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 IC₅₀ 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; Mehlihorn 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).
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°C in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂.

**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 (IC₅₀) 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 IC₅₀ 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 IC₅₀ 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. Regrowths after four days of treatment with drug-free media were indicated as viable (+) and died (-).](image)

![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.](image)
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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