RESEARCH ARTICLE

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

Mahmoud AboulLaila1,2, Amer Ragheb AbdEl-Aziz3, Soad Menshawy3, Naoaki Yokoyama1, Ikuo Igarashi1, Mohammad Al-Wabel4 and Mosaab Omar4,5

1National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-Cho, Obihiro, Hokkaido 080-8555, Japan; 2Department of Parasitology, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, Elbehera, Egypt; 3Department of Parasitology, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt; 4Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah, 51452 Qassim, Saudi Arabia; 5Department of Parasitology, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Qena, Egypt

*Corresponding author: mosaab.omr@vet.svu.edu.eg

ARTICLE HISTORY
Received: February 04, 2021
Revised: August 05, 2021
Accepted: August 29, 2021
Published online: August 31, 2021

Key words:
B. bovis
Coumermycin A1
in vitro
in vivo
RT-PCR
T. equi

ABSTRACT
Coumermycin A1, a coumarin antibiotic, has anticancer, antibacterial, antiviral, and antimalarial activities. We aimed to evaluate the anti-thielerial 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 100 µM 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 100 µM 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; AboulLaila et al., 2020). Piroplasmosis 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; AboulLaila 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 deoxyxugar 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
supplementation of the culture medium was provided for four days by 200 μl 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, 6 μL of uninfected equine or bovine RBCs were added to 14 μL 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 pretreated 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 (IC90) 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 TRI® 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) 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 storing 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 1×10⁷ *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 acetate12.5%). 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 acetate 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 0.1 µM (Fig. 1). Growth was suppressed on day 3, at concentrations of 100 µM (*B. bovis, B. bigemina*), 25 µM (*B. caballi*), and 10 µM (*T. equi*). Coumermycin A1 eliminated all *Babesia* species on day 3 and *T. equi* on day 1 of drug exposure at 100 µM. In vitro parasitic growth was found to be significantly suppressed by 5 nM of Diminazene acetate treatment (P<0.05). Diminazene acetate suppressed parasites at a concentration of 2000 nM, whereas 50 nM 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 10 µM (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 acetate showed no regeneration at 25 µM (B. *caballi*) or 1000 µM concentrations (B. *bovis, T. equi*).

![Fig. 1](https://example.com/fig1.png)

**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 (-).
and B. bigemina). The IC₅₀ 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 μM), as the negative control group had parasitemia comparable to that of untreated erythrocytes (not presented).

RT-PCR: The coumermycin A₁ at IC₅₀ 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 IC₅₀ values for T. equi and Babesia species than those of Diminazene aceturate, except for B. caballi. Coumermycin A₁ had an IC₅₀ value of 28 μM for *Plasmodium falciparum* (Divo et al., 1988), which was higher than the IC₅₀ 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. bovis, B. bigemina, and B. caballi. |
|---------------------------------|-----------------|-----------------|
| **IC₅₀ (nM)**                  | Coumermycin A₁  | Diminazene      |
| B. bovis                       | 70±1            | 300±30          |
| B. bigemina                    | 80±2            | 190±20          |
| B. caballi                     | 65±8            | 10±2            |
| T. equi                        | 57±3            | 710±15          |

* The 50% inhibitory concentration values are reported as drug concentrations in the nanomolar growth medium. It was calculated using a curve fitting technique on the 4th day of the in vitro culture. IC₅₀ values are presented as mean and SD for the 3 different experiments. * Divo et al. 1988; ND not determined

![Image](image-url)
Conclusions: In conclusion, coumermycin A1 inhibited growth effectively in *in-vitro* cultures of three Babesia species and *T. equi* and *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: Conceived and planned the experiments: MA; AA; MAO; SM conducted the experiments: MA; II; NY; MAO and Provided reagents/materials/analysis tools II, NY, MAAL, Wrote the manuscript: MA, MAO. All authors revised and accepted current manuscript version.

Funding: This study was sponsored by the Japan Society for the Promotion of Science (JSPS), as well as Ministry of Education, Culture, Science, and Technology, of Japan.

REFERENCES

Aboulaila M, El-Sayed SA, Omar MA, et al., 2020. Myrrh oil in vitro inhibitory growth on bovine and equine piroplasm parasites and Babesia microti of mice. Pathogens 9:173.

Aboulaila M, Abd El-Aziz A, Yokoyama N, et al., 2019. Luteolin: target validation in Babesia bovis by reverse transcription-polymerase chain reaction and in vivo inhibition of Babesia microti. J Clin Res Med Rep 1:103.

Aboulaila M, AbdEl-Aziz A, Mershawy S, et al., 2019b. Evaluation of the *in vitro* and *in vivo* inhibitory effects of enrofloxacin on the growth of Babesia Species and Theileria equi. J Drug Res Drug Abuse 1:3-6.

Aboulaila M, Abdelaziz A, Rizk M, et al., 2019c. Evaluation of the *in vitro* and *in vivo* inhibitory effects of quercetin on the growth of Babesia and Theileria parasites. Damanhour J Vet Sci 2:23-7.

Aboulaila M, Munkhjargal T, Sivakumar T, et al., 2012. Apiplastin-targeting antibacterials inhibit the growth of Babesia parasites. Antimicrob Agents Chemother 56:3196-206.

Aboulaila M, Nakamura K, Govind Y, et al., 2010. Evaluation of the *in vitro* growth-inhibitory effect of epofoxacin on Babesia parasites. Vet Parasitol 167:19-27.

Akram IN, Parveen T, Abrar A, et al., 2019. Molecular detection of Babesia microti in dogs and cat blood samples collected from Punjab (Pakistan). Trop Biomed 36:304-9.

Bazik SE, Beshishty AM, Akazi L, et al., 2020. Gas chromatography-mass spectrometry analysis, phytochemical screening and antiprotozoal effects of the methanolic Viola tricolor and acetic anhydride extracts. BMC Complement Med Ther 20:87.

Balkaya I, Utuk AE and Paskin FC, 2010. Prevalence of Theileria equi and Babesia caballi in Donkeys from Eastern Turkey in Winter Season, Pak Vet J, 30:245-6.

Bonilla TD, 2008. Cellular functions of DNA gyrase and conditional gene regulation in the malaria parasite Plasmodium falciparum. University of Florida.

Bork S, Okamura M, Boonchit S, et al., 2004. Identification of Babesia bovis L-lactate dehydrogenase as a potential chemotherapeutic target against bovine babesiosis. Mol Biochem Parasitol 136:165-72.

Brockelman C and Tan-ariya P, 1991. Development of an *in vitro* microtest to assess drug susceptibility of Babesia bovis and Babesia bigemina. J Parasitol 77:994-7.

Burlison JA and Blagg BS, 2006. Synthesis and evaluation of coumermycin A1 analogues that inhibit the Hsp90 protein folding machinery. Org Lett 8:4855-8.

Divo AA, Sartorelli AC, Patton CL, et al., 1988. Activity of fluoroquinolone antibiotics against *Plasmodium falciparum in vitro*. Antimicrob Agents Chemother 32:1182-6.

Hof H, 1986. The effect of coumermycin on experimental listeriosis. J Infect 13:17-23.

Ica A, VATANSEVER Z, YILDIRM A, et al., 2007: Investigation of ovine blood protozoa by reverse line blotting in the Kayseri region. Parasitology 49:90.

Jabbar A, Abbas T, Sandhu Z, et al., 2015. Tick-borne diseases of bovines in Pakistan: major scope for future research and improved control. Parasit Vectors 8:283.
Khor V, Yowell C, Dame JB, et al., 2005. Expression and characterization of the ATP-binding domain of a malarial Plasmodium vivax gene homologous to the B-subunit of the bacterial topoisomerase DNA gyrase. Mol Biochem Parasitol 140:107-17.

Kusuma BR, Peterson LB, Zhao H, et al., 2011. Targeting the heat shock protein 90 dimer with dimeric inhibitors. J Med Chem 54:6234-53.

Li SM, Westrich L, Schmidt J, et al., 2002. Methyltransferase genes in Streptomyces rishiriensis: new coumermycin derivatives from gene inactivation experiments. Microbiol 148:3317-26.

Matsuu A, Yamasaki M, Xuan X, et al., 2008. In vitro evaluation of the growth inhibitory activities of 15 drugs against Babesia gibsoni (Aomori strain). Vet Parasitol 157:1-8.

Munkhjargal T, AbouLaila M, Terkawi MA, et al., 2012. Inhibitory effects of pepstatin A and mefloquine on the growth of Babesia parasites. Am J Trop Med Hyg 87:68-688.

Nichterlein T and Hof H, 1991. Effect of various antibiotics on Listeria monocytogenes multiplying in L 292 cells. Infection 19 Suppl 4:S234-8.

Omar MA, Salama A, Elsify A, et al., 2016. Evaluation of in vitro inhibitory effect of enoxacin on Babesia and Theileria parasites. Exp Parasitol 161:62-7.

Rizk MA, El-Sayed SAE, AbouLaila M, et al., 2017. Evaluation of the inhibitory effect of N-acetyl-L-cysteine on Babesia and Theileria parasites. Exp Parasitol 179:43-8.

Rodriguez RI and Trees AJ, 1996. In vitro responsiveness of Babesia bovis to imidocarb dipropionate and the selection of a drug-adapted line. Vet Parasitol 62:35-41.

Salama AA, AbouLaila M, Terkawi MA, et al., 2014. Inhibitory effect of allicin on the growth of Babesia and Theileria equi parasites. Parasitol Res 113:275-83.

Tawfik AF, 1991. Effects of vancomycin, teicoplanin, daptomycin and coumermycin on normal immune capabilities. J Chemother 3:226-31.

Topcu Z, 2001. DNA topoisomerases as targets for anticancer drugs. J Clin Pharm Therapeut 26:405-16.

Vozzolo L, Loh B, Gane PJ, et al., 2010. Gyrase B inhibitor impairs HIV-1 replication by targeting Hsp90 and the capsid protein. J Biol Chem 285:39314-28.

Zhao HF, Boyd J, Jolicoeur N, et al., 2003. A coumermycin/novobiocin-regulated gene expression system. Hum Gene Ther 14:1619-29.