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Evaluation of *in vitro* and *in vivo* inhibitory effects of fusidic acid on *Babesia* and *Theileria* parasites

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ABSTRACT

Fusidic acid known to has antibacterial, antifungal, and antimalarial activities. Fusidic acid blocks translation elongation factor G gene in *Plasmodium falciparum*. In the present study, the inhibitory effects of fusidic acid on the *in vitro* growth of bovine and equine *Babesia* parasites were evaluated. The inhibitory effect of fusidic acid on the *in vivo* growth of *Babesia microti* was also assessed. The *in vitro* growth of four *Babesia* species that were tested was significantly inhibited ($P < 0.05$) by micromolar concentrations of fusidic acid (IC_{50} values = 144.8, 17.3, 33.3, and 56.25 μM for *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*, respectively). Combinations of fusidic acid with diminazene aceturate synergistically potentiated its inhibitory effects *in vitro* on *B. bovis* and *B. caballi*. In *B. microti*-infected mice, fusidic acid caused significant ($P < 0.05$) inhibition of the growth of *B. microti* at the dose of 500 mg/kg BW relative to control group. These results indicate that fusidic acid might be incorporated in treatment of babesiosis.

1. Introduction

Babesiosis is tick-transmitted disease caused by protozoa that comprise some of widespread parasites of erythrocytes in a wide range of wild and economically valuable domestic animals such as cattle and horses, and also in humans (Kuttler, 1988; Homer et al., 2000). Bovine babesiosis is caused by *Babesia bigemina*, *Babesia bovis*, *Babesia divergens* and *Babesia major*. Two species, *B. bovis* and *B. bigemina*, have a considerable impact on cattle health and productivity (Uilenberg, 1995). Equine piroplasmosis, caused by *Babesia equi* (recently re-classified to *Theileria equi* "*T. equi*") (Mehlhorn and Schein, 1998) and *Babesia caballi*, has emerged as an important protozoan infection from the veterinary and economic viewpoints (Schein, 1988). Clinical manifestations of babesiosis include malaise, fever, hemolytic anemia, hemoglobinuria, and edema (Roellinghoff and Rommel, 1994). *Babesia* is also zoonotic for humans. *Babesia microti* used to be identified most often as infecting rodents and humans in North America; however, it is now recognized as doing so throughout the world (Telford and Spielman, 1997). *B. microti* causes a relatively mild but persistent disease and has served as a useful experimental model for the analysis of animal and human babesiosis (AbouLaila et al., 2010a; Vannier et al., 2008).

Several chemicals were evaluated as chemotherapeutic drugs against babesiosis such as epoxomicin, ciprofloxacin, thiostrepton, and rifampicin (AbouLaila et al., 2010a, 2012), triclosan, and clodinafop-propargyl (Bork et al., 2003a,b)

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but they are not available for field animal treatment. The currently available drugs for treatment have toxic side effects such as quinuronium sulfate, imidocarb dipropionate, clindamycin phosphate (Mosqueda et al., 2012), therefore, the search for new drugs with low toxic effect to the animal was desired.

Fusidic acid is a steroid antibiotic derived from the fungus *Fusidium coccineum* (Verbist, 1990). It is often used in conjunction with rifampicin to treat severe Gram-positive bacterial infections, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Johanson and Hughes, 1994). Fusidic acid blocks the translation elongation factor G gene in bacteria (Zhang et al., 2005). Translation EF-G interacts consecutively with the ribosome during polypeptide synthesis and its function depends on guanosine 5′ triphosphate (GTP) binding and hydrolysis. After GTP hydrolysis, guanosine 5′ diphosphate GDP-bound EF-G dissociate from the ribosome. Fusidic acid targets translation EF-G and prevents the dissociation of both factors from the ribosome after GTP hydrolysis and thereby further protein synthesis (Johanson and Hughes, 1994; Hansson et al., 2005). Fusidic acid inhibited the growth of *Plasmodium falciparum* (*P. falciparum*) erythrocytic stages with immediate death effect (Johnson et al., 2011). The delayed-death phenotype is defined as the requirement for high initial drug concentrations to achieve moderate growth inhibition after 48 h and an approximate 10-fold-increased activity when the parasites have entered the second replication cycle at 96 h in case of *P. falciparum* (Aminake et al., 2011) and in case of *Babesia* parasites immediate death effect was observed in ciprofloxacin, thiostrepton, and rifampicin where the difference in 50% inhibitory concentration were nearly similar over 120 h (AbouLaila et al., 2012). There are 3 copies of elongation factor G genes found in *B. bovis* genome with similarities of 36.4% (Accession No. XM_001610419), 45.7% (Accession No. XM_001611121), and 47.5% (Accession No. XM_001610350) with *P. falciparum* EF G (Accession No. XM_001350688). Considering close biological similarities between *Plasmodium* and *Babesia* parasites, there is a strong motive for studying the chemotherapeutic potential of fusidic acid on *in vitro* inhibitory effect on babesiosis. Thus, the aim of the present study was to evaluate the inhibitory effect of fusidic acid upon the *in vitro* growth of bovine and equine *Babesia* parasites and *in vivo* on *B. microti*.

2. Materials and methods

2.1. Parasites

The Texas strain of *B. bovis* (Hines et al., 1992), Argentine strain of *B. bigemina* (Hotzel et al., 1997), USDA strain of *B. caballi* (Avarzed et al., 1997), USDA strain of *T. equi* (*B. equi*) (Bork et al., 2004), and Munich strain of *B. microti* (AbouLaila et al., 2012) were used in this study.

2.2. Culture conditions

Bovine and equine *Babesia* parasites used in this study were maintained in purified bovine or equine red blood cells (RBCs), using a microaerophilic stationary-phase culture system (Igarashi et al., 1994; AbouLaila et al., 2010a). Medium 199 was used for *B. bovis*, *B. bigemina*, and *T. equi* while RPMI 1640 used for *B. caballi* (both from Sigma–Aldrich, Tokyo, Japan). Media were supplemented with 40% normal bovine serum (for bovine *Babesia* isolates) or 40% normal equine serum (for equine *Babesi*a isolates), 60 U/ml of penicillin G, 60 μg/ml of streptomycin, and 0.15 μg/ml of amphotericin B (all three drugs from Sigma–Aldrich) were prepared and used in the culture media. Additionally, 13.6 μg of hypoxanthine (ICN Biomedicals Inc., Aurora, OH) per ml was added to the *B. equi* culture as a vital supplement. Serum-free GIT medium (Wako Pure Chemical Industrial, Ltd., Osaka, Japan) also was used for culturing *B. bovis* to assess the growth inhibitory effect of fusidic acid without serum (Bork et al., 2005; AbouLaila et al., 2010a).

2.3. Chemicals reagents

Fusidic acid was purchased from Sigma–Aldrich, Tokyo, Japan and used as a test drug. A working stock solution of 100 mM fusidic acid dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industrial, Ltd., Osaka, Japan) was prepared and stored at 4 °C until required for use. Diminazene aceturate was purchased from Ciba-Geigy Japan Limited, Tokyo, Japan and used as a comparator drug. Stock solution of 10 mM was prepared in distilled water and stored at −30 °C until use. Ciprofloxacin was purchased from Sigma–Aldrich, Tokyo, Japan and used also as a control drug in delayed death test. Stock solution of 20 mM was prepared in ethanol 70% and stored at −30 °C until use.

2.4. In vitro growth inhibition assay and drug combination test

The inhibitory effects of fusidic acid upon *Babesia* growth were tested using a modified version of an assay previously described by Bork et al. (Bork et al., 2003a, 2004; AbouLaila et al., 2010a). Parasite-infected RBCs were diluted with uninfected RBCs to obtain an RBC stock supply with 1% parasitemia. Twenty μl of RBCs with 1% parasitemia was dispensed into a 96-well microtiter plate (Nunc, Roskilde, Denmark) with 200 μl of the culture medium containing the indicated concentration of fusidic acid (5, 10, 25, 100, 200 and 400 μM) for *B. bovis*, 1, 5, 10, 50, 100, 200 and 400 μM for *B. bigemina*, 1, 10, 50, 200 and 400 μM for *B. caballi* and 1, 5, 10, 50, 100, 200 and 400 μM for *T. equi*. Diminazene aceturate was used at the concentrations of 5, 25, 50, 100, 500, 1000 and 2000 nM for all four parasites from the same cultures used for fusidic acid, and then incubated at 37 °C in a humidified multigas water-jacketed incubator. For experimental control, cultures without the drug and cultures containing only DMSO (0.06%, for fusidic acid), or distilled water (0.02%, for diminazene aceturate) were prepared. Combination therapies of fusidic acid and diminazene aceturate were tested in the *in vitro* cultures of *B. bovis* and *B. caballi* as models for bovine and equine *Babesia* parasites respectively. Fusidic acid/diminazene aceturate combinations (M1, M2, M3, M4, M5, M6, M7, M8 and M9) were prepared as previously described (Bork et al., 2003b; AbouLaila et al., 2010a)