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Evaluation of inhibitory effects of dipyridamole against bovine and equine piroplasmosis

Tuvshintulga, B., Sivakumar, T., Yokoyama, N. and Igarashi, I.*

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
*Corresponding author: Ikuo Igarashi; E-mail: igarcpmi@obihiro.ac.jp

ABSTRACT

Dipyridamole, an antiplatelet drug used for the secondary prevention and treatment of stroke, also has antiplasmodial activity and enhances the activity of chloroquine. We evaluated the inhibitory effects of dipyridamole on the growths of *Babesia* and *Theileria* parasites. The growth of *B. bovis* and *T. equi* was significantly inhibited at a 25 μM concentration of dipyridamole, while *B. bigemina* and *B. caballi* were significantly inhibited at 50 μM and 1 μM concentrations of dipyridamole, respectively, on day 3 of cultivation. The half maximal inhibitory concentration (IC_{50}) of dipyridamole was calculated at 39.5±4.5, 26.3±10.9, 13.2±3.6, and 23.5±0.5 μM on the growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*, respectively. In a viability assay, *B. bovis*, *B. bigemina*, and *T. equi* failed to grow at the previous treatment concentration of 100 μM of dipyridamole, while *B. caballi* had not grown at the previous treatment concentration of 50 μM of dipyridamole. As for *in vivo* inhibitory assay, treatment with 100 mg/kg of body weight dose of dipyridamole could not inhibit the *in vivo* growth of *B. microti*. In conclusion, dipyridamole might be used as a chemotherapeutic agent against bovine and equine piroplasm parasites. However, dipyridamole could not orally treat against *B. microti* infection.

Keywords: dipyridamole; *Babesia*; *Theileria*; *in vitro*; *in vivo*

INTRODUCTION

Bovine babesiosis and equine piroplasmoses are widely distributed worldwide, especially in tropical and subtropical regions. The cattle industry is vulnerable to costly problems and huge economic losses due to *Babesia bovis*, *B. bigemina*, and *B. divergens* (Bock *et al*., 2004), and the movement of horses between countries is restricted by infections of *B. caballi* and *Theileria equi* (Wise *et al*., 2013). These parasites are transmitted by hard ticks, and chemically controlling tick is the major method of preventing bovine and equine piroplasmoses. Nevertheless, tick control requires a large financial investment and prerequisites for success (Pegram *et al*., 2000). Accordingly, a highly protective vaccine and potent chemotherapy are needed for the prevention and treatment of bovine babesiosis and equine piroplasmoses. A vaccine is available only for bovine babesiosis; however, problems of vaccine production, such as storage and transportation for the attenuated vaccine and highly variable genetic diversity among piroplasmosis for recombinant and subunit vaccines, have not been completely solved (Bock *et al*., 2004). Harmful side effects, including pain at the injection site and residue in treated animals and relapse infection after treatment, have sometimes been reported with currently available drugs, such as diminazene aceturate and imidocarb dipropionate (Mosqueda *et al*., 2012). Therefore, potent chemotherapies with low side effects are needed to treat bovine and equine piroplasmoses.

Dipyridamole (DP) has long been used as a human medicine for the secondary prevention and treatment of stroke. This drug possesses antimalarial, antitumor, antileishmanial, and antitrypanosomal activities (Akaki *et al*., 2002a; 2002b; Hung *et al*., 2001; Johner *et al*., 2006; Seebeck *et al*., 2011). Regarding the selective potency of DP, it has been reported that the half maximal inhibitory concentration (IC_{50}) was calculated at 14 μM with the phosphodiesterase inhibition of *Trypanosoma brucei* (Seebeck *et al*., 2011), although DP has shown inhibitory effects on *Plasmodium falciparum* based on parasitic development in erythrocytes (Akaki *et al*., 2002b). It has also been reported that the apical ends of *P. falciparum* are changed by DP to inhibit the invasion of merozoites (Akaki *et al*., 2002a), a DP action whose mechanism is not fully understood. Moreover, 10 μM DP inhibits adenosine uptake by approximately 80%; however, equilibrative nucleoside transport is unknown (Frame *et al*., 2012). Purine bases, including adenine and guanine, might play a critical role in parasite biology (el Kouni, 2003), as the purine salvage pathway is incomplete in *B. bovis*, whereas adenosine kinases are absent in some *Theileria* parasites (Brayton *et al*., 2007). In the present study, we evaluated the growth-inhibiting effect of DP against *Babesia* and *Theileria* parasites.

MATERIALS AND METHODS

Parasites

In vitro growth of *B. bovis* (Texas strain), *B. bigemina* (Argentina strain), *B. caballi* (USDA strain), and *T. equi* (USDA strain) and *in vivo* growth of *B. microti* (Munich strain) were used.

In vitro growth inhibition assay

In vitro inhibitory assay was performed using 6 different concentrations (0.1, 1, 10, 25, 50, and 100 μM) of DP, following a previously described method with minor modification (Tuvshintulga *et al*., 2015). Twenty μl of infected RBCs with 1% parasitemia was added to 200 μl of media containing various concentrations of DP (P/N: D9766-1G; Sigma-Aldrich, Tokyo, Japan), 60% M199 medium for *B. bovis*, *B. bigemina*, and *T. equi*; RPMI 1640 medium for *B. caballi*; 40% bovine for *B. bovis* and *B. bigemina* or equine sera for *B. caballi* and *T. equi*; and 60 U/ml of penicillin G, 60 μg/ml of streptomycin, and 0.15 μg/ml of amphotericin B for all of the parasites. Only 13.6 μg/ml of hypoxanthine was supplemented into media for *T. equi*. In addition, fresh media and media with 0.1% dimethyl sulfoxide (DMSO) were used as controls. All reagents and chemicals were purchased from Sigma-Aldrich. Each mixture was added to a 96-well plate in triplicate. Parasites were cultivated at 37°C in 5% CO_2 and 5% O_2 in a humidified multigas water-jacketed incubator. The media were changed, and the parasitemia was monitored every day for 4 days using Giemsa-stained RBC smears. Approximately 2,000 RBCs were counted each smear for estimation of parasitemia. A less than 0.05 score of *P* value was considered as a statistical significant difference between treated and untreated groups using a Student's *t-test* analysis (unpaired two-tails method) in Microsoft Excel 2007.

Viability test

In the viability assay, parasites were sub-cultivated to add to fresh media containing 16 μl of fresh RBCs by 6 μl of previously treated infected RBCs. Parasitemia was monitored every day for 10 days by Giemsa-stained RBC smears.

***In vivo* growth inhibition assay**

For the *in vivo* inhibitory assay, 10 million *B. microti*-infected RBCs were intraperitoneally injected in 15 female BALB/c mice when mice were 8-week-old (CLEA, Tokyo, Japan). Mice were divided into 3 groups of 5 mice each. When parasitemia reached 1% in all mice, the first and second groups were orally administrated 0.2 ml of a 0.1% DMSO solution and a 100 mg/kg body weight dose of DP, while the third group was intraperitoneally injected with a 25 mg/kg body weight dose of diminazene aceturate for 5 consecutive days between day 3 to 7 post-infection. Parasitemia in all mice was monitored every 2 days for 32 days by Giemsa-stained blood smears. The effect of the drug was statistically analyzed between untreated and treated groups. All animal experiments were carried out in accordance with the ethical standards relating to the care and management of experimental animals set by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (Animal experimental approval number: 25-152).

Table 1. IC_{50} values of dipyridamole on the growth of parasites

Parasites	IC_{50} (μM)
B. bovis	39.5±4.5
B. bigemina	26.3±10.9
B. caballi	13.2±3.6
T. equi	23.5±0.5
P. falciparum	0.03[a]
Leishmania major (promastigote)	44.7±12.2 μM[b]

[a]Akaki *et al*., 2002a; [b]Johner *et al*., 2006

RESULTS AND DISCUSSION

Growth of *B. bovis* and *T. equi* was significantly inhibited by a 25 μM concentration of DP, while *B. bigemina* and *B. caballi* were significantly inhibited by 50 μM and 1 μM concentrations of DP, respectively, on the third day of cultivation (Fig. 1). The loss of typical shapes was observed in 100 μM-treated parasites on the second day of cultivation as compared to the untreated control groups (Figs. 2 and 3), which the loss of typical shape of parasite into RBC was distinguished when object as a parasite is stained with purple (nucleic acid) and lilac (cytoplasm) coloration. The IC_{50} values of DP on the growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were estimated to be 39.5±4.5, 26.3±10.9, 13.2±3.6, and 23.5±0.5 μM, respectively, on day 3 of cultivation (Table 1). Although *B. caballi* was most susceptible to inhibition by DP as compared to the other tested species in this study, the IC_{50} value of DP on *B. caballi* was approximately 440 times higher than the IC_{50} value of DP on *Plasmodium falciparum* (Table 1; Akaki *et al*., 2002b), whereas the IC_{50} value of DP on *B. bovis*, which is the highest value in this study, was similar to the IC_{50} of DP on *Leishmania major* (Johner *et al*., 2006). In a viability assay, *B. bovis*, *B. bigemina*, and *T. equi* previously treated with 100 μM DP failed to grow in fresh media, whereas *B. caballi* previously treated with 50 μM DP did not grow (Fig. 1). Our predication that DP could inhibit growth of parasites, was confirmed, suggesting that it might be

related to the inhibition of adenosine transporters in *Babesia* and *Theileria* parasites (Brayton *et al*., 2007). However, the mode of dipyridamole action on these parasites should be confirmed in future studies. The *in vivo* growth of *B. microti* was not inhibited by a 100-mg/kg body weight dose of DP administered orally (Fig. 4). However, the peak parasitemia was observed in DP-treated mice on day 10 post-infection, while that was in untreated mice on day 8 post-infection. It is likely that the time of peak parasitemia in mice was delayed by DP treatment. On the other hand, a high dose of DP might be required to inhibit the growth of *B. microti* in mice; however, a limited LD_{50} value of DP at 700 mg/kg in normal B6D2F1 mice was reported (Barberl-Heyob *et al*., 1992). In addition, when using oral administration, the drug can be destroyed by gastric juices; therefore, DP might not be administered orally. In conclusion, we evaluated the inhibitory effect of DP on four piroplasm parasites *in vitro* and *B. microti in vivo* and determined that DP might be an acceptable chemotherapeutic agent against bovine babesiosis and equine piroplasmosis. However, the oral administration of DP is an improper treatment against *B. microti* infection.

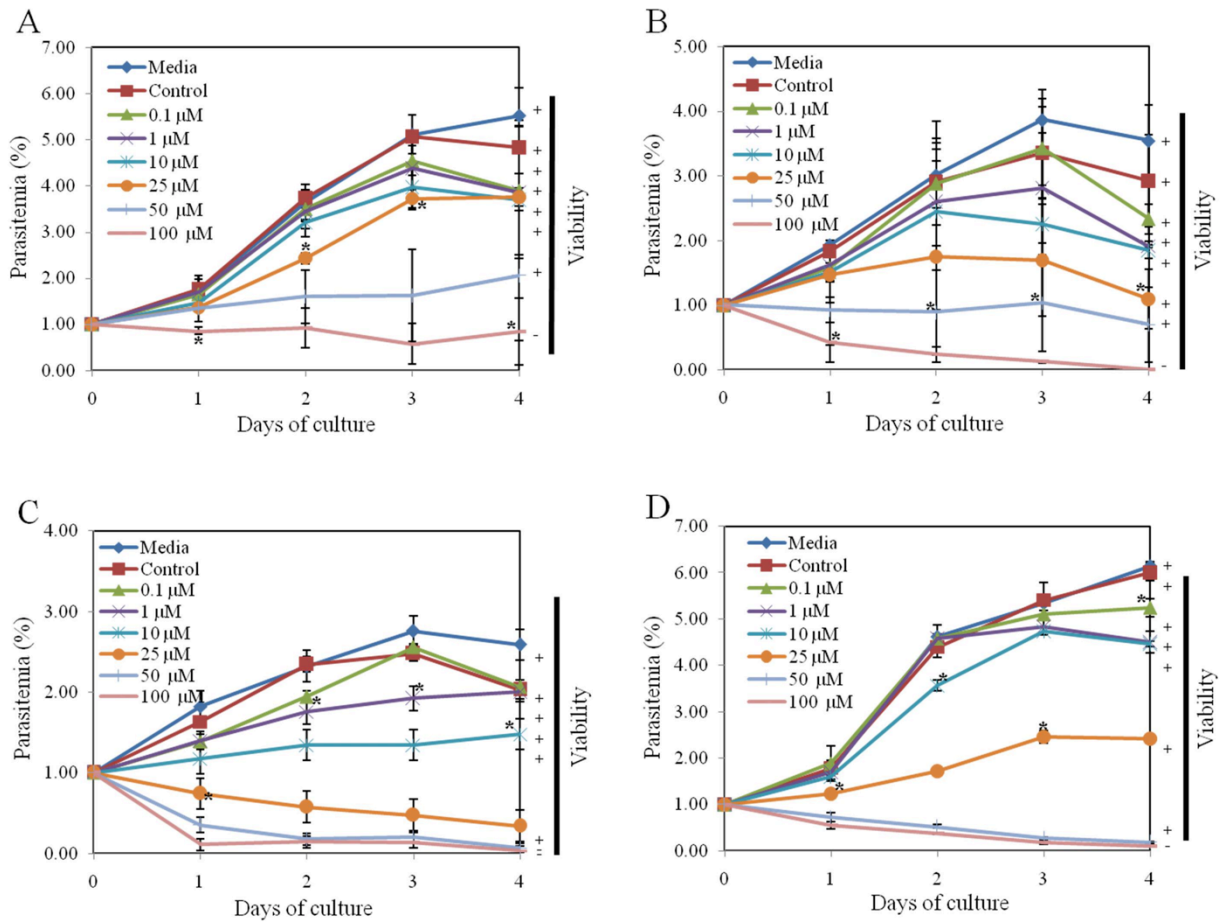

Fig. 1. *In vitro* growth of *B. bovis* (A), *B. bigemina* (B), *B. caballi* (C), and *T. equi* (D) was inhibited by various concentrations of dipyridamole. Three separate trials were analyzed to obtain the standard deviation. Asterisks indicate statistically significant differences ($P<0.05$) in comparison.

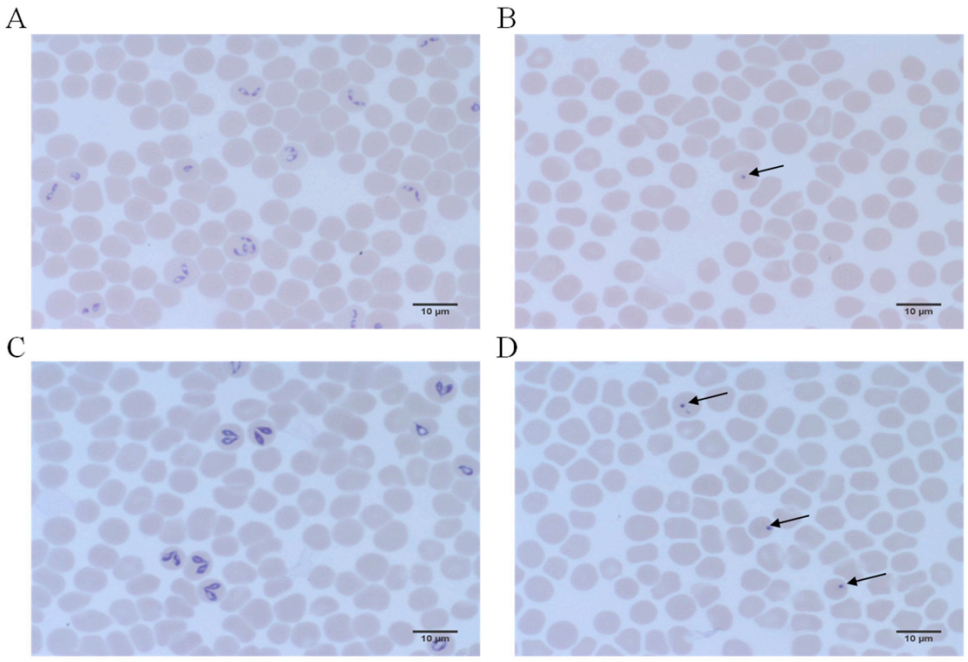

Fig. 2. *In vitro* growth of *B*. *bovis* (A) and *B*. *bigemina* (C) under no-treatement and *in vitro* growth of *B*. *bovis* (B) and *B*. *bigemina* (D) under treatment with a 100-μM concentration of dipyridamole. Arrows indicate some parasites that have lost their typical shape.

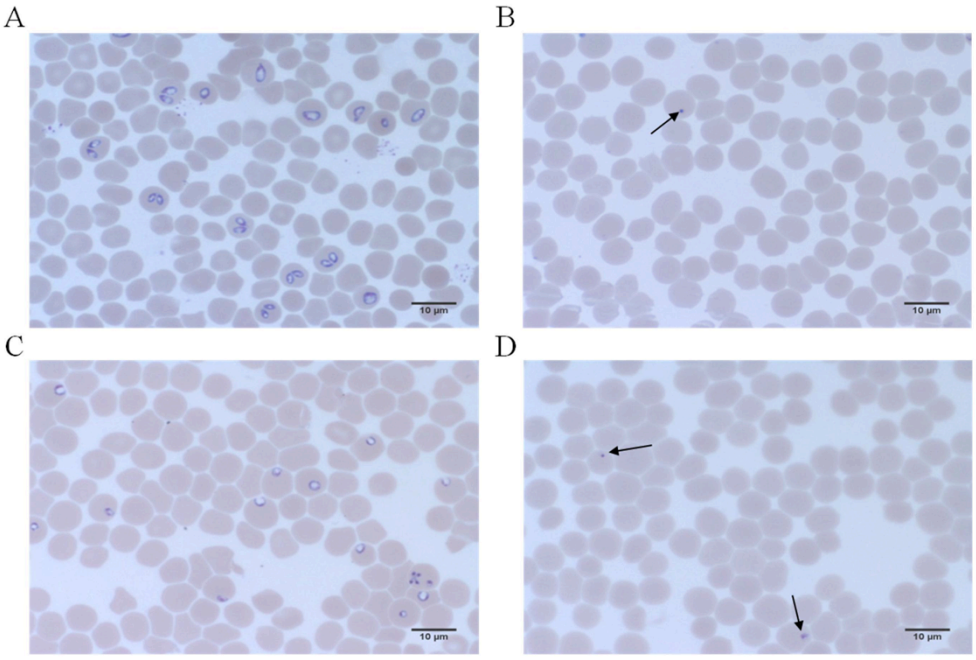

Fig. 3. *In vitro* growth of *B*. *caballi* (A) and *T*. *equi* (C) under no-treatement and *in vitro* growth of *B*. *caballi* (B) and *T*. *equi* (D) under treatment with a 100-μM concentration of dipyridamole. Arrows indicate some parasites that have lost their typical shape.

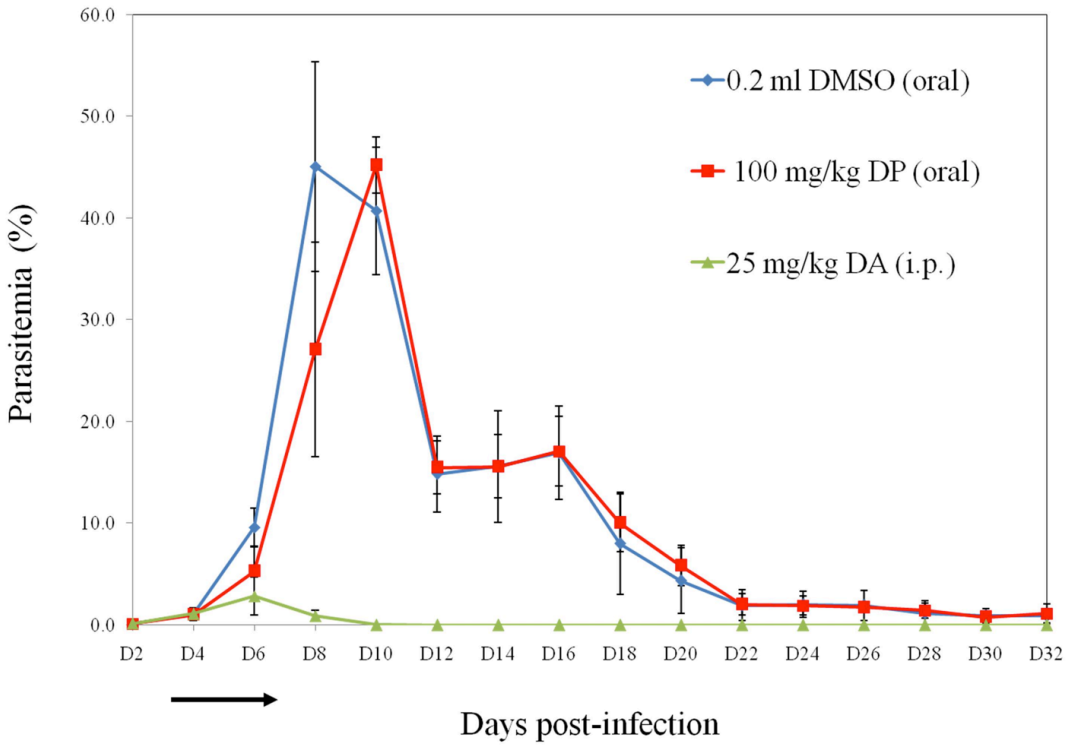

Fig. 4. *In vivo* growth of *B. microti* in mice treated with a 100-mg/kg body weight dose of dipyridamole (DP), a 25-mg/kg body weight dose of diminazene aceturate (DA), and 0.2 ml of 0.1% DMSO. The arrow with one head indicates 5 consecutive days of treatment on day 3 to 7 post-infection.

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CONFLICT OF INTEREST

All authors declare no actual or potential conflicts of interest.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript is approved by all authors, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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