Oral Administration of Azithromycin Ameliorates Trypanosomosis in *Trypanosoma congolense*-infected Mice

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Abstract

Animal trypanosomosis is a devastating parasitic disease that is of economic importance to livestock production. The infection includes animal African trypanosomosis, surra and dourine. The treatment is based solely on few compounds that were discovered decades ago and which are associated with severe toxicity. Furthermore, it is likely that the parasite has developed resistance towards them. Thus, there is an urgent need for new, accessible and less toxic drugs.

Azithromycin is an antibiotic with documented efficacy against *Toxoplasma, Babesia* and *Plasmodium*. The current study investigated its effects against animal trypanosomes. An *in vitro* system was used to determine the trypanocidal effects of azithromycin against *T. congolense, T. b. brucei* and *T. evansi*, and cytotoxicity in MDBK and NIH 3T3 cells. Furthermore, the trypanocidal effects of azithromycin were investigated in *T. congolense*-infected mice.

In vitro, azithromycin had an IC_{50} of 0.19 ± 0.17; 3.69 ± 2.26; 1.81 ± 1.82 µg/ml against *T. congolense, T. b. brucei* and *T. evansi,* respectively. No cytotoxic effects were observed in MDBK and NIH 3T3 cells. The efficacy of orally administered azithromycin was investigated in short-term and long-term treatment protocols. Although the short-term treatment protocol showed no curative effects, the survival rate of the mice was significantly prolonged ($p < 0.001$) in comparison to the control group. The long-term treatment yielded satisfying curative effects with doses of 300 mg/kg and 400 mg/kg achieving 80% and 100% survival, respectively. In conclusion, long-term oral azithromycin treatment has trypanocidal effects against *T. congolense.*

Keywords Animal trypanosomosis; Azithromycin; Oral administration; *Trypanosoma congolense*.

Introduction

Animal trypanosomosis is a spectrum of diseases that includes animal African trypanosomosis (AAT), surra and dourine. AAT, which is commonly referred to as nagana, is caused by *Trypanosoma congolense, T. brucei brucei* and *T. vivax* (Morrison et al. 2016). In addition to AAT, surra is caused by *T. evansi* and dourine caused by *T. equiperdum* (Desquesnes et al. 2013). Animal trypanosomosis is a devastating parasitic infection that results in major losses in livestock productivity in affected countries. The clinical symptoms include (but are not limited to) anemia, loss of condition and emaciation; untreated cases are usually fatal (Jaiswal 2015; Steverding 2010). Nagana is widely distributed in sub-Saharan Africa in accordance with the distribution of the insect vector, the tsetse fly (genus: *Glossina*) which is widely distributed on the African continent, particularly from the Tropic of Capricorn to the sub-Saharan region. More than 30 African countries are exposed to the infection. Several vectors, including biting flies such as the tabanids and stomoxyine transmit surra, while dourine is transmitted by infected animals—especially equids—through coitus and is therefore widely distributed in all areas in which equines are kept. Animal trypanosomosis affects a wide range of hosts including goats, camels, horses, donkeys, pigs and other species; however, cattle remain the main host for the infection (Desquesnes et al. 2013; Morrison et al. 2016; Steverding 2010). Even though *T. congolense* is a causative agent of AAT in livestock, the range of hosts also includes animals such as dogs; however, cases involving such animals are rarely reported (Lisulo et al. 2014; Watier-Grillot et al. 2013).

The control of the animal trypanosomosis is based on three factors: vector control, the breeding of trypanotolerant breeds, and the usage of trypanocides. Vector control can be

expensive when used on a large scale. Thus far, the total eradication of the vector has only been successful in a small and delimited area of Zanzibar (Vreysen et al. 2014). Secondly, the breeding of trypanotolerant livestock is costly and requires a prolonged period of time; thus, the only current available option is the usage of trypanocides (Clausen et al. 2010; d'leteren 1998; Mwai et al. 2015; Yaro et al. 2016). A number of the chemotherapeutic compounds that are currently available were established decades ago and have already been utilized for some time. The important problems with the currently available chemotherapeutic compounds include severe side effects and the resistance of the parasites, which has developed due to the extensive usage of the drugs (Steverding 2010). Resistance has become widespread and is out of control due to either the under-dosing of animals or the misdiagnosis of animals by veterinarians who lack the skills to conduct correct diagnostic examinations. Finally, the trypanocidal compounds that are in current use are chemically related, which has exacerbated the problem of cross-resistance. The compounds that are currently available for the treatment of both animal and human trypanosomosis are administered intramuscularly, intravenously or intraperitoneally. Thus, in the present study, we aimed to develop an effective orally administered drug with fewer side effects.

Azithromycin (AZM), $C_{38}H_{72}N_2O_{12} \cdot 2H_2O$, is a macrolide antibacterial agent that is used in treatment of various bacterial infections (Parnham et al. 2014). This compound has recently received a great deal of scientific attention due to its outstanding efficacy in the treatment of bacterial infections and other parasitic infections, including *Babesia*, *Toxoplasma gondii* and *Plasmodium* spp. (Birkenheuer et al. 2004; Blais 1993; Wilson et al. 2015). As a widely used compound, it is generally considered safe for use by humans and is reported to be associated with fewer side effects in comparison to other macrolides. Most importantly, however, macrolide antibiotics are generally affordable (Wilson et al. 2015). The main mechanism of action of macrolide antibacterial agents against bacteria is understood to be the

inhibition of protein synthesis. AZM exhibits the same mechanism against other organisms, including *Toxoplasma* and *Plasmodium* (Blais 1993; Wilson et al. 2015). In addition to the well-established long half-life, the compound accumulates in phagocytes, which enhances the delivery of high concentrations of the drug to the site of infection (Parnham et al. 2014).

The first objective of the study was to determine the *in vitro* effects of AZM *against* animal trypanosome species (*T. congolense, T. b. brucei* and *T. evansi*), and to determine the efficacy of orally administered azithromycin in *T. congolense*-infected mice.

Materials and methods

Parasites and *in vitro* culture

T. congolense IL3000, a savannah type strain isolated near the Kenya/Tanzania border in 1966; *T. evansi* Tansui and *T. b. brucei* GUTat 3.1 strains were maintained in the bloodstream form (BSF), and propagated at 33°C and 37°C, respectively, in air using HMI-9 medium (Hirumi and Hirumi 1991). The medium is composed of Iscove's modified Dulbecco's medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 20% heat inactivated fetal bovine serum (HI-FBS), 60 mM HEPES (Sigma-Aldrich), 1 mM pyruvic acid sodium salt (Sigma-Aldrich), 0.1 mM bathocuproine (Sigma-Aldrich), 1 mM hypoxanthine and 16 µM thymidine (HT supplement: Thermo Fisher Scientific K.K., Yokohama, Japan), 10 µg/L insulin, 5.5 µg/L transferrin and 6.7 ng/l sodium selenite (ITS-X: Thermo Fisher Scientific), 0.0001% 2-β-mercaptoethanol (Sigma-Aldrich), 0.4 g/L (Sigma-Aldrich) and 2 mM L-cysteine (Sigma-Aldrich). The cultures were maintained by replacing the entire supernatant with fresh medium every other day (Suganuma et al. 2014).

Cell line cultures

The Mardin-Darby bovine kidney (NBL-1 strain: JCRB cell bank) and mouse embryonic fibroblast, NIH 3T3 (Courtesy of Prof. Makoto Igarashi, National Research Center for Protozoan Diseases, Obihiro, Japan) cell lines were revived from the liquid nitrogen-preserved stock of Obihiro University of Agriculture and Veterinary Medicine, Japan. The cells were cultured in Minimum Essential Medium Eagle (MEM) suspended with 10% HI-FBS at 37˚C in an incubator under 5% CO_2. The cells were maintained by replacing the medium with fresh medium 2 days before the cells become confluent.

The *in vitro* evaluation of trypanocidal effects of AZM

T. congolense, T. b. brucei and *T. evansi* were seeded at 1×10^5; 1×10^4 and 2×10^4 cells/mL, respectively in a Nunc™ 96-well optical bottom plate (Thermo Fisher Scientific) and exposed to various concentration of AZM (0.32 to 25 µg/mL; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The plates were incubated at 33°C (*T. congolense*) and 37°C (*T. b. brucei* and *T. evansi*) for 72 h. Subsequently, 25 µL of CellTiter-Glo™ Luminescent Cell Viability Assay reagent (Promega Japan, Tokyo, Japan) was added to evaluate intracellular ATP concentration. The plate was shaken for 2 min (500 shakes/min) using a MS3 basic plate shaker (IKA® Japan K.K., Osaka, Japan) to facilitate cell lysis and the release of intracellular ATP. The plates were further incubated for 10 min at room temperature and were subsequently read using a GloMax® -Multi+ Detection System plate reader (Promega Japan) (Suganuma et al. 2014). The experiments were conducted in triplicates.

The *in vitro* cytotoxicity tests

Mammalian cell lines (MDBK and NIH 3T3) were seeded separately at a concentration of 1×10^5 cells/mL in a 96 well microtiter plate (Thermo Fisher Scientific) and exposed to various concentrations of AZM (0.32 to 25 µg/mL). The surviving cell fraction was determined by a CCK-8 (Dojindo Laboratories., Kumamoto, Japan) assay by adding 10 µL of CCK- 8 reagent onto the plates at 72 h after incubation. The plates were further incubated for an additional 2 hours at 37°C. Cell viability was determined using an ELISA reader (MTP 500, Corona electric, Japan), according to the amount of formazan that formed, which was determined based on the absorbance at 450 nm (Lou et al. 2010; Weyermann et al. 2005). The experiments were conducted in triplicates.

The selectivity index was calculated to allow for the possible identification of compounds with high efficacy and low cytotoxicity. This parameter reflects the quantity of the compound that is active against the pathogen but which is not toxic towards the host cells. The following formula was used:

Selectivity index = Minimum toxic concentration (μg/mL)/minimum inhibitory concentration (μg/mL)

where the minimum toxic concentration is the compound concentration that inhibited 50% of the cell growth, while the minimum inhibitory concentration was the concentration that inhibited the proliferation of the parasite by 50%.

Animal experiments

Healthy female BALB/c mice (CLEA Japan Inc., Tokyo, Japan) weighing 20–30 g were used in the study. The mice were kept in the animal house of Obihiro University of Agriculture and Veterinary Medicine, Japan. The animals acclimatized in plastic cages in an air conditioned environment and were maintained at 25 ± 2°C with 60 ± 10% relative humidity under a 12-h light and dark cycle for one week before commencing the experiments. All of the animals had *ad libitum* access to normal chow and water. The experiment was approved by the animal ethics committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval Nos. 28-129 and 28-169).

The trypanocidal effects of AZM on *T. congolense in vivo*

Short-term treatment

The virulent *T. congolense* IL3000 strain was propagated in mice and used for infection. The parasites were passaged twice in mice before the experiment. The mice were intraperitoneally infected with 100 μL of *T. congolense* strain at 5×10^3 parasites/mouse inoculated with PSG. The mice were randomly divided into 7 groups of 5 mice as follows: Group I (control group), the mice were infected but not treated; group II (positive control group), the mice were infected and treated with diminazene aceturate (DA) (3.5 mg/kg, intraperitoneally); Groups III, IV, V, VI and VII (the test groups), the mice were infected and orally treated using a feeding needle with 50, 100, 200, 300 and 400 mg/kg AZM, respectively. Treatment was initiated at 48 hours post-infection and was maintained for 7 consecutive days. The treatments were freshly prepared each day. The surviving mice were observed for 90 days while the others were observed until death. Each day, the parasitemia was evaluated and the effects of treatment were monitored using wet blood smears. Each slide was prepared with fresh blood collected from the tail vein (magnification: 400×). The experiments were conducted in duplicate.

Long term treatment

The long-term trypanocidal effects of AZM were further investigated in *T. congolense*-infected mice. The mice were divided into 8 groups of 5 mice each, as follows: Group I (the healthy control group), the mice were not infected or treated; Group II (the vehicle control group), the mice were infected and treated with vehicle; and Group III (the positive control group), the mice were infected and treated with DA; Groups IV, V, VI, VII, and VIII (the treatment groups), the mice were orally treated with 50, 100, 200, 300 or 400 mg/kg, respectively, of AZM using a feeding needle. Treatment was initiated at 48 hours post-infection and was maintained for 28 consecutive days. The parasitemia and the body weight of the mice

were recorded twice a week, while the blood parameters were evaluated once a week. The objective of this particular treatment was to determine the duration for which the *T. congolense*-infected mice depended on AZM. These experiments were conducted in duplicate.

The hematological parameters

The blood samples that were collected were subjected to a blood count using an automatic hematology analyzer (Celltac α, Nihon Kohden, Tokyo, Japan). The red blood cell (RBC), hematocrit (HCT), mean corpuscular hemoglobin (MCH), hemoglobin (HGB), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and platelet (PLT) count were analyzed.

Statistical analysis

The results were expressed as the mean ± standard deviation (S.D.) for the number of repeated trials indicated in each experiment. The statistical analyses were conducted in the acute phase of infection. The *t*-test was used for intergroup comparisons between the treated and non-treated groups. The survival curves were constructed using the Kaplan-Meier method and the curves were compared using a log-rank test. All of the data were compiled using GraphPad Prism Software program (version 5.0, GraphPad Software Inc., CA, USA). P values of < 0.05 were considered to indicate statistical significance.

Results

The *in vitro* trypanocidal activity of AZM

The activity of AZM was assessed *in vitro* after 72 h incubation of *T. congolense, T. b. brucei* or *T. evansi* BSF with the treatment regimens. The IC_{50} of AZM for *T. congolense, T. b. brucei* and *T. evansi* was 0.19 ± 0.17; 3.69 ± 2.26 and 1.81 ± 1.82 (mean [in µg/mL] ± S.D), respectively. AZM was less cytotoxic to MDBK and NIH 3T3 cells with an LC_{50} of >25 µg/ml in both cell types. Based on the IC_{50} and LC_{50} values that were obtained, the selectivity index against *T. congolense* was >131.58.

The *in vivo* trypanocidal effects of short-term treatment

In the present study, the prepatent period of *T. congolense* was 3 days. Oral treatment with AZM resulted in a moderate trypanocidal effect (determined based on parasitemia) in comparison to the control in which parasitemia increased from day 3 to day 8 (Fig. 1). All of the treated groups showed a dose-dependent trypanocidal effect with the initial clearance of the parasites from the peripheral circulation in all the groups; however, this was followed by a relapse, which resulted in the rapid growth of the parasites (Fig. 1). At 50 mg/kg, the parasite growth in the acute phase of the infection was slightly suppressed in comparison to the control group, with significant differences on days 4, 7, 5 and 8 ($p < 0.05$). In contrast, parasite growth in the AZM 100, 200, 300, 400 mg/kg groups and the diminazene aceturate group from day 3 to day 8 was significantly lower in comparison to the control group ($p < 0.05$; Fig. 1). There was a subsequent relapse in the 100, 200, 300 and 400 mg/kg groups on days 9, 10, 11 and 14, respectively (Fig. 1). The survival rate was significantly prolonged in all of the treated groups ($p < 0.001$). A number of mice survived in the AZM 200, 300 and 400 mg/kg groups, while all of the mice in the control and the AZM 50 and 100 mg/kg groups died (Fig. 2).

The mice that survived for 90 days were considered to have been cured. In all of the treated groups, survival was significantly prolonged in comparison to the control group ($p < 0.001$). The mice in the control group all died within 9 days of the experiment while those in the AZM 50 and 100 mg/kg groups died on days 15 and 16, respectively. In the AZM 200, 300 and 400 mg/kg groups, 30%, 30% and 20% of the mice survived, respectively, throughout the whole study period (Fig. 2).

The long-term treatment test

Body weight

A significant body weight increase was observed in the AZM 300 mg/kg group comparison to the non-treated control group ($p < 0.001$) while a decrease was observed in the AZM 200 mg/kg group ($p < 0.05$). The other groups showed no significant differences in comparison to the non-treated control group (Fig. 3).

Parasitemia

After the prepatent period, parasitemia increased steadily in the control and AZM 50 mg/kg group, while parasitemia was suppressed for an extended period (before eventually relapsing) in the other groups (Fig. 4). At a dose of 100 mg/kg, relapse was observed during day 8 of treatment, with rapid parasite growth observed between day 13 and 14, which led to the death of 80% of the mice (Fig. 5). The surviving mice relapsed and died before the next parasite count. At a dose of 200 mg/kg, relapse was observed on day 13 with rapid parasite growth that led to the death of 90% of the mice in this group (Fig. 5). Although the single surviving mouse survived to the end of the experiment, it showed a high level of parasitemia. The mice in the

AZM 300, 400 mg/kg and DA groups initially showed parasites in their peripheral circulation. Although these parasites were eliminated during the treatment period, a relapse occurred at one week after the treatment in the AZM 300 mg/kg group, which led to the death of 20% of the mice (Fig. 5).

Survival curve

The survival of the mice was significantly prolonged ($p < 0.001$) in all the treated groups. In contrast, the control mice all died on day 10, while the mice in the AZM 50 mg/kg and 100 mg/kg groups died on days 16 and day 21, respectively (Fig. 5). One of the mice in the AZM 200 mg/kg group survived to day 31 while the rest died during the treatment period. All mice in the AZM 300 mg/kg, 400 mg/kg and DA groups survived until the last day of the treatment; however, two of the mice in the AZM 300 mg/kg group relapsed and died on day 38. The rest of the mice were considered cured as no trypanosomosis-related symptoms were observed and no parasites were observed microscopically for a period of 90 days.

Blood parameters

The blood parameters related to anemia (specifically, the red blood cell [RBC] count; hemoglobin [HGB] level and the hematocrit [HCT] ratio) in the control and treated groups were compared to those of healthy mice. A non-significant decrease - which had been anticipated - was observed in the RBC of the control group. In contrast, a significant ($p < 0.05$) decrease was observed in the RBC count of the AZM 50 mg/kg group within the first week of infection. Significant decreases were also observed in the HGB levels of the control, and the AZM 50, 100 and 300 mg/kg groups ($p < 0.05$; $p < 0.0001$) (Table 1). The parameters of the treated groups also fluctuated; however, they remained within the normal ranges throughout

the study period (Table 1). In week 3, the HCT values of the AZM 300 and 400 mg/kg groups showed a significant decrease ($p < 0.05$); however, the values increased to within the normal range in the fourth week of the study (Table 1).

1 **Discussion**

2 AZM showed greater trypanocidal efficacy against *T. congolense* than *T. b. brucei* and
3 *T. evansi in vitro*. AZM is a well-known protein synthesis inhibitor that has shown efficacy
4 against *Toxoplasma gondii* (Blais 1993) and *Plasmodium falciparum* (Wilson et al. 2015). The
5 mode of action exhibited by AZM on trypanosomes is not yet known, however, the
6 development of a vacuole was observed in the current study on AZM treated *T. congolense*
7 parasites as shown in the online resource (Fig A1), therefore indicating the need for further
8 studies in order to address this issue. The heterogeneous efficacy against the tested
9 *Trypanosoma* species may therefore be attributed to protein synthesis, since *T. congolense*
10 (Subgenus *Nannomonas*) differs—both morphologically and taxonomically—from *T. b. brucei*
11 and *T. evansi* (*Trypanozoon*). However, further studies will be needed to confirm this
12 hypothesis. *In vitro* toxicity tests were conducted using MDBK and NIH 3T3 cells to determine
13 the potential of AZM to induce cell death. No toxicity was observed in either of the cell types
14 after 72 hours of AZM treatment. This resulted in an LC_{50} of >25 μg/ml. AZM is a well-studied
15 compound that is used to treat a variety of infections and which is generally considered safe;
16 thus, the results of the present study were in agreement with previous studies (Parnham et al.
17 2014; Wilson et al. 2015).

18 Mice infected with *T. congolense* savannah strain IL 3000 were treated both orally and
19 intraperitoneally in the current study, in order to determine the trypanocidal effect of AZM *in*
20 *vivo*. AZM showed mild to moderate efficacy against *T. congolense* BSF when it was
21 administered orally and short-term treatment cured some of the mice in the AZM 200, 300 and
22 400 mg/kg groups (Fig. 1 and 2). According to a previous report (Boonleang 2007), AZM is
23 readily and rapidly absorbed from the gastrointestinal tract and distributed to the body tissue
24 with excellent tissue penetration, which makes the oral route the preferred route of
25 administration for this compound. In addition, AZM is generally stable in acidic media, such

26 as the gastric environment. Another benefit is the greater oral bioavailability, which is
27 associated with the prolonged elimination of the compound from the animal body due to the
28 well-documented half-life (57 to 68 hours). When AZM was administered intraperitoneally
29 none of the mice were cured and all died (data not shown). Signs of toxicity also occurred in
30 association with the intraperitoneal tests in the present study, which resulted in loss of body
31 weight, loss of appetite, rough hair coat and hunched back in the AZM 400 mg/kg group
32 (Parasuraman 2011). Few studies have investigated the pharmacokinetics of intraperitoneally
33 administered AZM.

34 There are numerous factors that are likely to influence the effectiveness of orally
35 administered drugs in comparison to other routes of administrations. AZM has been shown to
36 be effective when administered orally; however, not all of the tested concentrations were
37 effective. This might be associated with factors such as drug-nutrient or food interaction(s)
38 (Bushra 2011; Foulds 1996). Bushra *et al.*, (2011) stated that AZM absorption is negatively
39 affected by the intake of food, which reduces the total bioavailability by 43%. This factor might
40 be associated with the failure of other subliminal concentrations. Conversely, other studies
41 have claimed that the intake of food does not affect the bioavailability of AZM in any way,
42 unless antacids are consumed together with the compound (Foulds 1996; Zuckerman 2004).
43 Under-dosing is another factor that could account for the treatment failure. The doses of AZM
44 that are administered for babesiosis, toxoplasmosis and bacterial infections range from 500 and
45 1,000 mg/kg daily for a prescribed number of days (Chico et al. 2008; Parnham et al. 2014). In
46 contrast, the highest dose that was administered in the present study was 400 mg/kg.

47 In the treatment groups, the parasite burden was relieved for a number of days. In
48 contrast, patent parasites were observed in the control group throughout the study. However,
49 although some of the mice did not relapse, the cessation of treatment was followed by relapse
50 in all of the treatment groups. Relapse led to a rapid increase in parasitemia; in all cases of

relapse, this resulted in the death within 3 days of the relapse. The mechanism underlying the effect of AZM on bacteria is well documented: AZM inhibits protein synthesis in the bacteria; thus, AZM possesses a bacteriostatic effect rather than a bactericidal effect (Dorfman et al. 2008). This mechanism has also been documented in *Toxoplasma* and *Plasmodium* studies (Blais 1993; Wilson et al. 2015). Thus, this mechanism was likely to have been responsible for the effects in the present study, given the fact that parasitemia was initially suppressed (in all of the treated groups) and that relapse occurred when the treatment stopped. The inability of the compound to clear the parasites from the peripheral blood circulation could be one of the reasons for the relapse. The second factor is that the compound is readily absorbed by the body tissues, which means that the concentration of the compound in the plasma drops rapidly as the concentration in the cells increases (Boonleang 2007). AZM was not suitable as a prophylactic agent for the same reason (data not shown)—this was evidenced by the fact that all of the test mice died. Few mice were cured from infection when mice were treated with oral AZM for 7 days at doses of 200, 300 and 400 mg/kg.

Even though AZM is a bacteriostatic agent, several studies have claimed that it is likely to show bactericidal effects over longer treatment periods and at increased concentrations (Dorfman et al. 2008). Thus, we also investigated the effects of long-term treatment in the mice infected with the *T. congolense* savannah isolate. Throughout the experiment, the body weight, parasitemia, blood parameters and clinical signs were taken into consideration. In line with our hypothesis, the survival of the mice was significantly prolonged in all of the treated groups, moreover, 80% and 100% of the mice in 300 and 400 mg/kg groups survived. These mice were monitored for 90 days without showing any signs of parasitemia in the peripheral circulation; they were therefore considered cured. The body weight of the surviving mice in these two groups was within the normal range. Their blood parameters also fluctuated within the normal ranges. No trypanosome-related symptoms were observed in these groups until the mice were

76 sacrificed; the blood parameters also remained within the normal ranges, which might indicate
77 that azithromycin ameliorated anemia, by minimizing the number of parasites, then eventually
78 clearing the parasites from the peripheral circulation of the surviving mice. Thus, the
79 hypothesis that AZM is trypanostatic and that the trypanocidal effects are concentration- and
80 duration-dependent, was confirmed.

81 In conclusion, AZM possessed *in vitro* trypanocidal effects against *T. congolense, T. b*
82 *brucei* and *T evansi* parasites *in vitro* without any cytotoxic effects in MDBK and NIH 3T3
83 cells. Oral treatment with AZM showed a marked trypanocidal effect against *T. congolense*
84 savannah isolate. The *T. congolense* infection not only affects livestock, it is also a life-
85 threatening infection in indigenous African dogs. Thus, oral AZM treatment might be a better
86 solution for the treatment of dogs. We plan to investigate the efficacy of oral AZM treatment
87 against *T. b. brucei,* which may provide a rough overview of its application against a human
88 pathogen. This is the first study to demonstrate the efficacy of oral AZM against *T. congolense*
89 in mice. Subsequent studies will be aimed at determining the efficacy of oral AZM against *T.*
90 *congolense* and to investigate whether it is a suitable treatment for the definitive hosts of *T.*
91 *congolense.*

92

93 **Conflict of interest**

94 The authors declared no conflict of interest.

95

96 **Ethical approval**

97 All procedures performed in studies involving animals were in accordance with the
98 ethical standards of Obihiro University of Agriculture and Veterinary Medicine, Japan animal
99 committee (Approval Nos. 28-129 and 28-169).

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175 **Figure legends**

176 **Fig. 1** The evaluation of parasitemia in mice infected with *T. congolense* and orally treated
177 with different concentrations of AZM. *$p < 0.05$ at 100 mg/kg on days 4, 5, 7 and 8 while the
178 parasitemia of all of the other groups showed significant differences in comparison to the
179 control group from days 3 to 8. The data are expressed as the mean ± S.D

180

181 **Fig. 2** Survival curves of mice infected with *T. congolense* and orally treated with different
182 concentrations of AZM. The survival rate was significantly different from that of the control
183 group (n = 10), ** $p < 0.001$ (Log-rank test)

184

185 **Fig. 3** The effect of 28 days of oral treatment with AZM on the body weight of *T. congolense*-
186 infected mice (n = 10). The data are presented as the mean ± S.D

187

188 **Fig. 4** The evaluation of parasitemia in mice infected with *T. congolense* and orally treated
189 with different concentrations of AZM. *$p < 0.05$ at 100 mg/kg on days 5, 7 and 9 while the
190 parasitemia of all of the other groups showed significant differences in comparison to the
191 control group from days 3 to 9. The data expressed as the mean ± S.D

192

193 **Fig. 5** The survival curves of mice infected with *T. congolense* and orally treated with different

194 concentrations of AZM for 28 days. The survival rate was significantly different from the

195 control group (n = 10), ** $p < 0.001$ (Log-rank test)

196

197 **Table notes**

198 **Table 1** The effects of 28 consecutive days of oral treatment with AZM on the hematological

199 parameters of *T. congolense*-infected mice

200

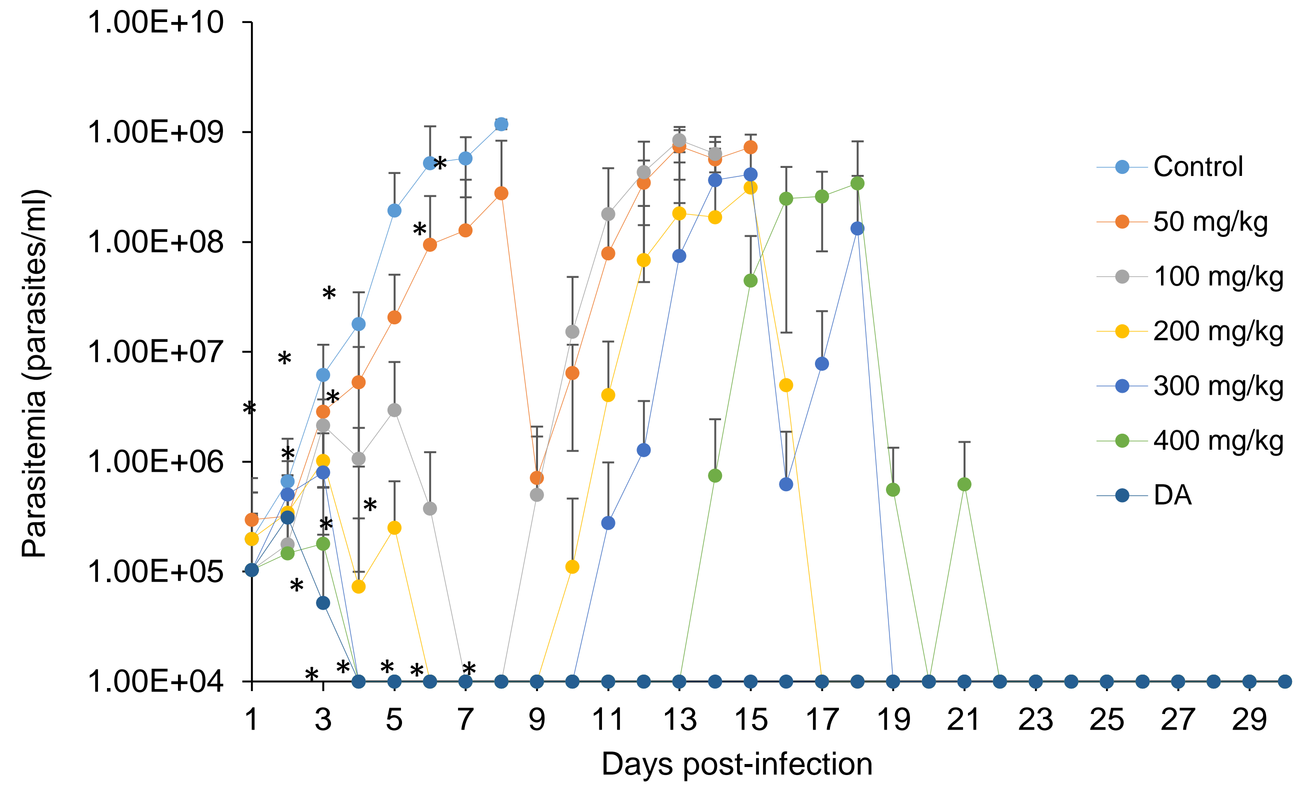

Fig. 1

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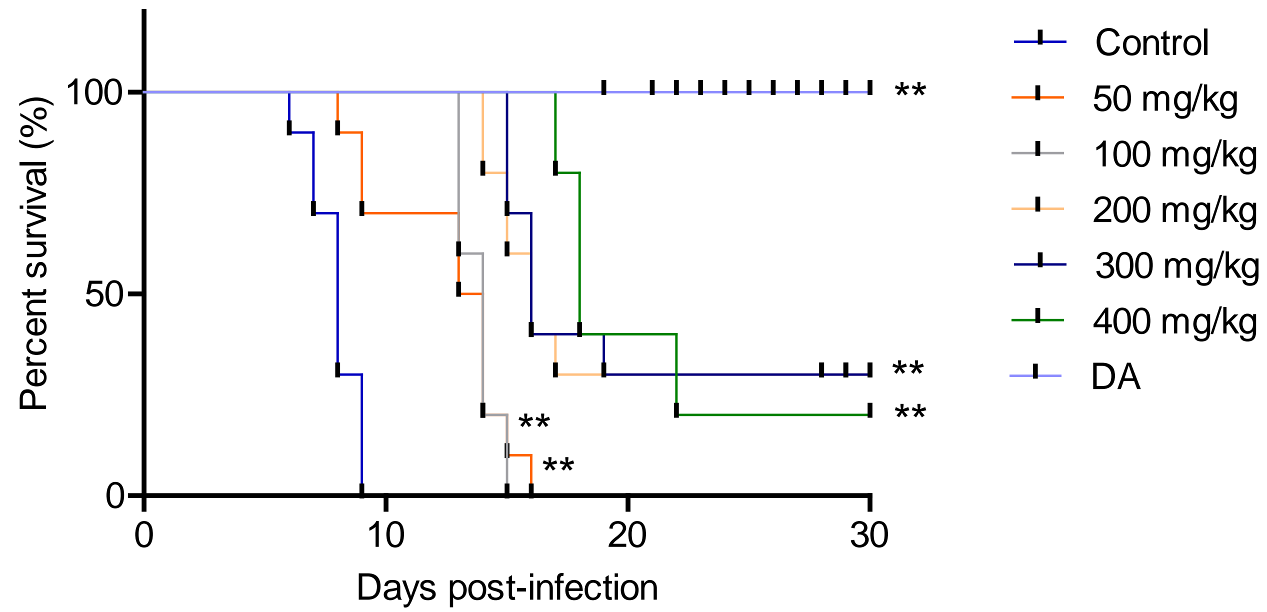

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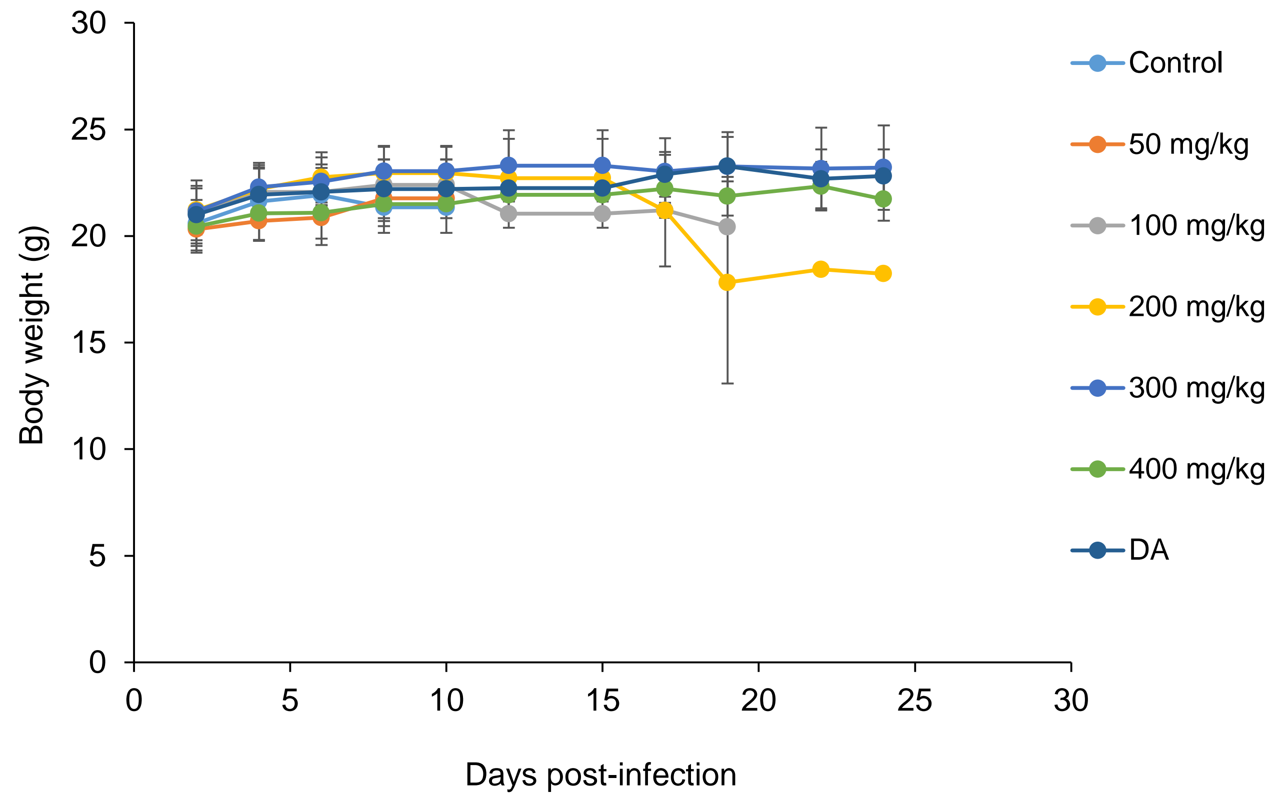

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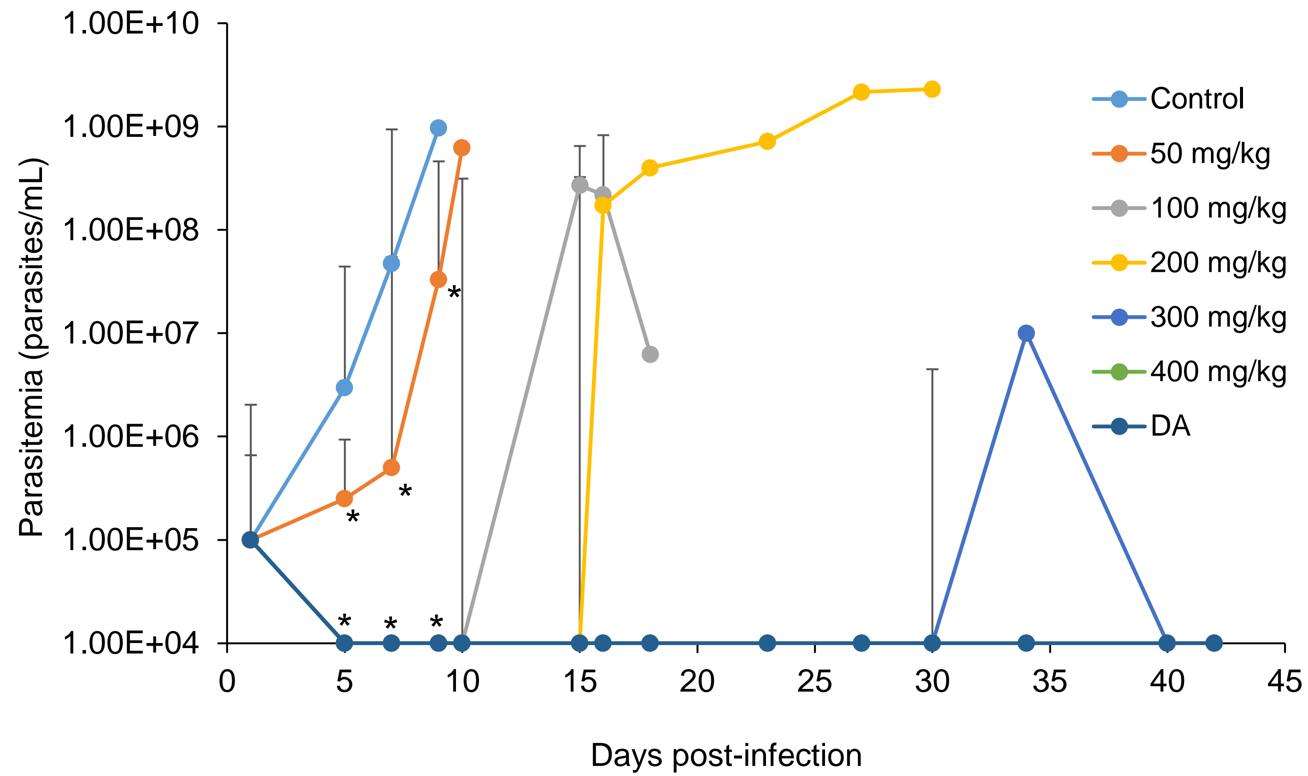

Fig. 4

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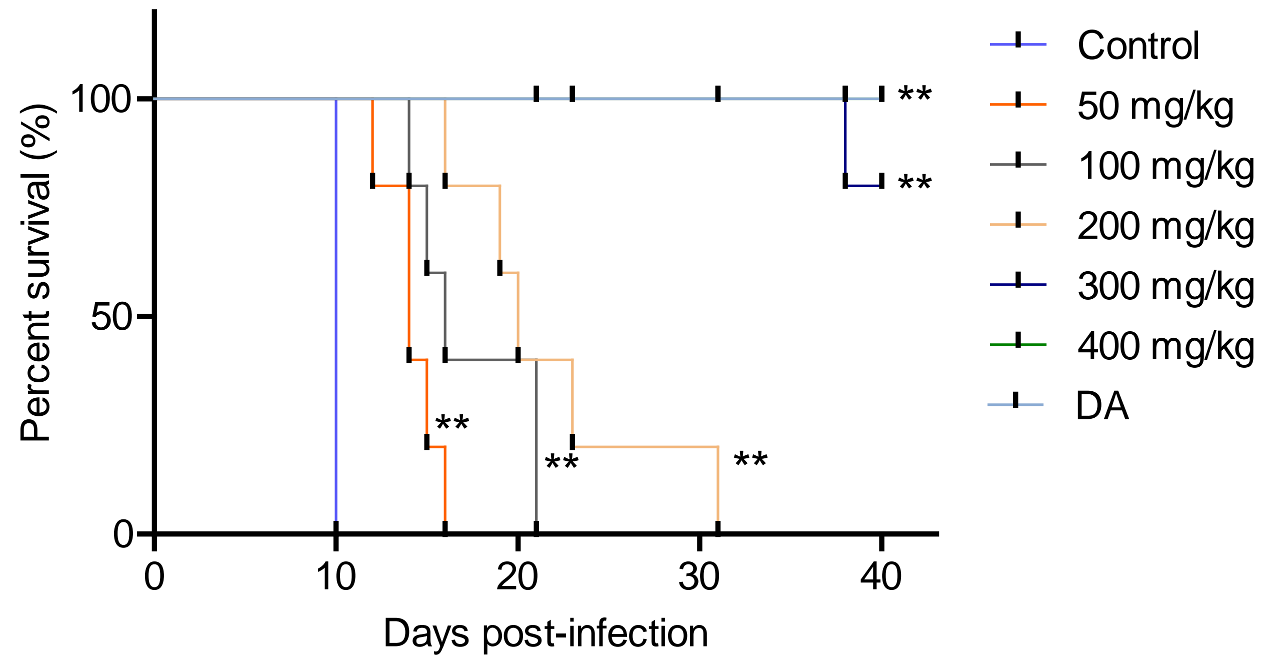

Fig. 5

Color

Table 1. The effects of 28 consecutive days of oral treatment with AZM on the hematological parameters of *T. congolense*-infected mice.

RBC (10^4/µl)								
Groups	**Healthy**	**Control**	**50 mg/kg**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**400 mg/kg**	**DA**
Week 1	878.60 ± 52.27	931.80 ± 44.57	992.40 ± 37.19*	934.80 ± 22.65	878.40 ± 57.82	956.80 ± 46.92	901.6 ± 115.89	915.80 ± 85.32
Week 2	983.00 ± 202.32	-	764.50 ± 16.82*	918.20 ± 120.43	862.60 ± 67.77	941.80 ± 22.25	844.80 ± 82.43	872.60 ± 29.18
Week 3	936.00 ± 33.64	-	-	-	-	846.80 ± 54.32	831.6 ± 68.41	865.20 ± 865.2
Week 4	888.80 ± 108.91	-	-	-	-	934.60 ± 86.81	832,6 ± 84,76	831.00 ± 68.57
Week 5	922.80 ± 43.15	-	-	-	-	911.75 ± 63.85	850.4 ± 94.07	877.00 ± 29.97
HGB (g/dl)								
Groups	**Healthy**	**Control**	**50 mg/kg**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**400 mg/kg**	**DA**
Week 1	12.86 ± 0.75	14.70 ± 0.54*	15.62 ± 0.50***	14.56 ± 0.40*	13.78 ± 0.89	14.80 ± 0.73**	14.12 ± 1.60	14.00 ± 1.13
Week 2	13.16 ± 1.57	-	12.18 ± 2.36	14.40 ± 1.88	13.64 ± 1.24	14.82 ± 0.39	13.48 ± 1.26	13.52 ± 0.53
Week 3	13.68 ± 0.41	-	-	-	-	13.14 ± 0.81	12.94 ± 0.96	13.32 ± 13.32
Week 4	13.44 ± 1.51	-	-	-	-	14.54 ± 1.31	12.70 ± 1,24	12.70 ± 0.89
Week 5	13.98 ± 0.72	-	-	-	-	13.70 ± 13.70	12.76 ± 1.36	13.28 ± 0.40
HCT (%)								
Groups	**Healthy**	**Control**	**50 mg/kg**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**400 mg/kg**	**DA**
Week 1	42.08 ± 2.67	43.14 ± 1.95	45.78 ± 1.86	43.44 ± 0.70	40.60 ± 3.00	43.90 ± 2.46	41.40 ± 4.83	42.30 ± 3.89
Week 2	42.72 ± 4.15	-	35.63 ± 7.21	42.66 ± 5.21	39.62 ± 3.16	43.54 ± 0.47	39.10 ± 3.56	40.46 ± 1.53
Week 3	44.74 ± 2.12	-	-	-	-	39.02 ± 2.35*	38.28 ± 2.98**	40.06 ± 40.06
Week 4	42.22 ± 4.96	-	-	-	-	43,20 ± 3,53	37.98 ± 3.71	38.18 ± 2.84
Week 5	43.34 ± 2.36	-	-	-	-	42.15 ± 42.15	39.04 ± 4.06	40.24 ± 1.34

The values are presented as the mean ± S.D. Asterisks indicate a statistically significant difference between the control and the AZM-treated group *$p < 0.05$; **$p < 0,001$; *** $p < 0.0001$. – Dead group