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Research Briefs

Title

Babesia bovis: Effects of Cysteine Protease Inhibitors on *In Vitro* Growth

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Abstract

In the present study, we examined the effects of four kinds of cysteine protease inhibitors (E64, E64d, leupeptin, and ALLN) on the *in vitro* asexual growth of *Babesia bovis*. Of these, only the lipophilic inhibitors, E64d and ALLN, were found to effectively inhibit the growth of *B. bovis*. In further experiments, E64d, but not ALLN, significantly suppressed the parasite’s invasion of host erythrocytes, while both chemicals, especially ALLN, inhibited the parasite’s replication within the infected erythrocytes. These data suggested the presence of cysteine protease(s) derived from *B. bovis*, in which the protease(s) would play important roles in the erythrocyte invasion and/or replication processes of the parasite.

Key words: *Babesia bovis*, cysteine protease, inhibitor, invasion, replication.

Babesia bovis, an obligatory intraerythrocytic parasite of the phylum Apicomplexa, is a major causative agent of bovine babesiosis, which causes severe clinical symptoms, such as fever, anemia, and cerebral dysfunctions, in cattle due to their asexual growth (Homer *et al*., 2000). The disease often results in great economic losses in the livestock industry worldwide (Brown and Palmer, 1999), and effective strategies are desired for eradicating babesiosis.

Cysteine proteases are known to play vital roles in the growth of several protozoan parasites. In *Trypanosoma* spp., for example, the cysteine protease-specific inhibitors impair their host cell invasion and arrest the intracellular development (Meirelles *et al*., 1992) or kill the cultured blood stream forms of the parasites (Troeberg *et al*., 1999). In *Plasmodium* spp., several protozoan cysteine proteases are involved in the erythrocyte invasion (Greenbaum *et al*., 2002), the degradations of hemoglobin and erythrocyte cytoskeletal proteins (Sijwali and Rosenthal, 2004; Takakuwa, 2001), and the final erythrocyte rupture for their egression (Wickham *et al*., 2003). Cysteine protease inhibitors have been studied for the development of new chemotherapeutic measures for trypanosomiasis (Engel *et al*., 1998), malaria (Olson *et al*., 1999), schistosomiasis (Wasilewski *et al*., 1996), and leishmaniasis (Das *et al*., 2001). However, no cysteine protease has been reported in *Babesia* parasites, except for “cys1,” which is a cathepsin-like cysteine protease found in *Babesia* (*Theileria*) *equi* (Eakin *et al*., 1990; Holman *et al*., 2002; Mehlhorn and Schein, 1998).

In the present study, we examined the inhibitory effects of four commercial cysteine protease inhibitors, E64 (*trans*-Epoxy-succinyl-L-leucylamido(4-guanidino)butane), E64d ((2S,3S)-*trans*-Epoxy-succinyl-L-leucylamido-3-methylbutane ethyl ester), leupeptin, and ALLN (*N*-acetyl-leucine-leucine-norleucinal), on the *in vitro* asexual growth of cultured *B. bovis*. These inhibitors are known to inhibit thiol proteases, such as calpain and cathepsin, but only leupeptin can also inhibit serine proteases (Carole and Wang, 1998). Furthermore, E64d and ALLN have membrane permeability, while E64 and leupeptin do not (Holman *et al*., 2002). These four inhibitors have been widely used in the research of the cysteine proteases of many protozoa, including a *Plasmodium* parasite (Dahl and Rosenthal, 2005), which is closely related to the *Babesia* parasite.

The Texas strain of *B. bovis* was cultured in purified bovine erythrocytes (RBCs) using a serum-free GIT medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described previously (Bork *et al.*, 2005). The growth-inhibition test of four cysteine protease inhibitors followed previously described methods for measuring the drug activity (Okubo *et al.*, 2006a; Bork *et al.*, 2003) with some modifications. Briefly, infected RBCs were diluted with non-infected RBCs to obtain 0.5% parasitemia. Fifty μl of the infected RBC mixture was subsequently suspended in 450 μl of a culture medium supplemented with 1 (or 20) - 220 μM of E64, E64d, leupeptin, or ALLN. The suspension was placed in a 48-well culture plate (Nunc A/S, Roskilde, Denmark) and then incubated in a humidified multigas water-jacketed incubator at 37°C in 5% CO_2, 5% O_2, and 90% N_2 for 3 days. In parallel, normal cultures, which were added with the same final volume of DMSO (solvent) instead of each chemical, were prepared as the control. The culture medium was replaced daily with 450 μl of the fresh medium containing the indicated concentration of each chemical.

Out of the four tested cysteine protease inhibitors, only the lipophilic inhibitors, E64d and ALLN, significantly affected the asexual growth of *B. bovis* at the concentration of 50 μM (Fig. 1). On the other hand, the hydrophilic inhibitors, E64 and leupeptin, did not affect the growth of the parasite in the range of 20 - 200 μM (Fig. 1; data not shown). The IC_{50} values of E64d and ALLN were calculated to be 29.9 and 25.1 μM, respectively, on the basis of the inhibitory rates (%) of E64d and ALLN at various concentrations within 1 - 220 μM (Table 1). These data suggest that *B. bovis* has cysteine protease(s). On the other hand, E64 and leupeptin did not show any effects on the asexual growth of *B. bovis* in the present study. One possible explanation of the negative results is that the target cysteine protease(s) might exist in the parasite's body; therefore, the membrane non-permeable agents could not reach it. Another possible explanation is that the E64 and leupeptin inhibited the target cysteine protease(s) but the protease(s) did not play an essential role in the asexual growth of the parasite. In *P. falciparum*, for example, targeted disruption of the cysteine protease, falcipain 1, did not influence their asexual growth (Saliha *et al.*, 2004).

Next, we investigated the effects of E64d and ALLN on the erythrocyte invasion of *B. bovis* by an *in vitro* invasion test using high-voltage pulsing, as described previously (Okubo *et al.*, 2006a; Franssen *et al.*, 2003), with some modifications. Briefly, after *B. bovis*-infected RBCs were suspended in an equal volume of a GIT medium, the mixture of 400 μl was subjected to five intermittent (10 sec, 4°C) high-voltage pulses (1.5 kV, 400 Ω, 25 μF) in a BioRad Gene Pulser II (Hercules, CA, USA) with a 0.2 cm pulser cuvette (BioRad) to rupture all of the infected and non-infected RBCs exclusively and prepare extraerythrocytic (free) parasites. The samples were then suspended in a GIT medium supplemented with E64d or ALLN at the indicated concentrations (20, 50, and 100 μM) or with the same final concentration of DMSO for the control. After a low centrifugation at 700 x g for 3 min, the pellet was resuspended in the same medium supplemented with E64d, ALLN, or DMSO and then transferred into a 96-well culture plate (Nunc A/S) with non-infected RBCs at a 10% packed cell volume. After the incubation at 37°C for 1 h, the number of infected RBCs was counted out of a total 5,000 RBCs in the Giemsa-stained smears, and the invasion efficiency was calculated as the percentage of parasitemia in the culture with E64d or ALLN to that with a DMSO medium control (100%).

As a result, the erythrocyte invasion activity of the parasite was significantly inhibited in the presence of 100 and 50 μM E64d ($P = 0.010$ and 0.045, respectively) (Fig. 2A). In contrast, none of the concentrations (100, 50, and 20 μM) of ALLN showed any significant inhibitory effects relative to the value of the control (Fig. 2B). Although the other two inhibitors (E64 and leupeptin) were also tested on the invasion and following replication tests, no inhibitory effects on these activities were noted (data not shown). This finding suggests the presence of an anonymous but functional babesial cysteine protease(s) that appears to be essential for the parasite's invasion to host RBCs. In contrast, the lack of effect of ALLN might be explained by the lack of activity of this inhibitor over the particular cysteine protease(s). In *P. falciparum*, it is known that a parasite's cysteine protease, falcipain 1, plays a specific role in host cell invasion and is also inhibited by E64d (Greenbaum *et al.*, 2002; Dahl and Rosenthal, 2005). Our data will be useful for elucidating the molecular mechanism of the erythrocyte invasion of *Babesia* parasites in future.

Finally, the effects of E64d and ALLN on the intraerythrocytic replication of *B. bovis* were also evaluated using the high-voltage pulsing method described previously (Okubo *et al.*, 2006b). After high-voltage pulsing, free parasites were incubated with normal RBCs at 37°C. When the parasites were incubated for 30 min, almost all parasites invaded the RBCs and were observed to form rings on the stained smears (data not shown, Okubo *et al*., 2006b). At that time, the infected RBCs were washed with the GIT medium and then incubated with the indicated concentrations (20, 50, and 100 μM) of E64d or ALLN or with the same final concentration of DMSO for the control, at 37°C for 5 h. After the incubation, Giemsa-stained smears were prepared, and the replication activity was calculated as the ratio of the number of divided parasite-containing RBCs to the entire population of infected RBCs (100%), among which more than 200 of the infected RBCs were monitored. The divided parasite-containing RBCs are infected RBCs with more than two replicated parasites before their egression (data not shown).

As shown in Figure 3, supplements with 100 and 50 μM E64d had stronger inhibitory effects on the parasite's replication than the control culture (P = 0.0019 and 0.018, respectively) (Fig. 3A). Moreover, 100, 50, and 20 μM ALLN had more significant inhibitory effects on the replication activity (P = 0.00026, 0.00029, and 0.00038, respectively) (Fig. 3B) than E64d. In *P. falciparum*, the parasite's cysteine proteases have been reported to play a vital role in host hemoglobin degradation (Sijwali and Rosenthal, 2004). Both E64d and ALLN were reported to block the intracellular processing of two parasite's cysteine proteases, falcipain-2 and -3 (Dahl and Rosenthal, 2005), which function to degrade the host hemoglobin in the intracellular stage (Sijwali *et al.*, 2001; Shenai *et al.*, 2000). Accordingly, it is also possible that the anonymous babesial cysteine protease could digest the host hemoglobin for their metabolism even if the phenomenon has not been observed in *Babesia* parasites.

Consequently, our data strongly suggested the presence of cysteine protease(s) derived from *B. bovis*, in which the protease(s) would play important roles in the erythrocyte invasion and/or replication processes of *B. bovis*. However, since there is no report about the identification of *B. bovis* cysteine protease(s) related to their asexual growth or even the homologous proteins, the exact role of babesial cysteine protease is still not clear. Progress of the genome project is expected to lead to the elucidation of the *B. bovis* cysteine protease. Further research will be necessary to elucidate the molecular mechanism of the asexual growth cycle of *Babesia* parasites as well as for developing novel anti-babesial drugs in the future.

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Figure Legends

Fig. 1. Effects of four cysteine protease inhibitors on the *in vitro* growth of *B. bovis*. Each value represents the mean ± standard deviation (SD) in 3 wells for each 50 μM chemical (E64, E64d, leupeptin, or ALLN) in 3 separate experiments. The asterisks indicate significant differences (*P < 0.05, **P < 0.005) between the inhibitor- and DMSO control-treated parasitemia analyzed using an independent Student's t-test.

Fig. 2. Effects of E64d (**A**) and ALLN (**B**) on the erythrocyte invasion of *B. bovis*. Relative values are expressed as the percentage of the parasitemia in the culture with E64d or ALLN to that in the DMSO medium control (Con: 100%) in an *in vitro* invasion test. Each value represents the mean ± SD in 3 wells for each concentration of chemicals in 3 separate experiments. The asterisks indicate significant differences (*P < 0.05) on the values between the chemical- and DMSO control-treated cultures analyzed using an independent Student's t-test.

Fig. 3. Effects of E64d (**A**) and ALLN (**B**) on the intraerythrocytic replication of *B. bovis*. Relative values are expressed as the rates of divided parasite-containing RBCs to all infected RBCs. Each value represents the mean ± SD in 3 wells for each concentration of chemicals in 3 separate experiments. The asterisks indicate significant differences (*P < 0.05, **P < 0.005) on the values between the chemical- and DMSO control-treated cultures analyzed using an independent Student's t-test.

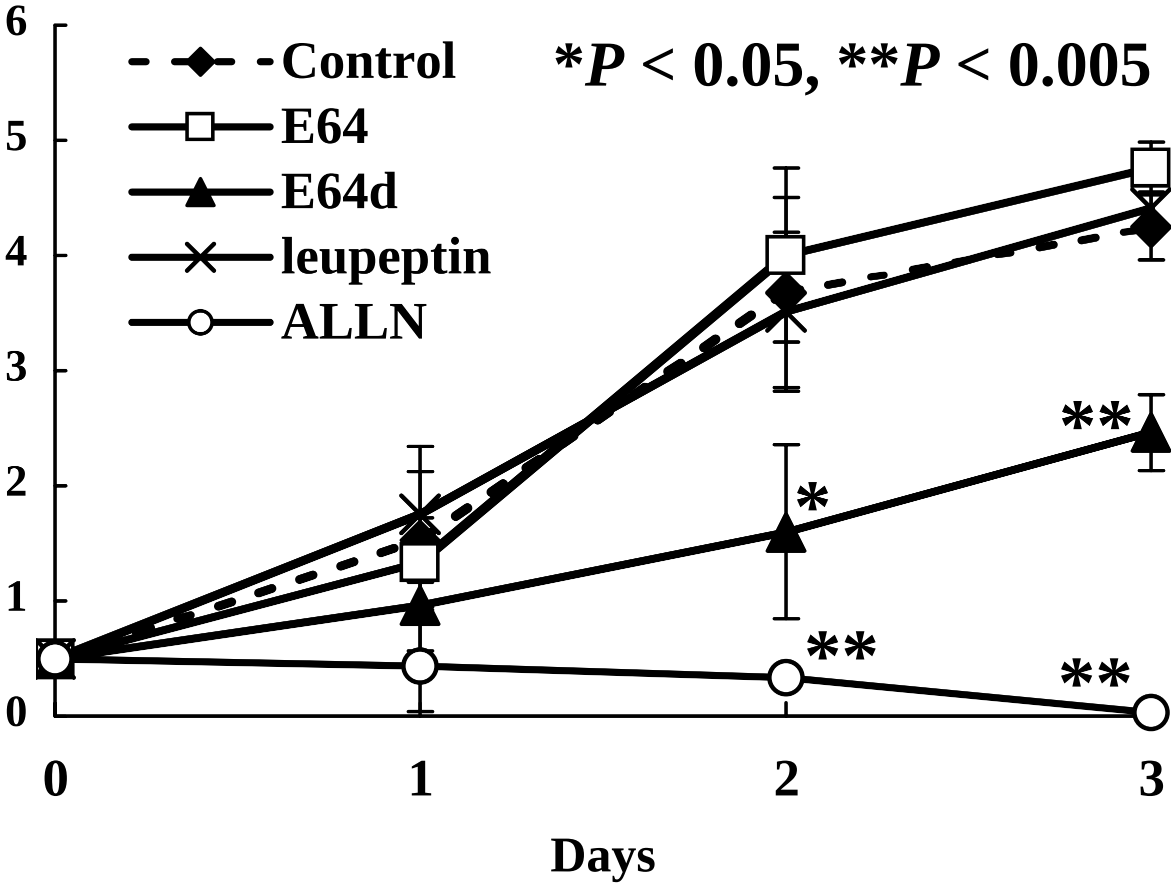

Fig. 1. Okubo *et al.*

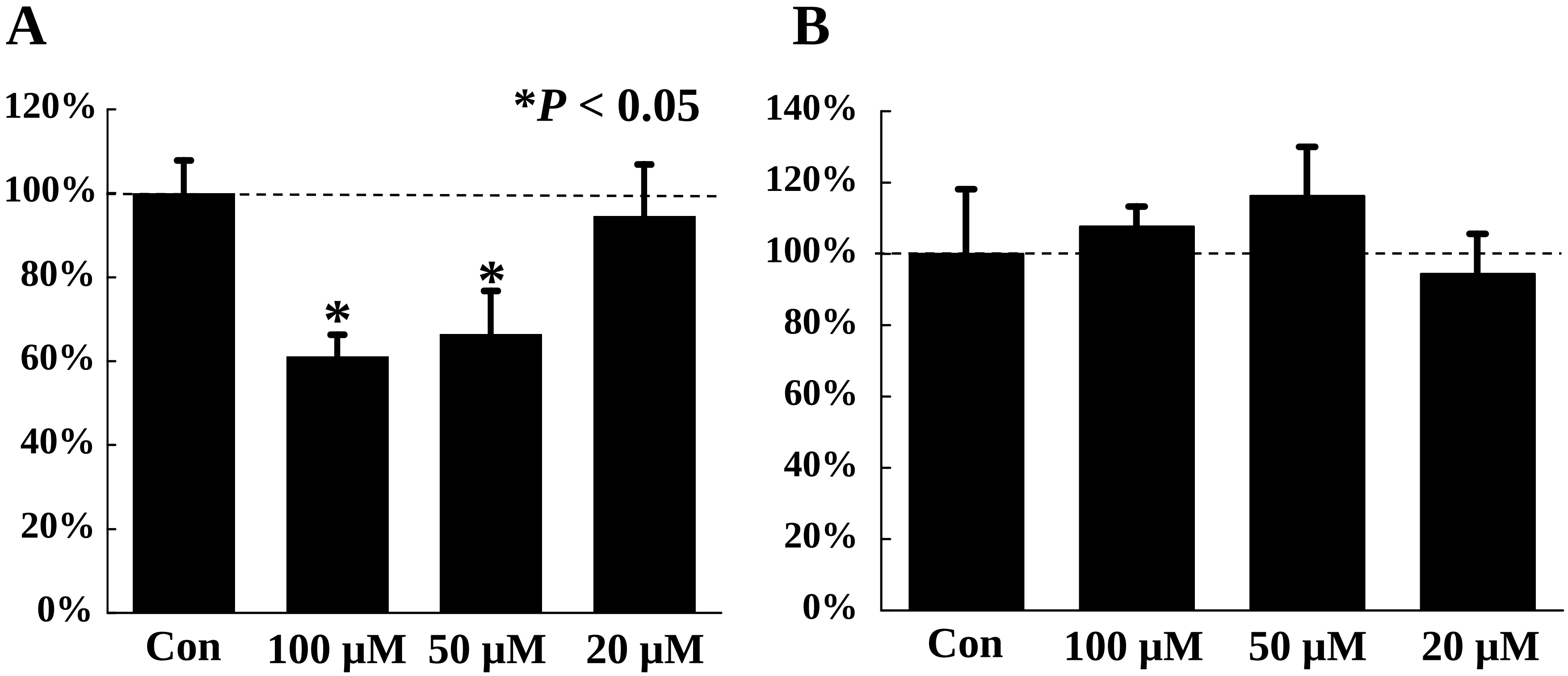

Fig. 2. Okubo *et al.*

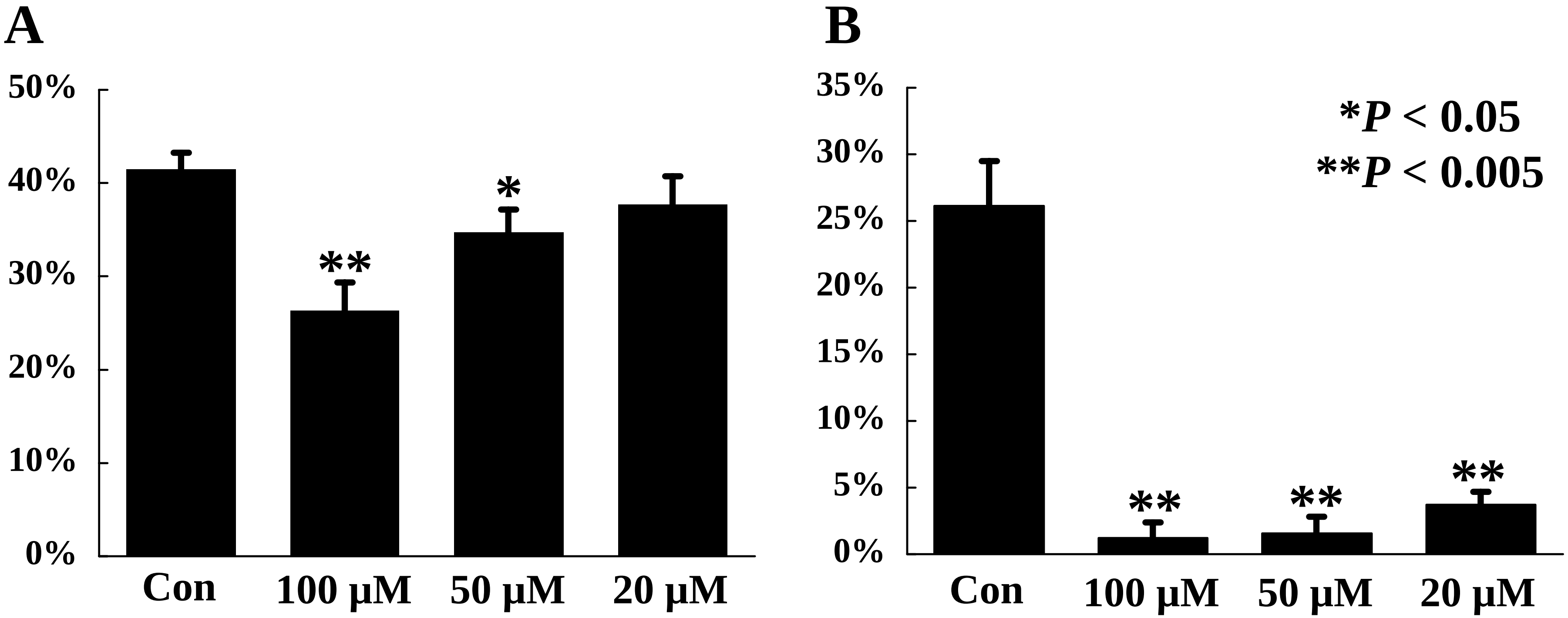

Fig. 3. Okubo *et al.*