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In vitro inhibitory effect of fosmidomycin on the asexual growth of *Babesia bovis* and *Babesia bigemina*

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ABSTRACT

The *in vitro* inhibitory effect of fosmidomycin was evaluated against the asexual growth of bovine *Babesia* parasites (*Babesia bovis* and *Babesia bigemina*). In the cultures with these parasites, severe reduction of parasite growth was observed by the addition of 1.25 μg/ml of the final drug concentration. Under these conditions, *B. bigemina* was completely cleared until the third day of cultivation. On the other hand, *B. bovis* was completely eliminated on the fourth day when the concentration was 6.25 μg/ml in the culture. These parasites failed to grow again when a drug-free medium was substituted for the subsequent cultivation. The IC_{50} values of fosmidomycin against *B. bovis* were determined as 0.88 and 0.63 μg/ml in the *in vitro* cultures with serum-containing M199 and serum-free GIT media, respectively, while it was 0.55 μg/ml against *B. bigemina* in a serum-containing M199 medium. Severe morphological changes, such as pycnotic and degenerative changes, were preferably observed in the treated parasites. These results suggest that fosmidomycin can be a potent chemotherapeutic agent against bovine babesiosis.

Key words: Fosmidomycin, *Babesia bovis*, *Babesia bigemina*, asexual growth, *in vitro* culture

INTRODUCTION

Babesiosis is an intraerythrocytic protozoan disease caused by members of the genus *Babesia* and transmitted by ixodid ticks (Homer *et al*., 2000). The *Babesia* parasites infect many wild and domestic animals and induce babesiosis. Among them, bovine babesiosis is mainly caused by *Babesia bovis* and *B. bigemina* and often leads to enormous economic losses to the livestock industry in tropical and subtropical regions of the world (De Waal and Combrink, 2006). At present, over half a billion of the world's cattle population is at risk for babesiosis (McCosker, 1981). The clinical symptoms of bovine babesiosis vary depending on the causative species of *Babesia*. The clinical signs of *B. bigemina* infection are fever, hemoglobinuria, and acute anemia, and parasitemia is often as high as 30% in infected cattle. On the other hand, in addition to the above clinical signs, cerebral or nervous signs characterize the *B. bovis* infection with lower parasitemia (Shkap *et al*., 2005). Both infections tend to be very dangerous if the infected cattle do not receive any chemotherapeutic treatment. The strategies adopted to control the disease can be categorized into three: vaccination, vector control, and chemotherapy (Ralph *et al*., 2001). Although several antibabesial drugs have been used to reduce the development of the clinical disease, serious problems presented by babesiosis remain unchanged (Bork *et al*., 2003). Therefore, continuous search for effective new drugs is essential in order to control babesiosis. Several novel antibabesial drugs, such as triclosan (Bork *et al*., 2003), artesunate, pyrimethamine, pamaquine (Nagai *et al*., 2003), heparin (Bork *et al*., 2004), imidazole derivatives,

staurosporine (Bork *et al*., 2006), cysteine protease inhibitors (Okubo *et al*., 2007), and atovaquone and azithromycin (Krause *et al*., 2000), have been successfully studied by using both *in vitro* and *in vivo* models.

Recently, fosmidomycin was tested as a novel antiprotozoan agent against *Plasmodium faliciparum*, which is a causative pathogen for human malaria (Jomaa *et al*., 1999). Fosmidomycin, originally known as a phosphonic acid antibiotic, was isolated from *Streptomyces lavendulae* and reported to show antibacterial activity against most Gram-negative bacteria (Murakawa *et al*., 1982). Afterward, it was successfully used in chemotherapeutic studies against bacterial infection in the urinary tract (Kanimoto and Greenwood, 1987). The previous studies demonstrated that fosmidomycin has very low toxicity and the drug was well tolerated by human even when given in the repeated doses of 8 g per day intravenously for 7 days (Kuemmerle *et al*., 1987). Interestingly, Jomaa *et al*. (1999) demonstrated that fosmidomycin is also active against *Plasmodium* parasites. fosmidomycin and its derivative, FR-900098, were found to inhibit the *in vitro* growth of *P. falciparum* in culture (Jomaa *et al*., 1999) as well as the *in vivo* growth of *P. vinkei* in infected mice (Ortmann *et al*., 2003). Furthermore, clinical trials using fosmidomycin have been conducted in Thailand, and the results have proved the efficacy and safety of fosmidomycin in therapeutic application against human malaria (Wiesner *et al*., 2002; Lell *et al*., 2003).

Babesia and *Plasmodium* parasites share many common features, and several antimalarial agents have been successfully tested for the *Babesia* parasites. Therefore, the present study was designed to evaluate the possible inhibitory effect of fosmidomycin on the growth of *B. bovis* and *B. bigemina* by determining their *in vitro* dynamics.

MATERIALS AND METHODS

In vitro cultivation of *Babesia* parasites

The Texas strain of *B. bovis* (Hines *et al*., 1992) and the Argentina strain of *B. bigemina* (Hotzel *et al*., 1997) were used in the present study. The parasites were grown in bovine red blood cells (RBC) using a previously established continuous micro-aerophilous stationary phase culture system (Igarashi *et al*., 1998; Vega *et al*., 1985). For the cultivation of *B. bovis*, two types of culture media, M199 and GIT (both from Sigma-Aldrich, Tokyo, Japan), were used. The M199-based cultured medium was prepared by supplementing with 40% bovine serum (BS). In contrast, the GIT medium was previously established in order to grow the *B. bovis* even in the absence of BS (Bork *et al*., 2005; Jackson *et a*l., 2001). On the other hand, *B. bigemina* was grown only in the M199 culture medium with 40% BS. Culture plates for these parasites were incubated under 5% CO_2 and 5% O_2 at 37˚C (Bork *et al*., 2003).

Growth inhibitory assay

An *in vitro* growth inhibitory assay was adopted as described previously (Bork *et al*., 2003; Brockelman and Tan-ariya 1991; Igarashi *et al*., 1998). Fosmidomycin (Invitrogen, Carlsbad, CA, USA) was dissolved in autoclaved distilled water to achieve the concentration of 2 mg/ml and then kept at -20˚C until use. *Babesia bigemina*- and *B. bovis*-infected RBC were obtained from the cultures with parasitemia of approximately 6%. The parasitemia of all starting cultures used for drug evaluation was set to 1% by adding non-infected bovine RBC. Ninety six-well culture plates were used to conduct the inhibitory assay. Twenty micro liters of bovine RBC with 1% parasitemia and 200 µl of the indicated culture medium with the specific drug concentration were dispensed to each well and then incubated under above mentioned atmosphere. Culture media were replaced every 24 hours with 200 µl of fresh media containing the specific drug concentration. During the 4-day cultivation, blood smears were prepared from the cultures every 24 hours and then stained with a Giemsa

solution to observe the parasites under a light microscope. Parasitemia was calculated on the basis of the number of infected RBC among 1,000 total RBC (Bork *et al*., 2003). In addition, morphological changes were also observed after a 2-day cultivation. In a preliminary experiment, Fosmidomycin of 0.5, 1, 5, 10, 25, and 50 μg/ml was first evaluated against *B. bovis*. From the results, concentrations of 0.01, 0.05, 0.25, 1.25, 6.25, and 31.25 μg/ml of Fosmidomycin were tested against the growth of both *B. bovis* and *B. bigemina*. Drug-free culture media were used as the controls. The experiments were conducted in triplicate and repeated three times for each parasite.

Viability test

After the fourth day of treatment, 6 μl of the cultured RBC was collected from the treated and control cultures and re-incubated with 14 μl of non-infected RBC and 200 μl of drug-free indicated media in new wells. Media were subsequently replaced with fresh ones every 24 hours, and blood smears were also prepared. Giemsa-stained smears were observed under a light microscope to judge the presence of grown parasites until 5 days after the subsequent cultivation (Bork *et al*., 2003; Nagai *et al*., 2003).

Statistical analysis

Results were statistically analyzed using a Student's *t* test at $p < 0.05$ to find out any significant differences in parasitemia.

RESULTS

Effect of fosmidomycin on the *in vitro* growth of bovine *Babesia* parasites

Severe reductions of parasitemia were observed in the *in vitro* growth of *B. bovis* and *B. bigemina* when the cultures were treated with 1.25 μg/ml of fosmidomycin, as shown in Figures 1A and B, respectively. Complete inhibition was observed in the growth of *B. bigemina* on the third day of treatment with 1.25 μg/ml (Panel B), while *B. bovis* was completely suppressed on the fourth day of treatment with 6.25 μg/ml (Panel A). In addition, neither parasite re-grew when drug-free media were substituted from the treated media with 1.25, 6.25, and 31.25 μg/ml of the drug (Fig. 1). Furthermore, there were no significant differences in the inhibitory effect of fosmidomycin on the growth of *B. bovis* between serum-containing M199 and serum-free GIT media (data not shown).

From the growth rates, 50% inhibitory concentrations (IC_{50}) of fosmidomycin were calculated on the basis of the parasitemia dynamics under the indicated drug concentrations when control cultures without any drug exhibited the maximum growth of parasites (Bork *et al*., 2004). The IC_{50} values of fosmidomycin against *B. bovis* and *B. bigemina* in serum-containing M199 media were determined as 0.88 μg/ml (Panel A) and 0.55 μg/ml (Panel B), respectively, while that against *B. bovis* in serum-free GIT media was 0.63 μg/ml (data not shown).

Morphological changes of fosmidomycin-treated parasites

When the Giemsa-stained blood smears of the treated cultures were examined under a light microscope, severe changes were observed in the characteristic shapes of *Babesia* parasites (Figs. 2B and D) from the control morphology (Figs. 2A and C). Many of the treated parasites were observed to be devoid of internal structures, showing picnotic and degenerative appearances.

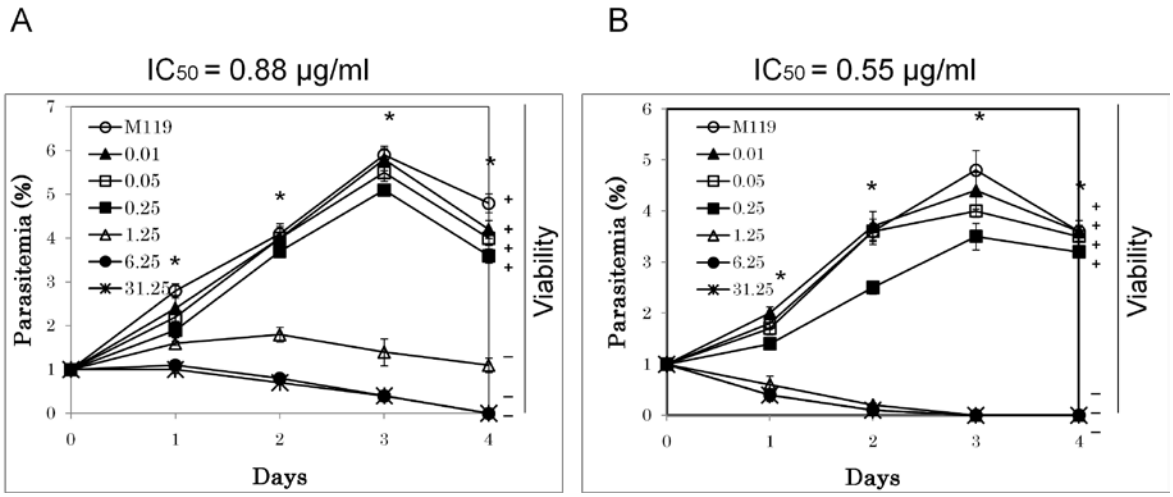

Fig. 1. Growth rates of *Babesia bovis* (A) and *Babesia bigemina* (B) in the presence of various concentrations of fosmidomycin with initial parasitemia of 1% in serum-containing M199 media. Each value shows the mean ± standard deviation (SD) of triplicate experiments. Parasite viability was monitored in the subcultures for 5 days in the absence of the drug: + = viable; - = dead. Asterisks indicate a significant difference ($P< 0.05$) between the 1.25 μg/ml fosmidomycin-treated and control groups.

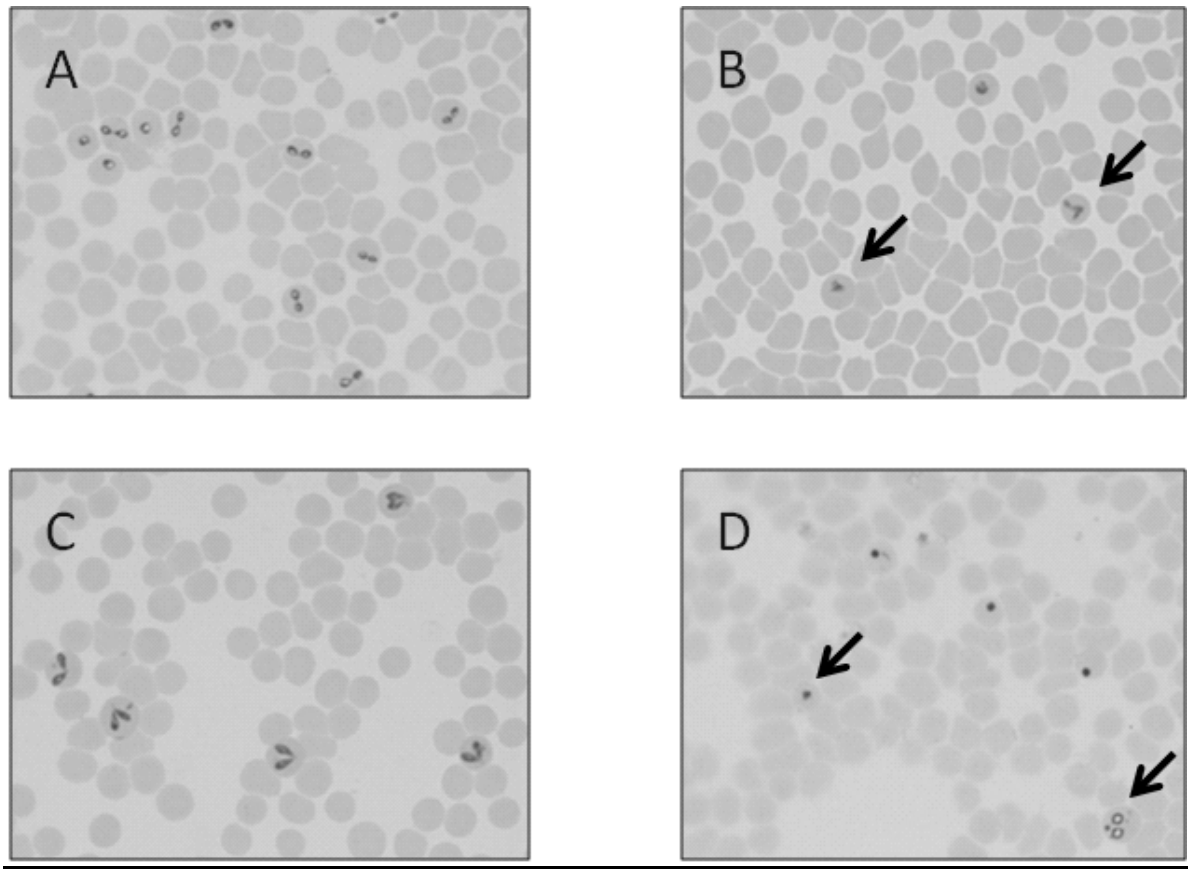

Fig. 2. Morphological changes in 1.25 μg/ml fosmidomycin-treated *Babesia bovis* (B) and *Babesia bigemina* (D) after a 2-day cultivation in comparison to the controls (A and C, respectively). Note the severe morphological changes of the treated parasites, as indicated by arrows.

DISCUSSION

Apicomplexan protozoa, including *Babesia* parasites, acquire a non-photosynthetic plastid, called an apicoplast, which contains an extrachromosomal DNA (McFadden and Roos, 1999). The apicoplast is likely derived from the ancestral endosysbiosis of *Cyanobacteria*, as all other plastids (Ralph *et al*., 2004). Because of the cyanobacterial heritage, many prokaryote-like and plant-like enzymes, which are different from those of the mammalian host, are present in the apicoplast of parasites (Gleeson, 2000). Therefore, the relative biological pathways can become excellent targets for drug construction (Waller and McFadden, 2005). Although the functions of the apicoplast are not well understood, it is clear that the apicoplast is indispensable for apicomplexan parasites (Ralph *et al*., 2001). The metabolic pathways in the apicoplast of *P. falciparum* are known to include heme biosynthesis (van Dooren *et al*., 2002), fatty acid biosynthesis (Waller *et al*., 1998), and isoprenoid precursor synthesis via the non-mevalonate pathway (Jomaa *et al*., 1999). From the genome project of *B. bovis*, only the relative enzymes to isoprenoid biosynthesis were found in the apicoplast of *B. bovis* (Brayton *et al*., 2007).

Isoprenoids, which consist of C5 isoprenoid units (Rohmer, 1999), influence many aspects of the metabolism, function, and membrane structure in living organisms (Wanke *et al*., 2001). In mammals and fungi, isopentenyl diphosphate (IPP), which is the universal isoprenoid precursor, is derived from the mevalonate pathway (Beytia and Porter, 1976). However, an alternative non-mevalonate pathway, which is also known as the methylerythritol phosphate or 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, is also present in bacteria, algae, and chloroplasts of higher plants (Rohmer *et al*., 1993). Importantly, the malaria genome project also identified the presence of the DOXP pathway in the apicoplast of *P. falciparum* (Jomaa *et al*., 1999). The initial step of the DOXP pathway is the formation of DOXP by the condensation of pyruvate and glyceraldehyde 3-phosphate. This reaction is catalyzed by DOXP synthase. Subsequently, DOXP is converted into a 2-C-methyl-D-erythritol 4-phosphate by the second enzyme, called “DOXP reductoisomerase” (Rohmer, 1999). Recent studies conducted against *Plasmodium* parasites indicated that fosmidomycin functions as a potent inhibitor of the enzyme, “DOXP reductoisomerase” (Jomma *et al*., 1999).

In the present study, fosmidomycin significantly inhibited the *in vitro* asexual growth of bovine *Babesia* parasites (*B. bovis* and *B. bigemina*). The IC50 values of fosmidomycin against these parasites (0.88 and 0.55 μg/ml, respectively) were slightly higher than those of previously tested *P. falciparum* strains (0.071 - 0.17 μg/ml) (Wiesner *et al*., 2002). However, in subsequent viability results with the cultures that had been treated with 1.25 μg/ml of fosmidomycin, the parasites failed to re-grow, indicating that the damage made by the drug was irreversible. The abnormal morphology of fosmidomycin-treated *Babesia* parasites also suggests that the drug has a certain inhibitory effect against bovine *Babesia* parasites. In contrast to our present results using bovine *Babesia* parasites, other studies, which were carried out with fosmidomycin against the *in vitro* growth of *Eimeria tenella* and *Toxaplasma gondii*, yielded poor results even at high concentrations of the drug (Clastre *et al*., 2007). Our present experiment clearly shows that fosmidomycin is a promising anti-babesial agent, since the safety and low toxicity of fosmidomycin have been proved in previous studies (Kuemmerle *et al*., 1987). However, *in vivo* experiments are necessary to reach any strong conclusions. In addition, previous trials indicated that a combination therapy of fosmidomycin with clindamycin was more effective than a mono-therapy against human malaria (Na-Bangchang *et al*., 2007). Therefore, further studies should be conducted to evaluate the efficacy of a combination therapy of fosmidomycin with other drugs.

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