Inhibitory Effects of Pepstatin A and Mefloquine on the Growth of *Babesia* Parasites

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Abstract. We evaluated the inhibitory effects of pepstatin A and mefloquine on the *in vitro* and *in vivo* growths of *Babesia* parasites. The *in vitro* growth of *Babesia bovis*, *B. bigemina*, *B. caballi*, and *B. equi* was significantly inhibited ($P < 0.05$) by micromolar concentrations of pepstatin A (50% inhibitory concentrations = 38.5, 36.5, 17.6, and 18.1 μM, respectively) and mefloquine (50% inhibitory concentrations = 59.7, 56.7, 20.7, and 4 μM, respectively). Furthermore, both reagents either alone at a concentration of 5 mg/kg or in combinations (2.5/2.5 and 5/5 mg/kg) for 10 days significantly inhibited the *in vivo* growth of *B. microti* in mice. Mefloquine treatment was highly effective and the combination treatments were less effective than other treatments. Therefore, mefloquine may antagonize the actions of pepstatin A against babesiosis and aspartic proteases may play an important role in the asexual growth cycle of *Babesia* parasites.

INTRODUCTION

Babesiosis is an infectious disease caused by an intraerythrocytic protozoon of the genus *Babesia*; the parasites are transmitted by the bite of ticks of family Ixodidae such as *Boophilus*, *Dermacentor*, *Rhipicephalus*, and *Haemaphysalis*.[1] The main clinical symptoms of babesiosis in infected animals include fever, hemolytic anemia, jaundice, hemoglobinuria, and edema.[2] A well-recognized disease of veterinary importance in cattle, horses, and dogs, babesiosis has also received increased attention as a globally distributed zoonosis in humans. In addition, serious economic losses have been caused by *Babesia* infections in the livestock and other industries.[3–5]

Although some anti-babesial agents have been used to control the disease, continuous searches for the development of new drugs against *Babesia* are caused by toxic side effects, repeated relapse of parasite infections, and the possibility of emerging drug-resistant parasites.[6] Several novel anti-babesial drugs, such as triclosan,[6] artesunate, pyrimethamine, pamaquine,[7] heparin,[8] imidazole derivatives, staurosporine,[9] and cysteine protease inhibitors,[10] have been successfully studied by using *in vitro* and *in vivo* models. However, these drugs have not been evaluated for field application. Therefore, development of new compounds that have chemotherapeutic effects against babesiosis with high specificity for the parasite and no side effects in the host is desired.

Aspartic proteases (APs) are a widely distributed family of enzymes among protozoan parasites, and several APs, including those of *Plasmodium falciparum* (plasmepsin),[11] *Eimeria tenella* (eimepsin),[12,13] *Cryptosporidium parvum* (cryptomepsin), and *Trypanosoma cruzi* (cruzipsin I and II),[14] have been characterized. Among them, the *P. falciparum* (plasmepsin)[11] enzyme of this class initiates the hemoglobin breakdown pathway that provides intraerythrocytic malaria parasites with nutritional resources. Inhibition of their activity results in the death of malaria parasites.[15–17] Pepstatin A, a potent inhibitor of AP, binds to the active site of plasmepsins in food vacuoles of *P. falciparum*,[18] prevents the degradation of hemoglobin, and kills malaria parasites. In *in vitro* studies, pepstatin A had a potent effect against cultured *P. falciparum*.[19,20] In an *in vivo* study using a murine malaria model, pepstatin A cured *P. vinckei*-infected mice.[20] Moreover, *P. vivax* AP is inhibited by pepstatin.[21]

Mefloquine is currently one of the recommended chemoprophylactic regimens for travelers visiting malaria-endemic areas.[22] Recently, mefloquine has been used for treatment[23,24] and prophylaxis[22,25] against *P. falciparum*. Mefloquine works by attacking parasites once they have entered erythrocytes by killing the parasites and preventing them from further multiplications. However, its exact mechanism is unknown. A possible explanation is that mefloquine, which is similar to chloroquine and quinine, appears to interfere with the ability of the parasite to metabolize and use erythrocyte hemoglobin.[26] Mefloquine might bind free heme, thus inhibiting the polymerization of heme or the swelling of food vacuoles. Conversely, mefloquine is believed to act by forming toxic heme complexes that damage parasitic food vacuoles. In addition, Mungthin and others[27] reported that combination of plasmepsin I, the Ro40-3488 inhibitor, with a drug such as chloroquine, amodiaquine, quinine, or mefloquine, resulted in antagonism between plasmepsin I inhibitor and chloroquine and mefloquine.

The class of enzymes known as APs has not yet been characterized in *Babesia* parasites. Additionally, *Babesia* parasites have similarities to malaria parasites and the AP target genes present in the *Babesia* genome sequence database.[28] Thus, the present study was conducted to evaluate possible inhibitory effects of pepstatin A and mefloquine, alone or combined, on the growth of bovine and equine *Babesia* parasites in *in vitro* and *B. microti*) in *in vivo* experiments.

MATERIALS AND METHODS

Parasites. The Texas strain of *B. bovis*, the Argentina strain of *B. bigemina*, the U.S. Department of Agriculture strains of *B. caballi* and *B. equi,* and the Munich strain of *B. microti* were used in this study. Parasites were grown in bovine and equine erythrocytes by using a continuous micro-aerophilous stationary phase culture system.[8] Medium M199 (for bovine *Babesia* and *B. equi*) and RPMI 1640 (for *B. caballi*) (both from Sigma-Aldrich, Tokyo, Japan) supplemented with 40% bovine serum (for bovine *Babesia*) or equine serum

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(for equine *Babesia*) were used in the culture media. Penicillin G (60 U/mL), streptomycin (60 µg/mL), and amphotericin B (0.15 µg/mL) were added to the culture media. Hypoxanthine (13.6 µg/mL) was added to *B. equi* culture.[29,30] *N*-Tris (hydroxymethyl) methyl-2-ainomethanesulfonic acid hemisodium salt (229 mg/mL) was added to bovine *Babesia* parasite cultures as a pH stabilizer (pH 7.2).[29,31,32] Culture plates for parasites were incubated in atmospheres of 5% CO_2 and 5% O_2 at 37°C.[29]

Mice. The Munich strain of *B. microti* was maintained by passage in blood of BALB/c mice. Twenty-four female BALB/c mice (8 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and were used for *in vivo* studies.

Chemical reagents. Pepstatin A (Isovaleryl-L-Val-L-Val-AHMHA-L-ALa-AHMHA (AHMHA= (3S, 4S)-4-amino-3-hydroxy-6-methyl-heptanoic acid) was obtained from Peptide Institute, Inc. (Osaka, Japan). Mefloquine (AS)-rel-a-(2R)-2-piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol monohydrochloride was obtained from Sigma-Aldrich. A working stock solution of 10 mM pepstatin A and mefloquine dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industrial, Ltd., Osaka, Japan) was prepared and stored at –20°C until use. Diminiazine aceturate was obtained from Ciba-Geigy Japan, Ltd. (Tokyo, Japan) and used as a comparator drug. A stock solution of 10 mM was prepared in distilled water and stored at –30°C until use. Tetracycline hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO) and used as a comparator drug. A stock solution of 20 mM tetracycline hydrochloride was prepared in distilled water and stored at –30°C until use.

***In vitro* growth inhibition assay and drug combination test.** The inhibitory effects of AP inhibitors upon *Babesia* growth were examined as described.[8] Parasite-infected erythrocytes were obtained from cultures with parasitemias of approximately 6–8%. Twenty microliters of erythrocytes with a parasitemia of 1% was dispensed into a 96-well microtiter plate (Nunc, Roskilde, Denmark) with 200 µL of the culture medium containing the indicated concentration of pepstatin A (5, 25, 50, 100, 250, and 500 µM) and mefloquine (1, 5, 25, 50, 100, and 500 µM) and then incubated at 37°C in a humidified multi-gas water-jacketed incubator. For the experimental control, cultures without the drug and cultures containing only 0.1% DMSO (for pepstatin A and mefloquine) were prepared. In addition, a solution of 2 µM diminazene aceturate and 100 µM tetracycline hydrochloride dissolved in distilled water, which is known to cause complete suppression of growth,[29] was used as a control. Three separate trials, consisting of triplicate experiments for individual drug concentrations, were performed over a period of four days. During the incubation period, the overlying culture medium was replaced daily with 200 µL of fresh medium containing the indicated concentration of pepstatin A and mefloquine. Combination therapies of pepstatin A/mefloquine were tested in *in vitro* cultures of *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi* as models for bovine and equine *Babesia* parasites. Pepstatin A/mefloquine combinations (C1, C2, C3, and C4) were prepared as described[29,33] with some modifications. The concentration of each drug used in the combination was not destructive to the parasites.

Concentrations of pepstatin A/mefloquine applied simultaneously to the cultures were as follows: *B. bovis* (C1, C2,

C3, and C4) = *B. bovis* (3/1.9, 3/3.9, 6/1.9, and 6/3.9 µM), *B. bigemina* (2.9/1.9, 2.9/3.8, 5.8/1.9, and 5.8/3.8 µM), *B. caballi* (2.6/2.2, 2.6/4.4, 5.2/2.2, and 5.2/4.4 µM), and *B. equi* (0.5/2.3, 0.5/4.6, 1/2.3, and 1/4.6 µM), respectively. Both reagents alone were tested in the *in vitro* cultures of all parasites by using the high and low concentrations of pepstatin A/mefloquine combination of each parasite as mentioned below. Concentrations of pepstatin A applied simultaneously to the cultures were as follows: *B. bovis* (P^a and P^b) = *B. bovis* (6 and 3 µM), *B. bigemina* (5.8 and 2.9 µM), *B. caballi* (5.2 and 2.6 µM), and *B. equi* (1 and 0.5 µM), respectively. The concentrations of mefloquine applied simultaneously to the cultures were as follows: *B. bovis* (M^a and M^b) = *B. bovis* (3.9 and 1.9 µM), *B. bigemina* (3.8 and 1.9 µM), *B. caballi* (4.4 and 2.2 µM), and *B. equi* (4.6 and 2.3 µM), respectively. Parasitemia were monitored daily by counting parasitized erythrocytes to approximately 1,000 erythrocytes in Giemsa-stained thin blood smears. The 50% inhibitory concentration (IC_{50}) values for the drugs upon growth of all parasites tested were calculated on the basis of the maximum growth of the DMSO control in the *in vitro* cell culture system by using interpolation after curve-fitting.

Viability test. After four days of treatment, 6 µL each of the control and drug-treated (at the various indicated concentrations) erythrocytes were mixed with 14 µL of parasite-free erythrocytes and suspended in fresh growth medium without drug supplementation. The plates were then incubated for five days. Culture medium was replaced daily, and parasite recrudescence was determined by using light microscopy to assess parasite viability.[8]

***In vivo* growth inhibition assay.** The *in vivo* growth inhibition assay for pepstatin A, mefloquine, and pepstatin A/mefloquine combinations (2.5 mg/kg/2.5 mg/kg and 5 mg/kg/5 mg/kg) was performed in BALB/c mice as described.[8,34] Twenty-eight week-old female BALB/c mice were divided into five groups of four mice and intraperitoneally inoculated with 1×10^6 *B. microti*-infected erythrocytes. In the first group, mefloquine dissolved in DMSO was administered at a dose rate of 5 mg/kg. In the second group, pepstatin A dissolved in DMSO was administered at a dose rate of 5 mg/kg. In the third group, a combination of pepstatin A and mefloquine (2.5 mg/kg of each) dissolved in DMSO was administered. In the fourth group, a combination of pepstatin A and mefloquine (5 mg/kg of each) dissolved in DMSO was administered. Phosphate-buffered saline containing 0.02% DMSO was administered to the fifth group as a placebo control.

When infected mice showed parasitemias of approximately 1%, mice in all experimental groups were administered daily intraperitoneal injections from days 3 to 8 post-infection. Levels of parasitemia in all mice were monitored daily until 30 days post-infection by examination of Giemsa-stained thin blood smears prepared from venous tail blood. All animal experiments were conducted in accordance with the Standard Relating to the Care and Management of Experimental Animals set by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan).

Statistical analysis. Differences in percentages of parasitemia for *in vitro* cultures and among groups of *in vivo* studies were analyzed by using the independent Student's *t*-test.[8,33] *P* values < 0.05 was considered statistically significant for all tests.

RESULTS

***In vitro* inhibitory effect of pepstatin A on *Babesia* parasites.** The *in vitro* growth of *B. bovis* (Figure 1A) and *B. bigemina* (Figure 1B) was significantly inhibited ($P < 0.05$) by 50 µM and 25 µM pepstatin A, respectively, and 5 µM pepstatin A significantly inhibited the growth of *B. caballi* (Figure 1C) and *B. equi* (Figure 1D). In the presence of 500 µM pepstatin A, growth of all parasites was completely suppressed. Complete suppression of parasites was observed with 2 µM diminazene aceturate (Figure 1A–C), and 100 µM tetracycline completely suppressed parasites (Figure 1C and D). Complete elimination of the growth of four species (*B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi*) from pepstatin A-treated cultures was observed on day 3 (Figure 1A–D). Complete elimination of the three parasites from diminazene aceturate–treated cultures was observed on day 3 of the treatment for *B. bovis*, *B. bigemina*, and *B. caballi* (Figure 1A–C).

The IC_{50} values of pepstatin A for growth inhibition of *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi* were 38.5, 36.5, 17.6, and 18.1 µM, respectively (Table 1). Subsequent viability

TABLE 1
IC_{50} values of mefloquine and pepstatin A for growth inhibition of different *Babesia* species

Organism	IC_{50} (µM)*		References
	Mefloquine	Pepstatin A	
B. bovis	59.7	38.5	Present study
B. bigemina	56.9	36.5	
B. caballi	20.7	17.6	
B. equi	4	18.1	
Plasmodium falciparum	0.028–0.13	4	19, 27, 34, and 35
Dog (neuron)	272	ND	36

*50% inhibitory concentration (IC_{50}) values are expressed as mefloquine and pepstatin A concentrations and are in micromolars of the growth medium and were determined on the day of maximum growth of the dimethylsulfoxide control *in vitro* culture by using a curve-fitting technique from three separate experiments. ND = not determined.

tests showed that there was no re-growth of the four species with pepstatin A at a concentration of 500 µM. Moreover, pepstatin A affected the morphology of *B. bovis* (Figure 2B), *B. bigemina* (Figure 2D), *B. caballi* (Figure 2F), and *B. equi* (Figure 2H) parasites in treated cultures. The pepstatin-treated cultures showed a high number of degenerated
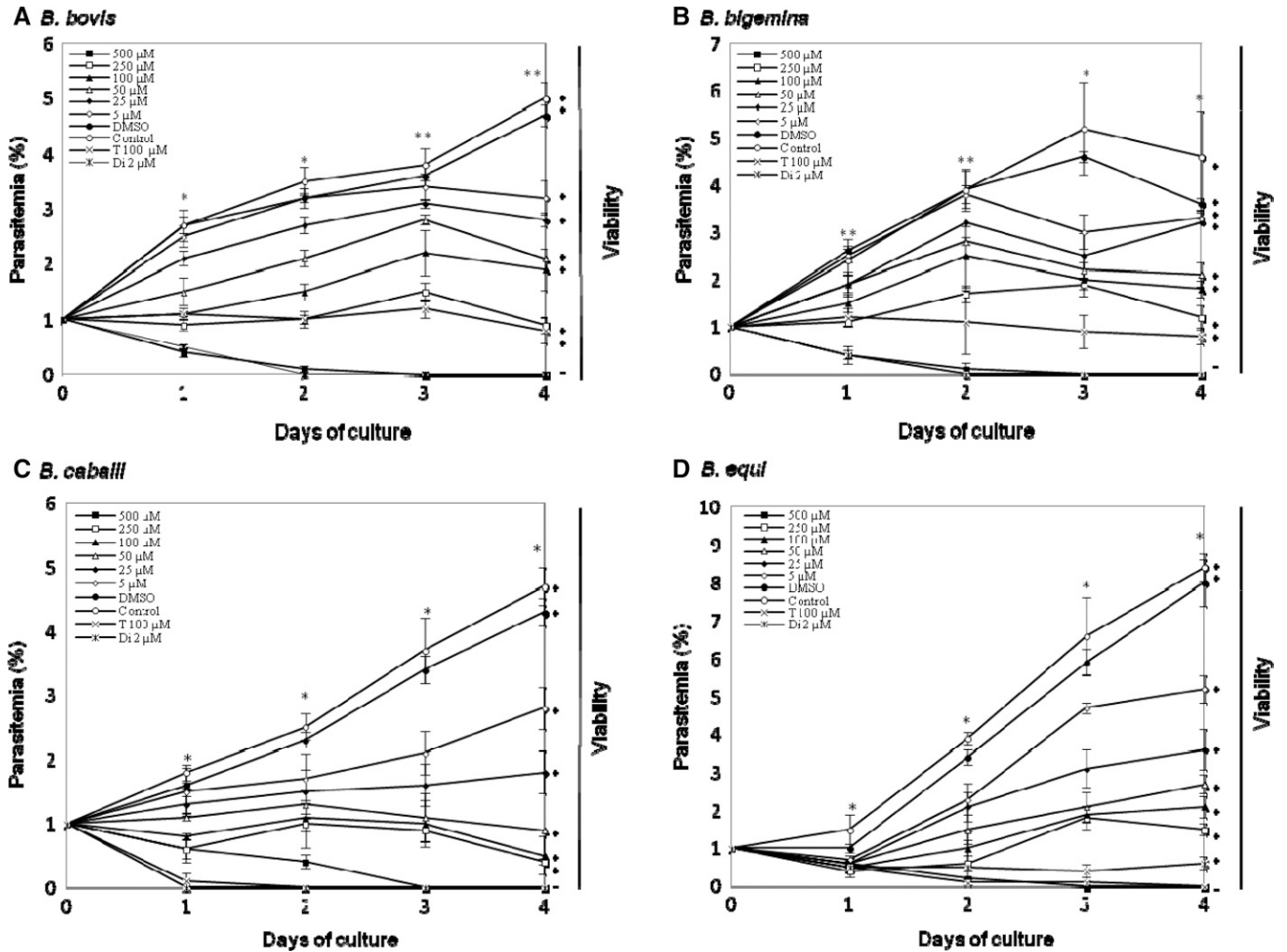

FIGURE 1.   Inhibitory effect of different concentrations of pepstatin A on the *in vitro* growth of *Babesia bovis* (**A**), *B. bigemina* (**B**), *B. caballi* (**C**), and *B. equi* (**D**). Diminazene aceturate (Di) and tetracycline hydrochloride (T) were used as positive controls. Each value represents the mean ± SD for experiments performed in triplicate. Curves represent results of one representative experiment of three separate replicates. Asterisks, (*) and (**), indicate statistically significant differences ($P < 0.05$, by Student's *t*-test) between 50 and 25 µM drug-treated cultures and the control cultures, respectively. DMSO = dimethylsulfoxide.
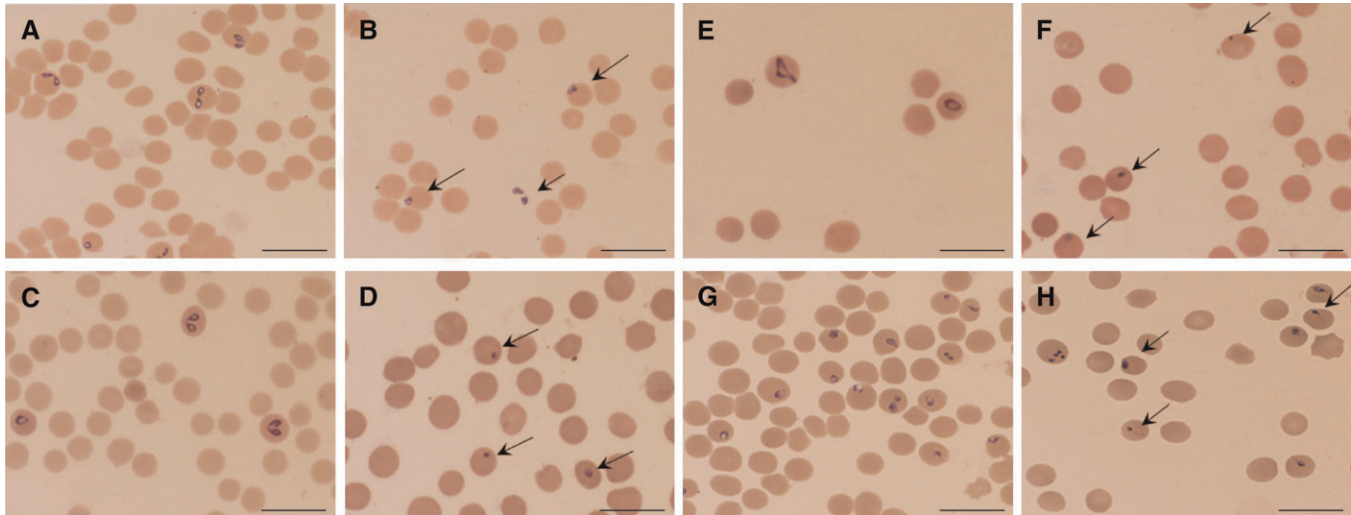
FIGURE 2. Light micrographs of pepstatin A-treated bovine and equine *Babesia* parasites in an *in vitro* culture. Micrographs were taken on day 3 of the experiment. *B. bovis*: control (**A**) and 25 μM pepstatin A (**B**). *B. bigemina:* control (**C**) and 50 μM pepstatin A (**D**). *B. caballi:* control (**E**) and 5 μM pepstatin A (**F**). *B. equi:* control (**G**) and 5 μM pepstatin A (**H**). Drug-treated cultures showed a higher number of degenerated parasites than control cultures. Scale bars = 10 μm.

parasites, which appeared to be dot-shaped when compared with those in control cultures. Some parasites appeared as abnormally multidividing forms.

***In vitro* inhibitory effect of mefloquine on *Babesia* parasites.** The growth of *B. bovis* (Figure 3A), *B. bigemina* (Figure 3B), *B. caballi* (Figure 3C), and *B. equi* (Figure 3D) was significantly inhibited ($P < 0.05$) by 1 μM mefloquine. In the presence of 100 μM of mefloquine, growth of all parasites was completely suppressed. In addition, *B. caballi* and *B. equi* were completely suppressed at concentrations of 50 and 25 μM, respectively. Complete suppression of diminazene aceturate–treated parasites was observed at a concentration of 2 μM (Figure 3A–C), and 100 μM tetracycline completely suppressed parasites (Figure 3C). After the mefloquine-treatment regimen, *Babesia* parasites were completely eliminated as early as day 1 (*B. caballi* and *B. equi*) and day 3 (*B. bovis* and *B. bigemina*) (Figure 3A–D). Complete elimination of the three parasites from diminazene aceturate–treated cultures was observed on day 1 (for *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi*) of treatment (Figure 3A–D), and tetracycline hydrochloride eliminated equine *Babesia* parasites on days 3 and 4 of treatment (Figure 3C and D). The IC_{50} values of mefloquine were 59.7, 56.9, 20.7, and 4 μM for *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi*, respectively (Table 1). Notably, *B. equi* was the most susceptible to mefloquine. Subsequent viability tests showed that there was no re-growth of the four species at a concentration of 100 μM mefloquine. Moreover, mefloquine affected the morphology of *B. bovis* (Figure 4B), *B. bigemina* (Figure 4D), *B. caballi* (Figure 4F), and *B. equi* (Figure 4H) parasites in treated cultures. The mefloquine-treated cultures showed a high number of degenerated parasites, which appeared dot-shaped when compared with those in control cultures. Abnormal multidividing forms were also observed.

***In vitro* inhibitory effect of combined compounds on *Babesia* parasites.** Combination therapies of pepstatin A and mefloquine were assessed in *in vitro* cultures of bovine (*B. bovis* and *B. bigemina*) and equine (*B. caballi* and *B. equi*) *Babesia* parasites. Drug combination experiments were performed

to evaluate potential synergistic or antagonistic effects. Pepstatin A/mefloquine combinations were prepared and applied simultaneously to cultures of *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi*. Although no complete elimination was observed in *in vitro* cultures of all parasites from any of combination-treated cultures during treatment, combination-treated cultures of all parasites showed significant inhibition compared with control cultures (Table 2). There was no significant different between both reagents alone and combinations treatment for *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi*. Subsequent viability tests showed that there was re-growth of bovine and equine *Babesia* parasites for all drug combinations used (Table 2).

***In vivo* effects of pepstatin A and mefloquine on *B. microti* infection.** To examine the effects of pepstatin A and mefloquine on rodent *Babesia*, infected mice were treated with pepstatin A and mefloquine either alone or in combinations. In the pepstatin A-, mefloquine-, and combination-treated groups, levels of parasitemia were significantly lower than those in the control group ($P < 0.05$). Peak parasitemia reached an mean of 23.5% and 26.1% when treated with pepstatin A/mefloquine combinations of 5 mg/kg/5 mg/kg and 2.5 mg/kg/2.5 mg/kg, respectively, and 22.8% in treatment with 5 mg/kg pepstatin A 8 days after inoculation (Figure 5). The mean for the mefloquine-treated (5 mg/kg) group was 17.1%, and the mean for the control group was 67.3% (DMSO) 9 days after inoculation (Figure 5). Parasites were completely eliminated on day 23 post-infection in the treated groups. Conversely, parasites were completely cleared on day 30 post-infection in the control group. There were significant differences ($P < 0.05$) between the control and treatment groups on days 5–14 post-infection (Figure 5).

DISCUSSION

Babesia, an intraerythrocytic protozoan parasite, is similar to the malarial parasite *P. falciparum*. *Plasmodium* spp. use hemoglobin for reproduction in infected erythrocytes. Hemoglobin degradation takes place in an acidic food vacuole of
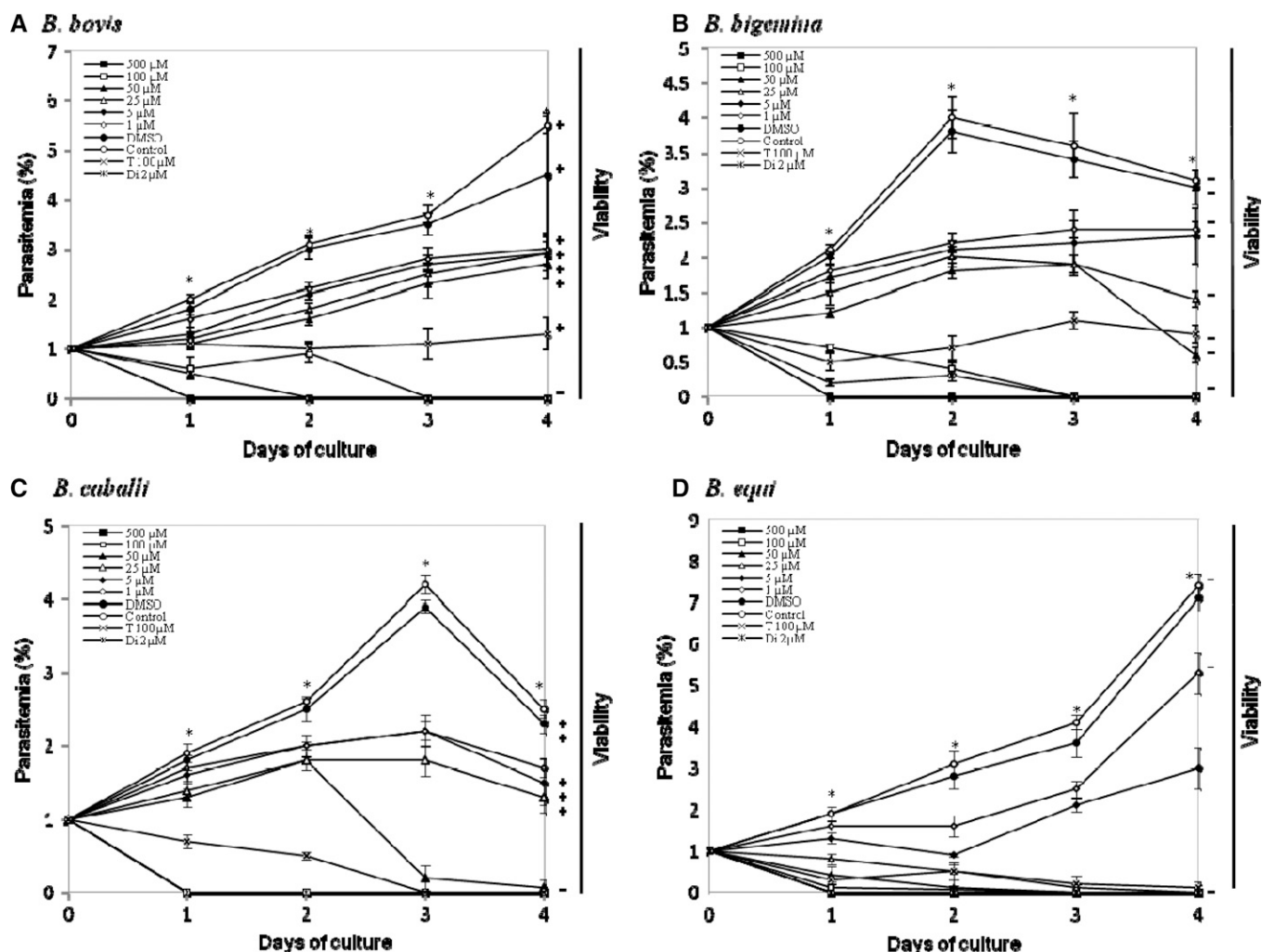
FIGURE 3. Inhibitory effects of different concentrations of mefloquine on the *in vitro* growth of *Babesia bovis* (**A**), *B. bigemina* (**B**), *B. caballi* (**C**), and *B. equi* (**D**). Diminazene aceturate (Di) and tetracycline hydrochloride (T) were used as positive controls. Each value represents the mean ± SD for experiments performed in triplicate. Curves represent the results of one representative experiment of three separate replicates. *Statistically significant differences ($P < 0.05$, by Student's *t*-test) between the drug-treated cultures and control cultures. DMSO = dimethylsulfoxide.

the parasite, and many current antimalarial drugs appear to disrupt important vacuolar functions.[16]

Aspartic protease inhibitors prevent degradation of hemoglobin and kill malaria parasites *in vitro* and *in vivo*.[18–21] However, in the *Babesia* parasite, these processes require further investigations. In the current study, AP inhibitor and anti-malarial drug were demonstrated to significantly inhibit *in vitro* and *in vivo* growth of *Babesia* parasites. Exposure of parasites to higher concentrations of both reagents completely suppressed the growth of bovine and equine *Babesia* parasites tested. Because treatment with DMSO only had no effect on parasitic growth, it this growth inhibition was likely caused by effects of both reagents.

The IC_{50} values of pepstatin A against bovine *Babesia* parasites (*B. bovis* and *B. bigemina*) were 38.5 μM and 36.5 μM, respectively, and those of equine *Babesia* parasites (*B. caballi* and *B. equi*) were 17.6 μM and 18.1 μM, respectively. In malaria parasites, 4 μM pepstatin A (IC_{50}) killed *P. falciparum* before trophozoite development and had a major effect on schizont maturation.[19]

The IC_{50} values of mefloquine against bovine *Babesia* parasites (*B. bovis* and *B. bigemina*) were 23 μM and 33.3 μM, respectively, and the values of mefloquine against equine *Babesia* parasites (*B. caballi* and *B. equi*) were 7.6 μM and 15 μM, respectively. Earlier studies showed that mefloquine was effective against *P. falciparum in vitro*, and showed low IC_{50} values of 28.7–130 nM.[27,34,35] Mefloquine showed high IC_{50} values (272 μM) for mammalian cells (dog neurons).[36]

Doses of pepstatin A and mefloquine for bovine babesiosis most effectively suppressed parasite growth compared with other drugs that have been tested for the treatment of babesiosis.[6,8,37] Conversely, effective doses of pepstatin A and mefloquine for bovine *Babesia* parasites were higher than those of other drugs tested in previous studies.[10,29,38–41] For equine *Babesia* parasites, the IC_{50} values of pepstatin A and mefloquine were similar to those reported in previous studies[6,7,16,42] but significantly lower than the IC_{50} values reported for equine *Babesia*.[8]

Moreover, the combination of pepstatin A and mefloquine produced antagonistic effects on *in vitro*-cultured parasites.
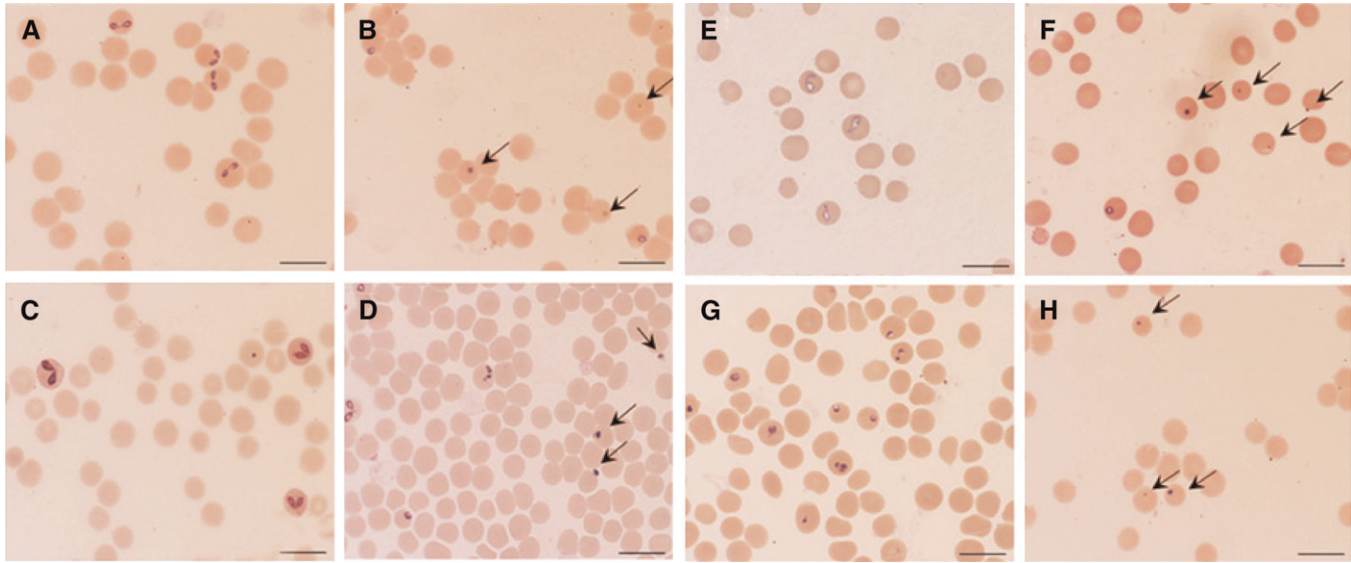
FIGURE 4. Light micrographs of mefloquine-treated bovine *Babesia* parasites in an *in vitro* culture. Micrographs were taken on day 3 of the experiment. *B. bovis*: control (**A**) and 1 μM mefloquine (**B**). *B. bigemina:* control (**C**) and 1 μM mefloquine (**D**). *B. caballi:* control (**E**) and 1 μM mefloquine (**F**). *B. equi:* control (**G**) and 1 μM mefloquine (**H**). Drug-treated cultures showed a higher number of degenerated parasites than control cultures. Scale bars = 10 μm.

It was previously reported that the plasmepsin inhibitor Ro40-4388 and the antimalarial drug chloroquine interacted antagonistically against *P. falciparum*.[17] Similar antagonistic actions between Ro40-4388 and mefloquine, quinine, amodiaquine, and halofantrine were found.[27] Conversely, AP inhibitors reduce hemoglobin degradation and subsequent release of heme, thus antagonizing the antimalarial activity of these drugs.

Furthermore, pepstatin A and mefloquine alone or in combination had inhibitory effects against *B. microti* infection in mice given single doses of 5 mg/kg, 5 mg/kg, or combinations of 2.5 mg/kg of each and 5 mg/kg of each during 6-day treatments. In the treated group, the parasitemia increased more slowly and achieved lower peaks compared with the parasitemia dynamics of the control group. In contrast, DMSO alone did not affect the growth of the parasites. Mefloquine was more effective than pepstatin A for treatment of infected mice, and pepstatin A/mefloquine combinations (2.5/2.5 mg/kg and 5/5 mg/kg) were less effective than each single treatment. The treated mice showed no signs of toxicity. As described in a previous study, *P. vinckei*-infected mice treated with 50 mg/kg of pepstatin A showed no toxic signs, and the *in vivo* effect was improved by combinations of AP and peptidyl cysteine inhibitors.[20] Mefloquine was used for treatment of mice infected with *Schistosoma mansoni* and *S. japonicum*[43] and of hamsters infected with *Opisthorchis viverrini*[44] at concentrations of 100–400 mg/kg for 2–4 weeks. There were no signs of toxicity observed in the murine model. Thus, pepstatin A (5 mg/kg) and mefloquine (5 mg/kg) did not induce any signs of toxicity. These results are consistent with those of Seminov and others,[20] who showed that 50 mg/kg of pepstatin A was used without any side effects. Therefore, mefloquine at this dose is not toxic to mice and might be used for treatment of babesiosis.

TABLE 2

Percentages of actual parasitemia and growth inhibition of combined applications of pepstatin A and mefloquine for *Babesia bovis*, *B. bigemina*, *B. caballi*, and *B. equi*

Pepstatin A/mefloquine (μM)	*B. bovis*			*B. bigemina*			*B. caballi*			*B. equi*		
	P %‡	I %§	V¶	P %×‡	I %§	V¶	P %‡	I %§	V¶	P %‡	I %§	V¶
A (medium)	6 ± 2.8	0	+	5.6 ± 1.7	0	+	4.5 ± 1.6	0	+	6 ± 2.5	0	+
B (0.0002% DMSO)	5.2 ± 1.9	0	+	4.7 ± 1.6	0	+	4.2 ± 1.2	0	+	5.7 ± 1.9	0	+
P (high)	1.7 ± 1.1	67.3#	+	2.2 ± 1.2	53.2#	+	1.6 ± 0.9	61.9#	+	1.3 ± 0.9	77.2#	+
P (low)	2.3 ± 1.5	55.8#	+	2.7 ± 1.8	42.6#	+	2.4 ± 1.1	42.9#	+	2.2 ± 1.3	61.4#	+
M (high)	1.4 ± 0.8	73.1#	+	2 ± 0.7	57.7#	+	0.6 ± 0.2	85.7#	+	0.3 ± 0.1	94.7#	+
M (low)	2.1 ± 0.7	59.6#	+	2.5 ± 1.3	46.8#	+	0.8 ± 0.5	81#	+	0.5 ± 0.1	91.2#	+
C1	2.8 ± 1.1	46.2#	+	3 ± 1.5	36.2#	+	2.2 ± 0.8	47.4#	+	2.4 ± 1.2	57.9#	+
C2	2.2 ± 0.9	57.7#	+	2.3 ± 1.1	51.1#	+	1.5 ± 0.6	62.5#	+	0.9 ± 0.6	84.2#	+
C3	1.9 ± 0.8	63.5#	+	2 ± 0.9	57.5#	+	0.9 ± 0.5	78.6#	+	0.7 ± 0.4	87.7#	+
C4	1.3 ± 0.5	75#	+	1.9 ± 0.5	59.6#	+	0.6 ± 0.1	85.7#	+	0.3 ± 0.03	94.7#	+

P% = percent actual parasitemia; I% = percent growth inhibition; V, viability testing; DMSO = dimethylsulfoxide.
A = each medium. and, B = DMSO (0.0002%). + indicated each medium (0.03%). P = pepstatin A for each parasite: (P high and P low) = *B. bovis* (6 and 3 μM), *B. bigemina* (5.8 and 2.9 μM), *B. caballi* (5.2 and 2.6 μM), and *B. equi* (1 and 0.5 μM); M = mefloquine for each parasite (M high and M low) = *B. bovis* (3.9 and 1.95 μM), *B. bigemina* (3.8 and 1.9 μM), *B. caballi* (4.4 and 2.2 μM), and *B. equi* (4.6 and 2.3μM). Pepstatin A/mefloquine combinations for each parasite: (C1, C2, C3, and C4) = *B. bovis* (3/1.9, 3/3.9, 6/1.9, and 6/3.9 μM), *B. bigemina* (2.9/1.9, 2.9/3.8, 5.8/1.9, and 5.8/3.8 μM), *B. caballi* (2.6/2.2, 2.6/4.4, 5.2/2.2, and 5.2/4.4 μM), and *B. equi* (0.5/2.3, 0.5/4.6, 1/2.3, and 1/4.6).
‡Percentage actual parasitemia is mean ± SD at day 3 of culture.
§Percentage growth inhibition was determined at day 3 of culture by compared with the control parasitemia.
¶Viability test indicates viability after 10 days of subsequent drug-free culture. + = viable.
#Significant difference (*P* < 0.05) between treated and control groups.
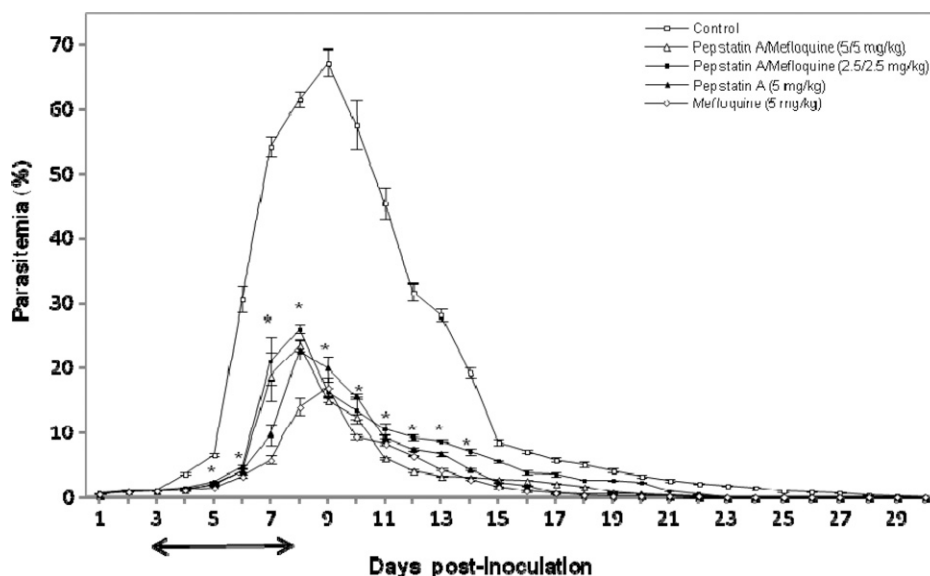
FIGURE 5. Inhibitory effect of combinations of pepstatin A and mefloquine (2.5 mg/kg of each and 5 mg/kg of each), pepstatin A (5 mg/kg), and mefloquine (5 mg/kg) on the *in vivo* growth of *Babesia* based on observations obtained for four mice per experimental group. Each value represents the mean ± SD. * = statistically significant differences ($P < 0.05$, by Student's *t*-test) between the treated groups and the control group. ↔ = time of inoculation or control reagent (dimethlysulfoxide) application of intraperitoneal pepstatin A, mefloquine, or a combination of pepstatin A and mefloquine.

Treatment with pepstatin A at 5 mg/kg, mefloquine at 5 mg/kg, and the combinations of 2.5/2.5 mg/kg and 5/5 mg/kg resulted in 66.1, 74.6, 61.2, and 65.1% inhibition, respectively, of the growth of *B. microti* in infected mice on days 9–10 post-infection, and parasites were completely eliminated on day 22 post-infection in the treated groups. Conversely, parasites were completely cleared on day 30 post-infection in the control group. In the mouse model, pepstatin A and mefloquine were more effective than other drugs tested in previous studies.[8,41] In addition, in combination treatment, the antiparasitic activity of pepstatin A and mefloquine was reduced to that of each single treatment in *in vitro* and *in vivo* studies, suggesting that mefloquine might antagonize actions of the inhibitor of AP, pepstatin A, against *Babesia* parasites.

The gene sequences of APs can be found in the *B. bovis* genome sequence database.[28] Therefore, *in vitro* and *in vivo* inhibition of these enzymes indicates an important role of APs in the growth cycle of *Babesia* parasites.

In conclusion, results of the present study showed that pepstatin A and mefloquine potently inhibit *Babesia* parasites *in vitro* and *in vivo*. Furthermore, pepstatin A might antagonize actions of mefloquine on the inhibitory effects of *in vitro* and *in vivo* combinations. This study also shows that AP has an important role in the growth cycle of *Babesia* parasites. Therefore, characterization, enzymatic activity, and effect of APs on hemoglobin degradation are needed.

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REFERENCES

1. Kuttler KL, 1988. World-wide impact of babesiosis. Ristic M, ed. *Babesiosis of Domestic Animals and Man*. Boca Raton, FL: CRC Press, 1–22.
2. Wright IG, Goodger BV, 1988. Pathogenesis of babesiosis. Ristic M, ed. *Babesiosis of Domestic Animals and Man*. Boca Raton, FL: CRC Press, 99–118.
3. Homer MJ, Aguilar-Delfin I, Telford III Sr, Krause PJ, Persing DH, 2000. Babesiosis. *Clin Microbiol Rev 13:* 451–469.
4. Kjemtrup AM, Conrad PA, 2000. Human babesiosis: an emerging tick-borne disease. *Int J Parasitol 30:* 1323–1337.
5. Vial HJ, Gorenflot A, 2006. Chemotherapy against babesiosis. *Vet Parasitol 138:* 147–160.
6. Bork S, Yokoyama N, Matsuo T, Claveria FG, Fujisaki K, Igarashi I, 2003a. Growth inhibitory effect of triclosan on equine and bovine *Babesia* parasites. *Am J Trop Med Hyg 68:* 334–340.
7. Nagai A, Yokoyama N, Matsuo T, Bork S, Hirata H, Xuan X, 2003. Growth-inhibitory effects of artesunate, pyrimethamine, and pamaquine against *Babesia equi* and *Babesia caballi* in *in vitro* cultures. *Antimicrob Agents Chemother 47:* 800–803.
8. Bork S, Yokoyama N, Ikehara Y, Kumar S, Sugimoto C, Igarashi I, 2004. Growth-inhibitory effect of heparin on *Babesia* parasites. *Antimicrob Agents Chemother 48:* 236–241.

9. Bork S, Das S, Okubo K, Yokoyama N, Igarashi I, 2006. Effect of protein kinase inhibitors on the *in vitro* growth of *Babesia bovis*. *Parasitology 132:* 775–779.

10. Okubo K, Yokoyama N, Govind Y, Alhassan A, Igarashi I, 2007. *Babesia bovis*: effects of cysteine protease inhibitors on *in vitro* growth. *Exp Parasitol 117:* 214–217.

11. Goldberg DE, Slater AFG, Beavis R, Chait B, Cerami A, Henderson GB, 1991. Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: a catabolic pathway initiated by a specific aspartic protease. *JEM 173:* 961–969.

12. Laurent F, Bourdieu C, Kaga M, Chilmonczyk S, Zgrzebski G, Yvore P, Pery P, 1993. Cloning and characterization of an *Eimeria acervulina* sporozoite gene homologous to aspartyl proteinases. *Mol Biochem Parasitol 62:* 303–312.

13. Jean L, Grosclaude J, Labbe M, Tomley F, Pery P, 2000. Differential localization of an *Eimeria tenella* aspartyl proteinase during the infection process. *Int J Parasitol 30:* 1099–1107.

14. Pinho R, Beltramini L, Alves C, De-Simone S, 2009. *Trypanosoma cruzi*: isolation and characterization of aspartyl proteases. *Exp Parasitol 122:* 128–133.

15. Francis SE, Gluzman IY, Oksman A, Knickerbocker A, Mueller R, Bryant ML, Sherman DR, Russell DG, Goldberg DE, 1994. Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. *EMBO J 13:* 306–317.

16. Silva AM, Lee AY, Gulnik SV, Majer P, Collins J, Bhat TN, 1996. Structure and inhibition of plasmepsin II, a hemoglobin-degrading enzyme from *Plasmodium falciparum*. *Proc Natl Acad Sci USA 93:* 10034–10039.

17. Moon RP, Tyas L, Certa U, Rupp K, Bur D, Jacquet C, Matile H, Loetscher H, Grueninger-Leitch F, Kay J, Dunn BM, Berry C, Ridley RG, 1997. Expression and characterisation of plasmepsin I from *Plasmodium falciparum*. *Eur J Biochem 244:* 552–560.

18. Banerjee R, Liu J, Beatty W, Pelosof L, Klemba M, Goldberg DE, 2002. Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. *Proc Natl Acad Sci USA 99:* 990–995.

19. Bailly E, Jambou R, Savel J, Jaureguiberry G, 1992. *Plasmodium falciparum:* differential sensitivity *in vitro* to E-64 (cysteine protease inhibitor) and pepstatin A (aspartyl protease inhibitor). *J Protozool 39:* 593–599.

20. Semenov A, Olson JE, Rosenthal PJ, 1998. Antimalarial synergy of cysteine and aspartic protease inhibitors. *Antimicrob Agents Chemother 42:* 2254–2258.

21. Sharma A, Eapen A, Subbararao SK, 2005. Purification and characterization of a hemoglobin degrading aspartic protease from the malarial parasite *Plasmodium vivax*. *J Biochem 138:* 71–78.

22. Palmer KJ, Holliday SM, Brogden RN, 1993. Mefloquine. A review of its antimalarial activity, pharmacokinetic properties and therapeutic efficacy. *Drugs 45:* 430–475.

23. Mockenhaupt FP, 1995. Mefloquine resistance in *Plasmodium falciparum*. *Parasitol Today 11:* 248–253.

24. Schlagenhauf P, 1999. Mefloquine for malaria chemoprophylaxis 1992–1998: a review. *J Travel Med 6:* 122–133.

25. Nevin RL, 2009. Epileptogenic potential of mefloquine chemoprophylaxis: a pathogenic hypothesis. *Malar J 8:* 188.

26. Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL, Ridley RG, 1998. An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials. *Biochem Pharmacol 55:* 727–736.

27. Mungthin M, Bray PG, Ridley RG, Ward SA, 1998. Central role of hemoglobin degradation in mechanisms of action of 4-aminoquinolines, quinoline methanols, and phenanthrene methanols. *Antimicrob Agents Chemother 42:* 2973–2977.

28. Brayton KA, Lau AO, Herndon DR, Hannick L, Kappmeyer LS, Berens SJ, 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. *PLoS Pathog 3:* 1401–1413.

29. AbouLaila M, Nakamura K, Govind Y, Yokoyama N, Igarashi I, 2010a. Evaluation of the *in vitro* growth-inhibitory effect of epoxomicin on *Babesia* parasites. *Vet Parasitol 167:* 19–27.

30. Zweygarth E, Just MC, de Waal DT, 1995. Continuous *in vitro* cultivation of erythrocytic stages of *Babesia equi*. *Parasitol Res 81:* 355–358.

31. Erp EE, Smith RD, Ristic M, Osorno BM, 1980. Optimization of the suspension culture method for *in vitro* cultivation of *Babesia bovis*. *Am J Vet Res 41:* 2059–2062.

32. AbouLaila M, Munkhjargal T, Sivakumar T, Ueno A, Nakano Y, Yokoyama M, Yoshinari T, Nagano D, Katayama K, El-Bahy N, Yokoyama N, Igarashi I, 2012. Apicoplast-targeting antibacterials inhibit the growth of *Babesia* parasites. *Antimicrob Agents Chemother 56:* 3196–3206.

33. Bork S, Yokoyama N, Matsuo T, Claveria FG, Fujisaki K, Igarashi I, 2003c. Clotrimazole, ketoconazole, and clodinafop-propargyl inhibit the *in vitro* growth of *Babesia bigemina* and *Babesia bovis* (Phylum Apicomplexa). *Parasitology 127:* 311–315.

34. Rogers WO, Sem R, Tero T, Chim P, Lim P, Muth S, Socheat D, Ariey F, Wongsrichanalai C, 2009. Failure of artesunate-mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia. *Malar J 8:* 10. doi:10.1186/1475-2875-8-10.

35. Wurtz N, Briolant S, Gil M, Parquet V, Henry M, Baret E, Amalvict R, Almeras L, Rogier C, Pradines B, 2010. Synergy of mefloquine activity with atorvastatin, but not chloroquine and monodesethylamodiaquine, and association with the *pfmdr1* gene. *J Antimicrob Chemother 65:* 1387–1394.

36. Lee HS, Go ML, 1996. Effects of mefloquine on Ca2+ uptake and release by dog brain microsomes. *Arch Int Pharmacodyn Ther 331:* 221–231.

37. Takabatake N, Hashiba S, Bork S, Okamura M, Yokoyama N, Igarashi I, 2004. Fucoidan inhibits the *in vitro* growth of *Babesia bovis*. *J Protozool Res 14:* 55–60.

38. Igarashi I, Njonge F, Kaneko Y, Nakamura Y, 1998. *Babesia bigemina*: *in vitro* and *in vivo* effects of curdlan sulfate on the growth of parasites. *Exp Parasitol 90:* 290–293.

39. Munkhjargal T, Aboulaila M, Sivakumar T, Yokoyama N, Igarashi I, 2009. Inhibitory effect of apicidin on *in vitro* and *in vivo* growth of *Babesia* parasites. *J Protozool Res 19:* 42–49.

40. Bork S, Yokoyama N, Matsuo T, Claveria FG, Fujisaki K, Igarashi I, 2003b. Clotrimazole, ketoconazole, and clodinafop-propargyl inhibit the *in vitro* growth of *Babesia bigemina* and *Babesia bovis* (Phylum Apicomplexa). *Parasitology 127:* 311–315.

41. AbouLaila M, Yokoyama N, Igarashi I, 2010b. Inhibitory effect of (-)-Epigallocatechin-3-gallate from green tea on the growth of *Babesia* parasites. *Parasitology 137:* 7858–7791.

42. AbouLaila M, Sivakumar T, Yokoyama N, Igarashi I, 2010d. Inhibitory effect of terpene nerolidol on the growth of *Babesia* parasites. *Parasitol Int 59:* 278–282.

43. Keiser J, Chollet J, Xiao SH, Mei JY, Jiao PY, Utzinger J, Tanner M, 2009. Mefloquine – an aminoalcohol with promising anti-schistosomal properties in mice. *PLoS Negl Trop Dis 3:* e350. doi:10.1371/journal.pntd.0000350.

44. Keiser J, Odermatt P, Tesana S, 2009. Dose-response relationships and tegumental surface alterations in *Opisthorchis viverrini* following treatment with mefloquine *in vivo* and *in vitro*. *Parasitol Res 105:* 261–266.