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Original papers

Evaluation of inhibitory effect of redox-active antimalarial drug against *Babesia microti* in mice

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ABSTRACT. Babesiosis is an emerging, tick-transmitted disease caused by the intraerythrocytic parasite *Babesia microti*. In immunocompetent individuals, *B. microti* infection quickly resolves after antibabesial treatment. Immunocompromised patients and those of advanced age experience chronic and relapsing babesiosis, accompanied by severe complications and often, a fatal outcome. In these individuals, *B. microti* infection may persist despite multiple courses of treatment with antiprotozoal drugs. The increasing incidence of human babesiosis caused by *B. microti,* coupled with a growing number of immunosuppressed people who do not respond to standard antibabesial therapy, emphasises the need for new therapeutics for this protozoan infection with more effective mechanisms of action. Plasmodione, namely 3-[4-(trifluoromethyl)benzyl]-menadione, acts as a redox cycler and disrupts the redox homeostasis of *Plasmodium*-infected erythrocytes. The present study was designed to evaluate the potential inhibitory effect of this novel antimalarial compound against intraerythrocytic stages of *B. microti* in mice. Our results demonstrate that plasmodione did not reduce the level of parasitemia in *B. microti*-infected mice, indicating that interfering with the parasite redox balance is not an effective strategy to restrict the division of this protozoan. The mechanism of parasite resistance to plasmodione may be based on the differences in the oxidative metabolisms of *Babesia* and *Plasmodium* parasites inside infected erythrocytes. The significance of our results is discussed in relation to the development of novel antibabesial drugs based on redox-active benzylmenadiones.

Key words: *Babesia microti*, babesiosis, plasmodione, benzylmenadiones, treatment

Introduction

Babesiosis is an emerging tick-borne disease caused by the intraerythrocytic parasite *Babesia microti* [1]. In spleen-intact, immunocompetent individuals, *B. microti* infection is typically asymptomatic or appears as a mild flu-like disease that quickly resolves spontaneously or after a single course of standard antibabesial therapy [2]. Immunocompromised patients and those of advanced age experience chronic and relapsing babesiosis requiring hospital admissions, and which is accompanied by severe complications and often, a fatal outcome. In these individuals, *B. microti* infection may persist despite multiple courses of treatment with standard antiprotozoal drugs [3]. The increasing incidence of human babesiosis caused by *B. microti,* coupled with a growing number of immunosuppressed people who live or travel in areas where babesiosis is endemic, emphasises the need for new therapeutics with more effective mechanisms of action. This need is all the greater, as the emergence of parasite resistance to standard antiprotozoal drugs has been observed in immunocompromised patients [4]. Therefore, a range of therapeutics with novel chemical entities that exploit new molecular targets and overcome established drug resistance mechanisms are needed.

Plasmodione, namely 3-[4-(trifluoromethyl) benzyl]-menadione, has been identified as a promising antimalarial agent [5,6]. The compound acts as a redox cycler and disrupts the redox homeostasis of *Plasmodium*-infected erythrocytes. Plasmodione is highly effective against asexual intraerythrocytic stages of *Plasmodium* parasites, as observed in *in vitro* and *in vivo* experiments [5].

Plasmodione also demonstrates a low potential to induce drug resistance and its antiplasmodial activity remains unaffected by the most common mechanisms of resistance to antimalarials in clinical use [6].

B. microti exhibits similarities in life cycle, developmental process and parasitic strategies to the protozoa of the genus *Plasmodium*, and the same mechanisms of immune response are implicated in protection against both *Babesia* and *Plasmodium* infections [7]. In fact, it has been proposed that babesiosis and malaria are conceptually identical diseases [8]. Therefore, antimalarial drugs are widely used for treatment of *B. microti* infection [1]. The present study was designed to explore the potential inhibitory effect of plasmodione, a novel antimalarial compound, against the asexual intraerythrocytic stages of *B. microti* in mice, the results of which may serve as grounds for establishing a new future drug-development strategy for redox-active benzylmenadiones.

Materials and Methods

Mice. Female BALB/c mice, eight weeks of age, were maintained under specific pathogen-free conditions and were allowed *ad libitum* access to drink and commercial pellet food. All experimental protocols were approved by the Local Ethical Committee.

Parasite and infection. *B. microti* King's 67 strain was maintained by weekly blood passage in BALB/c mice. The mice were infected with *B. microti* by intraperitoneal inoculation of 10^7 parasitized erythrocytes. A preliminary study was performed to determine the optimal infection dose and assess the kinetics of parasitemia in BALB/c mice. Mice were sacrificed on day 11 post-infection. Parasitemia was monitored by microscopic examination of Hemacolor®-stained (Merc/Millipore, Darmstad, Germany) thin blood smears and calculated as the number of parasitized erythrocytes versus the total number of erythrocytes. A minimum of 1,000 erythrocytes were counted and an erythrocyte infected with multiple parasites was counted as a single infected cell. The blood samples were obtained from the tail vein on days 1, 2, 3, 4 and 11 post-infection.

***In vivo* growth inhibition assay.** Plasmodione was freshly prepared as 70% Tween-80 and 30% ethanol stock solution in sterile phosphate-buffered saline (PBS; pH=7.2; Biowest, Nuaille, France) on the day of use. Twenty-four hours post-infection, *B. microti*-infected mice received the first treatment by the intraperitoneal route. The mice were further treated on days 2 to 4. The compound was tested with a daily dose of 30 mg/kg body weight. Control *B. microti*-infected mice were injected with methylene blue or PBS. Methylene blue was dissolved in deionized water (Millipore) at a dose of 15 mg/kg of body weight and then stored at 4°C

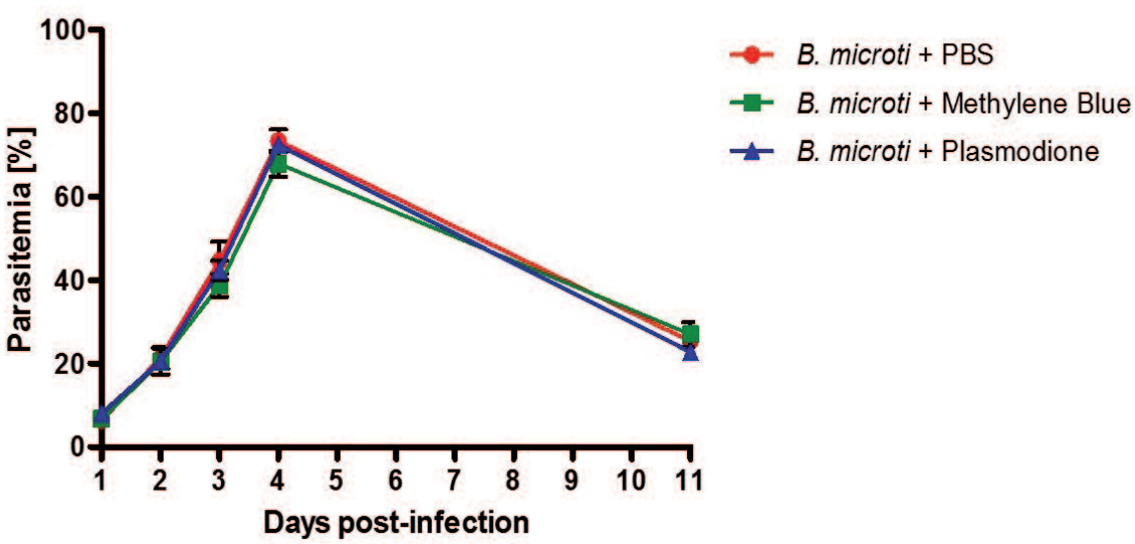

Fig. 1. The course of parasitemia in *B. microti*-infected mice after treatment with plasmodione. Mice were inoculated intraperitoneally with 10^7 parasitized erythrocytes and treated with plasmodione on days 1, 2, 3 and 4 post-infection. The level of parasitemia in *B. microti*-infected mice injected with plasmodione was compared with the level of parasitemia in *B. microti*-infected mice treated with methylene blue or injected with PBS. The results are expressed as a mean percent parasitemia (± SD) of 3 mice per group.

until further use for *in vivo* assay.

Statistical analysis. Statistical analysis was performed using the GraphPad InStat software (GraphPad Software Inc., La Jolla, CA, USA). All results are expressed as means (± SD). The significance of differences between parasitemia means were determined using the unpaired Student's *t*-test.

Results

To determine whether plasmodione inhibits the growth of *B. microti,* BALB/c mice were repeatedly injected with 30 mg/kg of the compound in PBS on days 1–4 post-infection. For comparison, control *B. microti*-infected mice were administrated with methylene blue. The level of parasitemia was assessed on days 1, 2, 3, 4 and 11 post-infection by determining the percentage of infected erythrocytes in peripheral blood. The *B. microti*-infected mice did not exhibit any significant decrease of parasitemia after treatment with plasmodione. The kinetics of parasitemia was almost identical with that observed in untreated *B. microti*-infected mice and was comparable with that in *B. microti*-infected mice injected with methylene blue (Fig. 1).

Discussion

Several compounds that suppress the growth of *Plasmodium* parasites have been successfully evaluated against protozoa of the genus *Babesia* [9–11]. Following on from this, the present study examines the effect of a novel antimalarial agent, plasmodione, against *B. microti* in mice. No previous study has examined the antibabesial activity of this compound. Based on two models of *Babesia* treatment, it was found that plasmodione did not reduce the level of parasitemia in *B. microti*-infected mice, indicating that interference with the parasite redox balance is not an effective strategy for restricting the development of this protozoan at the used dosage.

Plasmodione is known to be a NADPH-consuming redox cycler which causes parasite death by inducing excessive oxidative stress [5]. The compound prevents haem detoxification, resulting in free haem accumulation in the food vacuole and in membranes, which in concert with oxygen species, catalyses oxidation reactions and protein damage, leading to parasite death and infected erythrocyte phagocytosis [12]. Unlike protozoa of the genus *Plasmodium*, *B. microti* does not digest its host cell haemoglobin during the intraerythrocytic cycle as a source of essential nutrients; it is therefore not exposed to elevated fluxes of reactive oxygen species (ROS) created during the pro-oxidative process [13]. As plasmodione acts via haematin-catalysed oxidation reactions, *B. microti* cells remain unaffected during treatment.

Furthermore, the bioactivation of plasmodione catalysed by glutathione reductase is essential for antimalarial activity. Plasmodione is known to function as a redox cycler both in its oxidized and reduced forms. The oxidized form is reduced by NADPH in glutathione reductase-catalysed reactions occurring within the cytosols of infected erythrocytes. In its reduced form, the compound can convert methaemoglobin, the major nutrient of the *Plasmodium* parasites, to indigestible haemoglobin [5]. As for *Babesia* parasites, the previous study failed to find the encoding region of glutathione reductase [14]. This finding indicates that *B. microti* evades the action of ROS in a different manner than observed in *Plasmodium* parasites. Thus, it seems reasonable to suggest that plasmodione is not effective in killing the asexual intraerythrocytic stages of *B. microti* because the cascade of redox reactions, leading to bioactivation of the compound, is impaired by deficiencies in glutathione reductase.

Interest has recently been awakened in the antiplasmodial activities of methylene blue [15–17], the drug of choice in the treatment of methaemoglobinemia [18], especially as a companion drug in combination therapies [19–21]. Methylene blue has also been identified as an antibabesial agent that exhibits high inhibition efficiency on the *in vitro* growth of *Babesia bovis*, *Babesia bigemina*, *Babesia caballi* and *Theileria equi* [formerly *B. equi*] [22]. Similar to benzyl-menadiones, methylene blue seems to exert its anti-protozoal activity by causing a redox imbalance [23]. However, the treatment with 15 mg/kg/day of methylene blue in the present study did not reduce the level of parasitemia in BALB/c mice. It is possible that methylene blue suppresses *B. microti* growth in a dose-dependent manner. As the protozoa of the genus *Plasmodium* are relatively more susceptible to methylene blue than *Babesia* parasites, methylene blue administrated at a dose of 15 mg/kg/day acts effectively against *Plasmodium berghei*, but not *B. microti*. Tuvshintulga et al. [22] have reported that BALB/c mice infected with *B. microti* and treated with a daily dose of 50 mg/kg

methylene blue exhibited significantly reduced level of parasitemia on days 7–10 post-infection as compared to the control untreated group. Nevertheless, it should be noted that methylene blue treatment in this study resulted in 36% inhibition of *B. microti* growth in BALB/c mice [22], suggesting that methylene blue is poorly efficient against the parasite *in vivo*, even in a higher dose. The decreased susceptibility of *B. microti* to methylene blue therapy may be a result of its use of different oxidative metabolism pathways than those described for the genus *Plasmodium*. This may explain why combination therapy based on antimalarials and antibiotics is used for better infection outcome and, in very serious cases, leads to the need for blood transfusions [3,11,24–26].

To conclude, plasmodione is a promising antimalarial lead that exerts its antiplasmodial activity by interfering with a highly-complex and tightly-regulated redox network in the host-parasite unit. However, the compound fails to reduce the parasite load in mice infected with *B. microti*, indicating that the parasite is not susceptible to the compound. This finding suggests that redox-active benzylmenadiones should not be considered as potent antibabesial agents.

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