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Original Article

Inhibitory effects of 19 antiprotozoal drugs and antibiotics on *Babesia microti* infection in BALB/c mice

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Abstract

Introduction: Different results have been achieved in the evaluation of antiparasitic drug activity in Mongolian jirds, hamsters, and BALB/c mice infected with *Babesia microti*. The aims of the present study were to find a preferable method for drug screening and to re-evaluate the activity of several drugs against *B. microti*.

Methodology: The activity of 19 drugs on *B. microti*-infected BALB/c mice was evaluated. The study was built on Peters' four-day suppressive test, and the pathogenicity of the blood from the treated mice was also used as indicator.

Results: The results showed that 15 of the 19 drugs had little or no *in vivo* effect against *B. microti*. The inhibitory rates of atovaquone and azithromycin were high at all doses, but the microscopy-negative blood of recovered mice was still infectious. Similar to robenidine hydrochloride at 25 and 50 mg/kg, primaquine at 100 mg/kg had a 100% inhibitory rate. Robenidine hydrochloride achieved a 100% inhibitory rate at 100 mg/kg, and the blood of recovered mice did not result in parasitemia in subpassage experiments. Parasite-negative blood from mice treated with antimalarial drugs (clinically used for babesiosis) still caused parasitemia in subpassage experiments. This suggests that these drugs cannot eradicate the parasites.

Conclusions: Peters' four-day suppressive test and the pathogenicity of the blood from the treated mice are suitable methods for preliminary investigating possible drugs against *B. microti*. Considering that robenidine hydrochloride achieved the best activity against *B. microti* in BALB/c mice in our study, further studies are needed.

Key words: inhibitory effects; antiprotozoal drugs; antibiotics; *Babesia microti*; BALB/c mice

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Introduction

Babesiosis is a malaria-like tick-borne zoonotic disease caused by a protozoan of the genus *Babesia*, which parasitizes the erythrocytes of a wide range of mammalian hosts, including humans. It is transmitted by infected ticks from a vertebrate reservoir to humans or by blood transfusion from an infected individual [1,2]. Human babesial infections are mainly caused by *Babesia microti*, *Babesia divergens*, and *Babesia bovis*, which have distinct geographical distributions based on the presence of competent hosts [3]. The clinical symptoms of acute onset, which are similar to those of malaria, include fever, sweating, chills, and fatigue. Older, immunocompromised, and asplenic patients experience similar symptoms but with increased severity. Even in immunocompetent patients, babesiosis can persist for many months [3-8]. It can quickly become life threatening and cause death if proper clinical treatment is not administered on time. Retrospective surveys have shown that the case fatality rate is 5%–9% [5,7,8].

B. microti can infect many species of animals [9] and different strains of mice with different susceptibility to infection [10]. Previous studies compared the susceptibility of one outbred (CF1) and four inbred (BALB/c, C57, CBA, and C3H) strains of mice with human-origin *B. microti*. Intact C3H mice developed a significantly higher rate of parasitemia than did the other four strains, whereas splenectomized BALB/c developed significantly more parasitemia than did the other splenectomized strains. Splenectomized mice of all strains had higher rates of parasitemia than did their intact counterparts. Mice infected with *B. microti* produced transient high levels of parasitemia, but subsequently recovered from acute infection within 24 to 38 days. A self-limiting course was observed in all strains, whether splenectomized or intact [10].

Combination therapies, usually consisting of an antimalarial agent and an antibiotic, such as azithromycin/quinine [11], clindamycin/quinine [12], azithromycin/atovaquone [13,14], and atovaquone/proguanil [15], were recommended and applied clinically for the treatment of babesiosis. Generally, combination therapies can suppress parasitemia, and most patients do not relapse after standard antibabesial therapy. But immunosuppressed patients have been shown to experience relapse after therapy. *B. microti* may become resistant to azithromycin/atovaquone when this combined drug regimen is administered to highly immunocompromised patients [16].

In animal studies, *B. microti*-infected Mongolian jirds [17] and hamsters [18,19] were used as animal models to evaluate drug activity. Twenty selected antiprotozoal agents or their combinations were assessed against *B. microti* in Mongolian jirds [17]. At the highest nonfatal doses for five days, the pentaquine phosphates, melarsoprol, diminazene aceturate, 4-methylprimaquine (WR-181 023), and two other diamidine derivatives (WR 199 385 and WR 214 400) had good activity with inhibitory rates of 96.4%–99.9%. In a hamster model, 17 antiprotozoal drugs were evaluated [18]. Subpassage studies were undertaken for further evaluation of drug activity. WR238605 was superior to the other drugs because the blood from the hamsters treated with it failed to develop parasitemia six weeks after subinoculation, indicating that a parasitological cure had been achieved. The subpassage of blood was taken as the preferred method to assess drug activity. WR238605 belongs to the 8-aminoquinolines and is structurally similar to primaquine. In the same experiment, several well-recognized antimalarial drugs, including mefloquine, halofantrine, artesunate, and artelenic acid, exhibited little or no effect on parasitemia in a hamster model of *B. microti* infection. In contrast, diamidine, pyrroloquinazoline, and biguanide all showed > 95% suppression of parasitemia at three and seven days post-treatment. For diamidine, the test result was the same as in the jird model.

The preventative and therapeutic effects of atovaquone on babesiosis were evaluated, and they were compared with those of the combination of clindamycin/quinine in another experimental study, in which the hamster model was applied. Clindamycin plus quinine was effective, but less so than was atovaquone. In contrast, proguanil, another antimalarial that is usually used clinically, had no effect against the disease when used alone in the model [20].

At present, there is no unified understanding of a gold-standard drug or optimal treatment regimen for babesiosis. There is no recognized standard in animal models, methods, or practice for the evaluation of drug activity. Given that *B. microti* produces a self-limiting infection in mice [10], parasitemia rapidly declines and clears spontaneously within a short period. Thus, it might not be accurate to assess drug activity when erythrocyte infection rate (EIR) is low in both test and control groups. It is necessary to develop a rational method and an optimal animal model for drug screening or activity evaluation.

In the present study, BALB/c mice were used to observe the dynamic change in EIR in the peripheral blood of mice infected with *B. microti* and the pathogenicity/infectivity of the blood from self-limited and cured/recovered mice. Taking Peters' four-day suppressive test as a reference [21-23], combined with the infectivity of blood from mice post-treated as an additional indicator, we assessed 19 antiparasitic agents and antibiotics for their activity against *B. microti*.

Methodology

Parasites and animals

B. microti strain ATCC$^{\circ,R}$PRA-99 was provided by the Institute of Laboratory Zoology, Chinese Academy of Medical Sciences (Beijing, China). The non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China) were used as a reservoir for preserving *B. microti*. Female BALB/c mice (18 ± 2 g) were purchased from SLAC Laboratory Animal Co., Ltd., and were inoculated intraperitoneally with 0.2 mL blood containing 1×10^7 parasitized erythrocytes. The infected blood was collected, heparinized, and diluted with sterile saline to achieve the desired concentration.

Chemicals

Clindamycin and azithromycin were purchased from YuanQi Pharmchem Co., Ltd. (Wuhan, China); atovaquone hydrochloride was purchased from Hisoar Pharmaceutical Co., Ltd. (Hangzhou, China); artesunate, dihydroartemisinin, and artemether were purchased from Oukang Phytochemistry Technology Co., Ltd. (Chengdu, China); quinine, piperaquine, chloroquine, lumefantrine, primaquine, pyrimethamine, sulfadoxine, ivermectin, and robenidine hydrochloride were purchased from Galaxy

Chemical Co., Ltd. (Wuhan, Chian); mefloquine was purchased from Libang Pharmaceutical Co., Ltd. (Xi'an, China); proguanil hydrochloride was purchased from Swellxin Bio-Pharm Co. (Zhangjiagang, China); posaconazol was purchased from (Sigma Aldrich, St. Louis, USA); and pyronaridine was provided by the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Shanghai, China). The purity of all the drugs was > 99.5%. Piperaquine, primaquine, chloroquine, and pyronaridine were prepared as aqueous solutions at a concentration of 1.25 mg/mL in deionized water, and the others were prepared as suspensions at the same concentration in 5% soluble starch solution (10 mL/kg). All prepared drugs were stored at 4°C. Information about the name, structural traits, and clinical indications of drugs is shown in Table 1.

Observation of the dynamic change in EIR in B. microti BALB/c mice

Parasitemia of three mice was determined by EIR, based on the number of infected erythrocytes per 1,000 erythrocytes in Giemsa-stained thin blood smears that were prepared from a drop of blood collected from the tip of the tail. Microscopic examination of the thin blood smears was conducted every 2 or 3 days until 28–30 days post-infection. Blood without detectable parasitemia on 3 consecutive days was considered as negative. All the experiments were repeated three times.

Observation on the pathogenicity of blood from BALB/c mice with self-limited infection

The pathogenicity of blood was judged by subpassage studies [18]. Negative blood was collected by enucleating eyeballs of BALB/c mice that recovered from infection with *B. microti* at day 42. Collected blood was then heparinized, followed by intraperitoneal inoculation into three naive BALB/c mice for each drug. The animals were monitored by thin blood smear microscopy at an interval of 2 or 3 days starting from day 3 to evaluate the pathogenicity of their blood. The microscopic examination ceased if parasitemia was observed; otherwise, it continued to day 28. All the experiments were repeated three times.

Table 1. Categories, names, structure traits, and main indications of drugs screened in the study

Category	Name	Structural trait	Indication
Antimalarial	Artesunate	Artemisinins, containing peroxobridge	Malaria
	Dihydroartemisinin	Artemisinins, containing peroxobridge	Malaria
	Artemether	Artemisinins, containing peroxo bridge	Malaria
	Quinine	Quinoline, amino-alcohol	Malaria
	Piperaquine	Aminoquinolines	Malaria
	Chloroquine	Aminoquinolines	Malaria
	Primaquine	Aminoquinolines	Malaria
	Mefloquine	Quinoline, amino-alcohol	Malaria
	Lumefantrine	Fluorene, amino-alcohol	Malaria
	Pyronaridine	Benzonaphthyridine	Malaria
	Proguanil	Guanidine	Malaria (combined with atovaquone)
	Robenidine	Guanidine	Coccidiosis
	Pyrimethamine	Pyrimidine	Malaria
Antiparasitics (broad spectrum)	Ivermectin	Macrolides	Nematodiasis, epizoonosis, filariasis (microfilariae)
Anti-infective agents	Atovaquone hydrochloride	Naphthalenedione	Malaria, *Pneumocystis carinii* pneumonia (combined with proguanil)
Antibiotics	Clindamycin	Macrolides, erythromycins	Infectious diseases caused by *Staphylococcus aureus*, *Streptococcus* and anaerobic bacteria
	Azithromycin	Macrolides	Infectious diseases caused by *Staphylococcus aureus*, *gonococcus*, *Legionella*, Gram-negative bacteria, toxoplasmosis, *Treponema pallidum*, *etc.*
	Sulfadoxine	Sulfonamides	Infectious diseases caused by hemolytic *Streptococcus*, *Pneumococcus*, and *Shigella*, malaria (combined with pyrimethamine)
Antifungals	Posaconazol	Triazoles	Fungal infection, trypanosomiasis

Evaluation of anti-babesial potential of drugs

Taking Peters' four-day suppressive test as a reference, 25, 50, and 100 mg/kg/day doses were used for each drug. The control group consisted of mice receiving orally a 5% soluble starch solution, because there is no gold standard drug for this disease to date. The first day of inoculation was defined as day 0. Treatment was orally initiated at four hours post-infection (day 0) and then once daily up to day 3. Efficacy was measured by inhibitory rate (IR) calculated as follows:

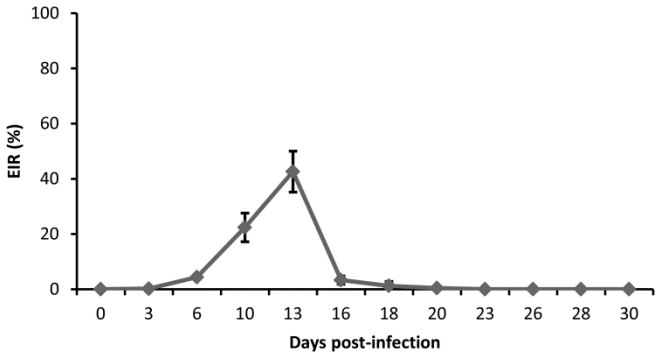
$$IR = \frac{\text{Average EIR of control group} - \text{Average EIR of drug group}}{\text{Average EIR of control group}} * 100$$

For the groups with no differences from the controls in EIR, observation ceased at days 9–11. For every experimental group, three mice were observed. All the experiments were repeated three times.

Pathogenicity of blood from treated and healed BALB/c mice

Pathogenicity of blood was taken as one of the indicators for evaluating the drugs' ability to lead to parasitological cure. The mice in the effective drug groups were maintained to 42 days post-inoculation, and the subpassage studies were carried out. The EIR was observed at intervals of 2 or 3 days starting from day 3 to evaluate drug efficacy. The microscopic examination ceased if parasitemia was observed; otherwise, it continued to day 28. For every experimental group, three mice were observed. In addition, all the experiments were repeated three times.

Figure 1. Dynamic curve of erythrocyte infection rate (EIR) in BABL/c mice infected with *B. microti*



Results

Dynamic change in EIR in BALB/c mice infected with B. microti

After BALB/c mice were infected with *B. microti*, parasitemia was observed from day 3 and reached a peak at day 10, followed by a rapid decline and then a slight fluctuation at a low level. Parasitemia began to be cleared at around day 18 and was removed completely at day 28. The dynamic change in EIR is shown in Figure 1. BALB/c mice infected with *B. microti* experienced a self-limiting infection that lasted for 26–28 days.

Pathogenicity of blood from BALB/c mice with self-limited infection

Blood from BALB/c mice with self-limited infection was inoculated into naive mice. Parasitemia appeared at day 3 and reached a peak at day 13, then declined sharply. The peak time of EIR in subpassage was delayed, but the trend was consistent with the last passage. The EIR values are shown in Table 2.

Table 2. Erythrocyte infection rate (EIR) of BABL/c mice inoculated blood from self-limited mice (n = 3)

Days post-inoculation	EIR (%)	
	Mean	**Standard deviation**
0	0.0	0.0
3	0.2	0.1
6	4.4	0.7
10	22.4	5.2
13	42.6	7.4
16	3.3	1.5
18	1.2	1.6
20	0.4	0.7
23	0.0	0.0
26	0.0	0.0
28	0.0	0.0
30	0.0	0.0

Activity of drugs against B. microti

Among the 19 drugs, azithromycin, atovaquone, primaquine, and robenidine hydrochloride showed good suppression of parasitemia. The EIR of azithromycin and atovaquone was in the range of 73.7%–99.0%, but complete clearance of parasitemia was not observed. Primaquine had a good EIR at daily doses of 25 and 50 mg/kg, but it also failed to eliminate parasites completely. At 100 mg/kg, the EIR of primaquine was 100% and parasitemia was not detected until day 21. The same phenomenon was also observed with treatment with 25 and 50 mg/kg of robenidine hydrochloride. Occurrence of parasitemia was delayed to days 21 and 25, respectively. Only the mice treated with 100 mg/kg of robenidine hydrochloride had 100% EIR, and protozoa were not detected until day 42 before the subpassage experiment. The EIR values of azithromycin, atovaquone, primaquine, and robenidine hydrochloride for *B. microti* in BALB/c mice are shown in Table 3.

Artesunate, dihydroartemisinin, lumefantrine, and sulfadoxine had low EIR. EIR of mice treated with pyrimethamine, quinine, artemether, piperaquine, pyronaridine, mefloquine, chloroquine, proguanil hydrochloride, clindamycin, ivermectin, and posaconazole did not differ from that of the control group.

Pathogenicity of blood from mice treated with azithromycin, atovaquone, and robenidine hydrochloride

The blood samples for subpassage studies were collected from mice in seven groups that were treated with azithromycin and atovaquone at three doses and robenidine hydrochloride at dose of 100 mg/kg. Protozoa were detected in all subpassaged mice with blood samples collected from the groups treated with azithromycin and atovaquone at days 3–5. Only the blood from the group treated with robenidine hydrochloride at a dose of 100 mg/kg did not produce parasitemia in further subpassage experiments. Parasitemia in animals treated with 100 mg/kg of primaquine and 25 and 50 mg/kg of robenidine hydrochloride was delayed but observed at days 21 and 25. Therefore, no subpassage experiments were undertaken for primaquine groups at all dosages and robenidine hydrochloride at 25 and 50 mg/kg.

Discussion

To build a model and evaluate drug activity against babesiosis in this preliminary study, the dynamic changes in infected erythrocytes in BALB/c mice were monitored by thin blood smear microscopy for one month. After reaching a peak at days 9–11, the protozoan density declined sharply, then fluctuated slightly at a low level. The infected mice underwent a process during which the symptoms of the animals changed from listlessness and trembling to normal, and the density of protozoa declined in the peripheral blood. However, NOD/SCID mice had no such symptoms and could live with a high EIR. Because *Babesia* can exist stably for a long time in NOD/SCID mice with a high EIR, these mice were used as a reservoir for preserving *B. microti* in our laboratory. We did not test drug activity against *Babesia* in NOD/SCID mice models, but whatever the outcome,

Table 3. *In vivo* inhibitory rate of azithromycin, atovaquone, primaquine, and robenidine to *B. microti* from days 9 and 11 post-inoculation and the detection status of protozoan in blood thereafter

Drug	Dosage (mg/kg•day×day)	Mean inhibitory rate on the days PI[a] (%)		Detection status of *Babesia* on the days PI[b]						
		9	11	14	16	18	21	23	25	28
Azithromycin	25×4	74.82	81.90	D	D	N	N	N	N	N
	50×4	95.71	97.50	D	D	N	N	N	N	N
	100×4	94.72	99.00	D	D	N	N	N	N	N
Atovaquone	25×4	76.89	73.70	D	D	N	N	N	N	N
	50×4	86.80	76.90	D	D	N	N	N	N	N
	100×4	93.40	90.60	D	D	N	N	N	N	N
Primaquine	25×4	78.33	74.00	D	D	N	N	N	N	N
	50×4	89.56	97.20	D	D	N	N	N	N	N
	100×4	100.00	100.00	N	N	N	D	D	D	D
Robenidine	25×4	100.00	100.00	N	N	N	D	D	D	D
	50×4	100.00	100.00	N	N	N	N	N	D	D
	100×4	100.00	100.00	N	N	N	N	N	N	N

[a] Results are expressed as percent inhibition of EIR in treated animals compared to untreated controls on day 9 and day 11 post infection; [b] Results are expressed as detection status of *B. babesia* in treated animals from day 14 to day 28; PI: post infection; N: protozoans were not detected; D: protozoans were detected

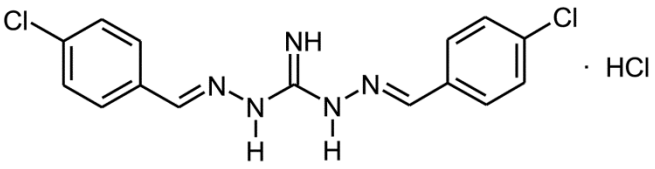
the mice are not an appropriate drug screening model because of their high cost. However, considering that babesiosis is an immunodeficiency-related disease, it is necessary to use this immunodeficient animal model for pharmacodynamic authentication.

There is no gold standard drug for the treatment of babesiosis; thus, we had only a negative control group that was infected with *B. microti* without treatment. There is no standard operational procedure for anti-babesial drug screening, and both *Babesia* and *Plasmodium* parasites have an identical location, with morphological and symptomatic similarities. Therefore, we used Peters' four-day inhibitory test as a reference for preliminary evaluation.

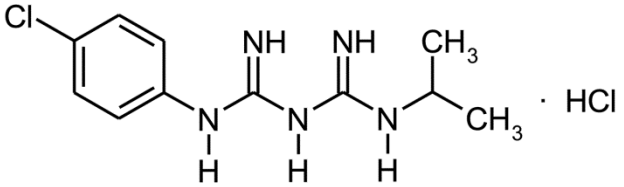
However, the occurrence of babesiosis differs from that of malaria in two aspects. First, the EIR of mice infected with *Babesia* was low before day 7 post-inoculation and declined sharply after day 13. It might not be accurate to calculate the inhibitory rates of drugs at such a low EIR before day 7 and after day 13. Thus, it is reasonable to choose the time from days 9 to 11 as a time window for the evaluation of drug activity. Second, blood from mice with self-limited infection is still infective even it is microscopically negative. Therefore, the virulence of blood from treated mice could also be taken as a criterion to evaluate drug activity, both in hamster and BALB/c mouse models. Among the 19 drugs, 15 were completely ineffective or had low inhibitory rates on *B. microti* in BALB/c mice, and some of the drugs were antimalarial agents usually used for the treatment of babesiosis. The EIR of artesunate was only 41.3% at a dose of 100 mg/kg, which is different from what was reported in other studies [24]. However, atovaquone displayed a higher inhibitory rate without completely clearing parasitemia in BALB/c mice, which was similar to findings in Mongolian gerbils [25] and hamsters [18,26]. Atovaquone and clindamycin had preferable inhibition in Peters' four-day suppressive tests, but the blood collected from the recovered mice was still infectious, even though it showed negative by thin blood smear microscopy. This finding might explain why protozoa cannot be completely cleared, resulting in patients having to accept repeated medication.

Primaquine, which is similar to WR 238605 in structure, had a good EIR at doses of 25 and 50 mg/kg and produced a 100% EIR at 100 mg/kg until day 21. However, it has no prospect of clinical use because the dose used in this experiment was close to its mean effective dose (ED_{50}) [1] and thorough clearance was not achieved. It follows that, if the drug were used at a safer dose for the treatment of babesiosis, it would be even less likely to achieve satisfactory results. However, azithromycin and atovaquone exhibited low toxicity, and they produced a high level of inhibition at safe doses, so they have potential for further development for clinical use.

Figure 2. Structures of robenidine hydrochloride and proguanil hydrochloride



(1) Robenidine hydrochloride



(2) Proguanil hydrochloride

We repeated the experiment for robenidine hydrochloride thrice and the results were consistent. Robenidine and proguanil are similar in structure (Figure 2) to a guanidine group, but their activity against *Babesia* is different. Therefore, it is necessary to explore further the structure-activity relationships of this family.

Conclusions

Robenidine hydrochloride achieved the best activity against *B. microti* in BALB/c mice in our study. Unfortunately, robenidine is currently only available for the treatment of coccidiosis in chickens and is not licensed for human use. Given the current situation in which there is a lack of drugs against babesiosis, it is necessary to explore the possibility of registering veterinary drugs for use in humans.

Peters' four-day test was used only for preliminary assessment of antibabesial activity. We also need to explore the reasonable treatment time and the best drug combinations based on systematic assessment.

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