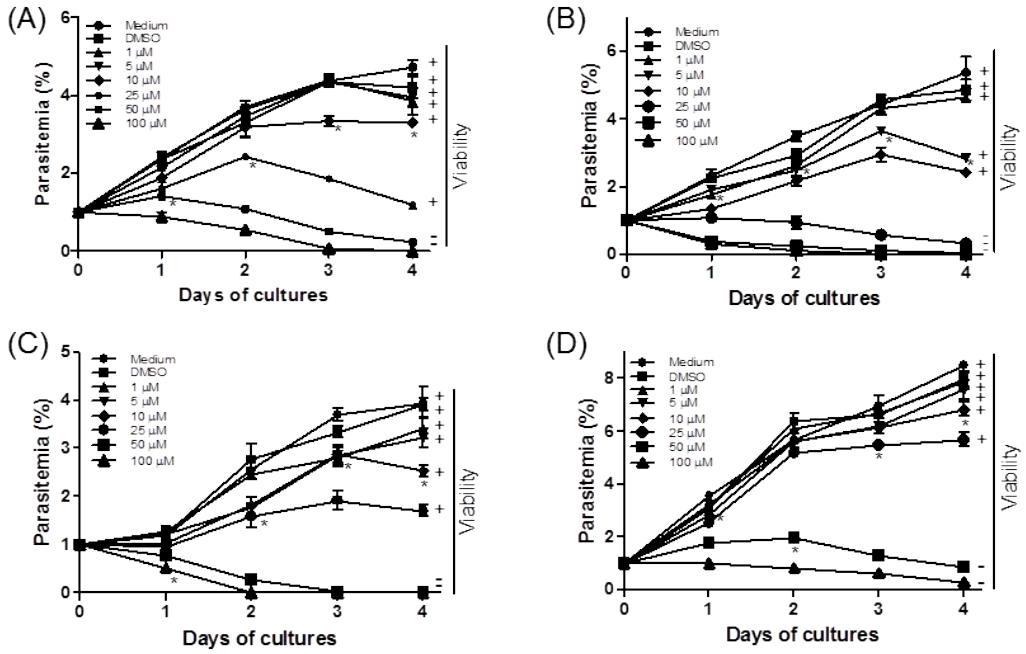

Fig. 1. Growth inhibitory effect of gedunin on *B. bovis* (A), *B. bigemina* (B), *B. caballi* (C), and *T. equi* (D). All of experiments were carried out using infected RBCs with 1% initial parasitemia for four days. Each value represents the mean ± standard deviation of three separate experiments in triplicate. Asterisks indicate a significant difference ($P < 0.05$) in parasitemia between control and drug-treated parasites. Re-growths after four days of treatment with drug-free media were indicated as viable (+) and died (-).

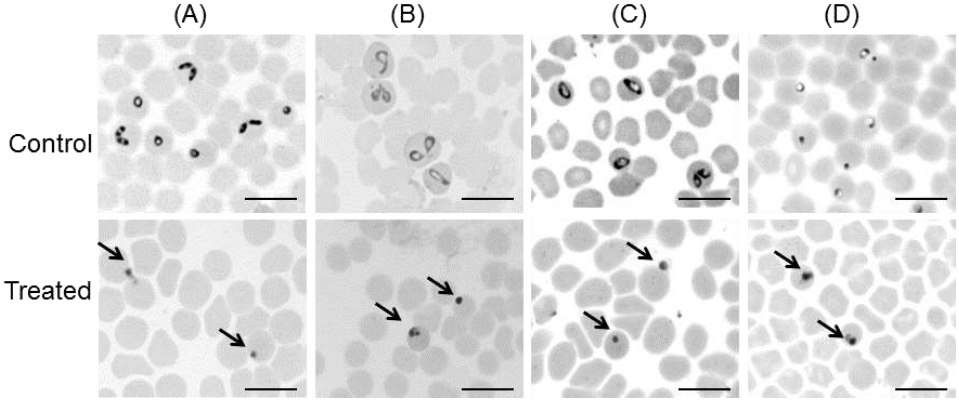

Fig. 2. The light microscopic appearance of *B. bovis* (A), *B. bigemina* (B), *B. caballi* (C), and *T. equi* (D) after 3 days of treatment with 25 μM gedunin. Note the severe morphological changes in the treated parasites compared to the controls. Arrows indicate the dot-like *Babesia* parasites and swollen *T. equi* that were treated with gedunin. Bar, 10 μm.

DISCUSSION

In recent years, several pharmacological compounds have been developed and evaluated for the treatment of babesiosis, but only a few drugs, such as imidocarb and diminazene aceturate, are currently in use (Mosqueda *et al*., 2012). The present study evaluated the inhibitory effect of gedunin on the *in vitro* growth of *Babesia* and *Theileria* parasites. Gedunin inhibited the growth of *Babesia* parasites *in vitro*, and the IC_{50} values were moderately low. The solvent dimethyl sulfoxide (DMSO) did not significantly affect the growth of *Babesia* parasites. This indicated that the inhibitory effect was due to the gedunin treatment.

Gedunin, which has the same activity as celastrol, inhibits the Hsp90 co-chaperone (Matts *et al*., 2011). A previous study reported that gedunin inhibits the function of Hsp90 by directly binding with p23, a co-chaperone protein, and causes cancer-cell death by inducing apoptosis. In addition, while moderately increased Hsp70 expression was observed in gedunin-treated cells, the expression pattern of Hsp27 remained the same (Patwardhan *et al*., 2013). Therefore, the client proteins of Hsp90 should be analyzed to demonstrate the specific activity of gedunin on *Babesia* and *Theileria* parasites. However, the client proteins of Hsp90 are yet to be identified in *Babesia* and *Theileria* spp. Alternatively, as a next step, Hsp70 and Hsp90 expression patterns in the gedunin-treated parasites must be examined.

Previously, 50% cytotoxicity concentration (CC_{50}) values of gedunin against vero cells were determined to be 440.4 µM (Misra *et al*., 2011). The IC_{50} values determined in the present study are higher than those for *P. falciparum* (1.3 – 1.6 µM) (Chianese *et al*., 2010) and cancer cells (3.2 – 8.8 µM) (Brandt *et al*., 2008). However, the IC_{50} values of gedunin against all the parasites tested in this study were well below the CC_{50} value, suggesting that gedunin might be available for safe therapeutic application against bovine and equine piroplasmosis. Although the studies on *P. falciparum* showed very low IC_{50} of gedunin against this parasite, the substance failed to provide the expected protection against the rodent malaria parasite *P. berghei*, in *in vivo* experiments (Bray *et al*., 1990). The authors suggested that the reasons for this might include the poor bioavailability after the drug administration. Therefore, it is of paramount importance to evaluate the *in vivo* effect of this chemical on *Babesia* and *Theileria* parasites. The previous studies showed that the poor bioavailability of curcumin, a natural product from *Curcuma longa* (turmeric), can be improved by adding piperine, a chemical from black pepper (Shoba *et al*., 1998). Therefore, such additives might be identified and used to increase the bioavailability of gedunin when conducting *in vivo* experiments. In conclusion, the present findings suggest that gedunin might be a potential therapeutic agent and that additional studies including *in vivo* experiments are essential.

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