Evaluation of the Inhibitory Effects of Coumermycin A1 on the Growth of Theileria and Babesia Parasites in vitro and in vivo

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ABSTRACT

Coumermycin A1, a coumarin antibiotic, has anticancer, antibacterial, antiviral, and antimalarial activities. We aimed to evaluate the anti-theilerial and anti-babesial activity of coumermycin A1 in mice in vivo. Coumermycin A1 efficacy was determined by the transcription of DNA gyrase, a type II DNA topoisomerase using reverse transcriptase-polymerase chain reaction (RT-PCR) transcription. Coumermycin A1 significantly inhibited the development of preliminary parasitemia (1%). Theileria equi and the Babesia species B. bigemina, B. bovis, and B. caballi were observed with IC50 values of 80, 70, 57, and 65 nM, respectively. Their development was remarkably inhibited at observed concentrations of 10, 25, 50, and 100µM for the studied organisms T. equi, and the Babesia species B. caballi, B. bovis, and B. bigemina, respectively. In the subsequent viability test, parasite re-growth was suppressed at 100µM for B. bigemina and B. bovis and at 50 µM for B. caballi and T. equi. Coumermycin A1 Treatment of B. bovis cultures with Coumermycin A1 completely suppressed the transcription of the DNA gyrase subunits B and A genes. In BALB/c mice, the development of Babesia microti was inhibited by 70.73% using 5 mg/kg of Coumermycin A1.

INTRODUCTION

Theileria and Babesia are the most common tick-borne blood parasites in animals, which provoke substantial economic losses to animal farms worldwide. Babesia parasites stimulate clinical syndromes like hemolytic anemia, fever, hemoglobinuria, jaundice, and edema. Babesia species, which are primarily common in tropical and sub-tropical regions, cause severe economic damage in the livestock industries in these areas (Jabbar et al., 2000; Ica et al., 2007; AbouLaila et al., 2020). Protoplasmosis in equines, caused by tick-borne protozoa T. equi and B. caballi, is blamed for huge economic losses in the equine industry (Balkaya et al., 2010; AbouLaila et al., 2020). Babesia microti infects humans and rodents in several areas, like USA, and Pakistan (Akram et al., 2019). Many anti-babesial drugs have been used for several years without success due to their toxic effects (Vial and Gorenflo, 2006). Therefore, novel anti-babesiosis medications with high parasite specificity and minimal host toxicity are urgently needed. Coumarins have 3-amino-4-hydroxy coumarin and a replaced deoxysugar molecule called noviose, which is required for their biological activities (Li et al., 2002). Coumermycin A1 comprises two of the coumarin-noviose nuclei that are connected via a 3-methyl-2, 4-dicarboxyl pyrole connector. It is a potent inhibitor of subunit B of the DNA gyrase enzyme in Plasmodium species (Divo et al., 1988; Khor et al., 2005). Moreover, it is an effective C-terminal inhibitor of 90 kDa heat shock proteins (Hsp90) in cancer cells and exhibits antiviral activity (Burlison and Blagg 2006; Vozzolo et al. 2010; Kusuma et al. 2011). Coumermycin A1 has anticancer (Topcu, 2001), antibacterial (Nichterlein and Hof, 1991), antiviral
MATERIALS AND METHODS

Chemical reagents: Coumermycin A1 (from Sigma-Aldrich USA) and a stock solution of 100mM DMSO (dimethyl sulfoxide) were stored at -30°C before their use. Diminazene aceturate, an anti-infective medication [(Ganaseg) (Ciba-Geigy Japan Ltd., Tokyo, Japan)], was dissolved within double-distilled water (DDW) as a positive control to obtain a 10mM operating stock solution and keep it at -30°C.

Babesia microti and mice: B. microti (Munich strain) was maintained in the laboratory through serial passage in BALB/c mice (AbouLaila et al., 2010). The BALB/c mice (thirty-two-month-old females) were obtained from the CLEA Japan Laboratory supply Company (Tokyo, Japan) to perform in vivo experiments.

In vitro Babesia cultivation: The chemotherapeutic effect of Coumermycin A1 against Babesia strains was assessed, including Texas strain B. bovis, Argentina strain B. bigemina and B. caballi. The species T. equi (Agriculture Department of the United States) was also examined in the present study (Bork et al., 2004). The parasites were cultured using either equine or bovine RBCs in a continuous microaerophilic fixed phase culture process. We used 60 U/ml of penicillin G, 60 µg/ml of streptomycin antibiotics, and 0.15 µg/ml of amphotericin B, and growth media (all from Sigma-Aldrich, Tokyo, Japan), which were complemented with equine or bovine serum.

For the T. equi culture, 13.6 mg/ml of hypoxanthine (ICN Biomedicals, Inc., Aurora, OH) was used as a supplement. For B, RPMI 1640 culture media was used for B. caballi, 40% horse serum, amphotericin B, and antibiotics were added as supplements (AbouLaila et al., 2010).

Assay on in vitro inhibition of growth: From cultures with 5% parasitemia, the Babesia species (B. bigemina, B. bovis, B. caballi) and T. equi were derived. They were then diluted to a starting parasitemia of 1% by using new red blood cells for the assays, the assay was done as mentioned in previous reports (Bork et al., 2004; AbouLaila et al., 2020). Using 96-well culture plates containing a 20 µl inoculum of pressed RBCs and 200µl of specific culture medium (with 0.1, 1, 5, 10, 25, 50, and 100 µM of coumermycin A1). Diminazene aceturate was used at 5, 10, 50, 100, 1000, or 2000µM (AbouLaila et al., 2010). For the negative control, cultures containing only solvents were developed (DMSO 0.01% for coumermycin A1 and double-distilled water (0.02%) for Diminazene aceturate). This procedure was performed three times in three triplicate wells. The controls were maintained at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen gas (N2). Daily supplementation of the culture medium was provided for four days by 200µl of the new medium, which contained the appropriate concentration of medication. Levels of parasitemia were determined using 1,000 Giemsa-stained smears of RBCs. On day 3 of in vitro culture, the 50% inhibitory concentration was determined using a curve-fitting technique called interpolation.

Testing viability: After day 4 of therapy, 6µL of uninfected equine or bovine RBCs were added to 14µL of previously treated RBC culture in 200 µl of a new growth medium. This medium was replaced daily for 10 days to determine the parasite's revival through microscopic evaluation (AbouLaila et al., 2010).

Effects of Coumermycin A1 on Host Erythrocytes: Toxic effects of coumermycin A1 on host RBCs have been previously assessed (AbouLaila et al., 2020). 100 µM coumermycin A1 was incubated with equine and bovine RBCs for three hours at 37°C. Then, RBCs were washed multiple times with drug-free media for 72 hours for the Babesia parasite cultivation process. Non-treated control cells were performed in the same way as the pre-treated group. The growth of Babesia and Theileria parasites in the pre-treated RBCs was noticed and then compared to the non-treated control cells.

Reverse Transcription Polymerase Chain reaction and Nucleic Acids Extraction: RT-PCR was determined the coumermycin A1 effect on the transcription of DNA gyrase subunits A and B genes (Aboulaila et al., 2012). Cultivation (24 well culture plates) of B. bovis was performed using bovine erythrocytes. Cultures were treated with coumermycin A1 (IC50) for eight hours (Aboulaila et al., 2012). The control group (negative) cultures contained only dimethyl sulfoxide (DMSO) 0.001% and were devoid of medication. After collecting erythrocytes from three wells, they were cleansed with phosphate-buffered saline (PBS) and centrifuged at 3000 rpm for five minutes. RNA was recovered entirely using the TR® chemical reagent (Sigma-Aldrich, USA), and its concentrations were determined spectrophotometrically (Thermo Fisher Scientific, Inc., USA) and stored at -80°C in a freezer.

RT-PCR was done by PrimeScript™ One-Step RT-PCR Kit Version 2 (Takara, Japan). Exactly 150 ng total RNA was obtained from both treated and control cultures and used to amplify (1) the A and B subunits of DNA gyrase of B. bovis and (2) the tubulin beta chain gene of B. bovis (AbouLaila et al., 2012) to control the targeted genes transcription in both cultures. The reverse-transcription reaction was conducted in a reaction volume of 50 µL at 50°C for 30 minutes. The procedure included the following steps: (1) denaturation for 2 minutes at 94°C, followed by 30 denaturation cycles at 94°C for 30 seconds; (2) primer annealing for 30 seconds at 50°C, 60°C and 54°C for gyrase A/B and tubulin beta genes; (3) primer annealing for either 3 minutes for the elongation of gyrase A/B or 2 minutes for the elongation of the tubulin beta chain genes at 72°C and (4) after staining with ethidium bromide, all PCR products were electrophoresed on a 2% agarose gel and observed on a UV transilluminator using a 1000 base pair DNA ladder marker.
**Assay of in vivo growth inhibition:** The in vivo test of coumermycin A1 inhibition of *B. microti* was assessed twice in BALB/c mice, as previously described with some slight variation (AbouLaila et al., 2010). Briefly, fifteen BALB/c female mice, eight-week-old, were divided into three groups of five and received intraperitoneally $1 \times 10^7$ *B. microti* infected RBCs. The subjects were observed, and once parasitemia reached 1%, they received treatment daily until the fifth day.

Dimethyl sulfoxide (DMSO) was used to dissolve the drugs (3% for coumermycin A1) and double-distilled water (DDW) (Diminazene aceturate 12.5%). Before injection, the phosphate buffer solution was diluted. For the negative control group, DMSO in phosphate buffer solution was administered (0.02%). In the first category, five mg/kg of coumermycin A1 was infused intraperitoneally in a 0.3 ml buffer solution (phosphate), whereas the second group received an intraperitoneal injection containing 0.3 ml of PBS involving 0.013% DDW. The third group received subcutaneous administration of Diminazene aceturate 25 mg/kg (Ganaseg, Japan Ciba-Geigy, Ltd.) in 0.1 ml DDW (AbouLaila et al., 2010).

Parasitemia was monitored daily for up to 20 days following infection using 1,000 RBCs in Giemsa-stained smears. All animal studies have been performed in compliance with the National Research Center for Protozoan Diseases’ Experimental Animal Care and Management Standard.

**Statistical analysis:** The student’s t-test determined significance in the statistical analysis using the JMP software program (SAS Institute, Inc., USA). This program was used for the statistical analysis of all data in this study. The threshold for statistical significance was set to $P<0.005$.

**RESULTS**

**In vitro growth inhibition:** Coumermycin A1 significantly inhibited parasitemia for all the studied strains at a concentration of 0.1µM (Fig. 1). Growth was suppressed on day 3, at concentrations of 100µM (*B. bovis, B. bigemina*), 25µM (*B. caballi*), and 10µM (*T. equi*). Coumermycin A1 eliminated all Babesia species on day 3 and *T. equi* on day 1 of drug exposure at 100µM. In vitro parasitic growth was found to be significantly suppressed by 5nM of Diminazene aceturate treatment ($P<0.05$). Diminazene aceturate suppressed parasites at a concentration of 2000nM, whereas 50nM was needed to inhibit *B. caballi* expansion. For ten days following medication withdrawal, parasites did not appear at 50 (B. bovis), 25 (B. caballi), 100 (B. bigemina), and 10µM (T. equi). Babesia species exposed to lower medication concentrations resumed growth once the medication was withdrawn; this was determined by enhanced parasitemia observed under a microscope. Parasites exposed to diminazene aceturate showed no regeneration at 25µM (B. caballi) or 1000µM concentrations (B. bovis, T. equi).

![Fig. 1: Graphs depicting the in vitro coumermycin A1 inhibitory effects on growth at different concentrations (A) B. bovis, (B) B. bigemina, (C) B. caballi and (D) T. equi. Each of the values is expressed as mean±SD. The curves demonstrate the findings of the 3 triplicate studies. An asterisk denotes any significant difference between coumermycin A1 treated and untreated cultures. Regrowth was determined as viability (+) after 10 days; death is denoted by (-).](image-url)
and *B. bigemina*. The IC\textsubscript{50} values for diminazene and Coumermycin A1 are shown in Table 1. The DMSO-treated group grew similarly to the control group within the cultures. The morphology of parasites in treated and untreated cultures was compared. Coumermycin A1 causes the parasites to swell without cytoplasm in *B. bovis* cultures (Fig. 2B), which corresponds to the DMSO group’s typical morphology (Fig. 2A). Compared to standard parasites in the DMSO-negative control cultures, parasites appeared degenerated in coumermycin A1-treated *B. bigemina* (Fig. 2C), *B. caballi* (Fig. 3B), and *T. equi* (not presented) cultures. Coumermycin A1 was found to be safe and nontoxic to host red blood cells (RBCs), even at the highest concentration (100\mu M), as the negative control group had parasitemia comparable to that of untreated erythrocytes (not presented).

**RT-PCR**: The coumermycin A1 at IC\textsubscript{50} concentration can inhibit mRNA transcripts of the DNA gyrase subunit B and subunit A genes in cultured *B. bovis*, but not in untreated parasites. The treatment possessed no consequence on the tubulin beta chain gene transcripts.

**In vivo effects of Coumermycin A1 on *B. microti***: There was a significant decrease in the parasitemia level in the treated group compared to the untreated group (P<0.05) between days 3 and 7 post-infection (Figure 4). The highest level of parasitemia (5.4\%) was observed on the fifth day following infection when treated with diminazene aceturate (25 mg/kg), and 12.75\% on the seventh day following infection when treated with 5 mg/kg coumermycin A1. Parasitemia was found to be 43.6\% in the untreated group (DMSO) on the sixth day post-infection (Fig. 4).

**DISCUSSION**

This work revealed that coumermycin A1 provoked a significant *in vitro* inhibition of the development of three *Babesia* species. Moreover, when *T. equi* parasites were exposed to higher drug concentrations, they were completely suppressed. *T. equi* was found to be more sensitive to coumermycin A1 compared to the three *Babesia* species. In this study, the coumermycin A1 showed lower IC\textsubscript{50} values for *T. equi* and *Babesia* species than those of Diminazene aceturate, except for *B. caballi*. Coumermycin A1 had an IC\textsubscript{50} value of 28 \mu M for *Plasmodium falciparum* (Divo et al., 1988), which was higher than the IC\textsubscript{50} values for *T. equi* and *Babesia* species.

**Table 1**: The 50\% inhibitory concentration values of coumermycin A1 and diminazene aceturate for the parasites *T. equi* and the *Babesia* species *B. bosii*, *B. bigemina*, and *B. caballi*.

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<thead>
<tr>
<th></th>
<th>Coumermycin A1</th>
<th>Diminazene</th>
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<tr>
<td><em>B. bosii</em></td>
<td>720±1</td>
<td>300±30</td>
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<tr>
<td><em>B. bigemina</em></td>
<td>80±2</td>
<td>190±20</td>
</tr>
<tr>
<td><em>B. caballi</em></td>
<td>65±8</td>
<td>19±2</td>
</tr>
<tr>
<td><em>T. equi</em></td>
<td>57±2</td>
<td>710±15</td>
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\textsuperscript{a} The 50\% inhibitory concentration values are represented as drug concentrations in the nanomolar growth medium. It was calculated using a curve fitting technique on the 4\textsuperscript{th} day of the *in vitro* culture. IC\textsubscript{50} values are reported as mean and SD for the 3 different experiments. \textsuperscript{b} Divo et al. 1988; ND not determined.

**Fig. 2**: These are light microscopic slides depicting coumermycin A1 (10 \mu M) treated cultures of; (A) *B. bosii* (control) and (B) treated, and (C) *B. bigemina* control while (D) represents the treated group. Treated cultures demonstrated an increased number than control cultures of swollen and degenerated parasites shown by arrows. Micrographs were drawn on day three of the procedure: bars scale, 10\mu M.

**Fig. 3**: Light microscopic images of *in vitro* *B. caballi* cultures treated with 10\mu M coumermycin A1. Image (A) represents the control group, and (B) is the coumermycin A1-treated cultures. Cultures treated with the medication a higher number of swollen and degenerated parasites shown by arrows when compared to the control (negative) group. Micrographs on the third day of therapy were taken. Scale bars, 10\mu M.

**Fig. 4**: This graph depicts the inhibitory effects of intraperitoneal infusion of diminazene aceturate 25 mg/kg and 5 mg/kg s.c coumermycin A1 in five mice that were part of the experimental community on the *in vivo* growth of *Babesia microti*. Values are represented as mean and standard deviation. An asterisks represents substantial differences (Student t-test; \* P<0.01). Post-inoculation between control groups treated with coumermycin A1 and dimethyl sulfoxide from days 3 to 7. The double-headed arrow indicates rehabilitation days.

The 50\% inhibitory concentration values of coumermycin A1 for *T. equi* and the *Babesia* species were found to be lower than previously tested antibabesial therapy (Bork et
Conclusions: In conclusion, coumermycin A1 inhibited growth effectively in *in-vitro* cultures of three *Babesia* species and *T. equi* and in *in vivo* growth of *B. microti* in mice. Coumermycin A1 might be considered as a safe and effective anti-piroplasm agent for theileriosis and babesiosis.

Authors contribution: MA, AA, MAO and SM conceived and planned the experiments. MA, II, NY and MAO conducted the experiments. II, NY and MAAI provided reagents/materials/analysis tools. MA and MAO wrote the manuscript. All authors revised and accepted current manuscript version.

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