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The effects of nitidine chloride and camptothecin on the growth of *Babesia* and *Theileria* parasites

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ABSTRACT

The treatment of bovine and equine piroplasmosis is limited to diminazene aceturate (DA) and imidocarb dipropionate. To address this challenge, we need to explore novel drug compounds and targets. Topoisomerases are potential drug targets because they play a vital role in solving topological errors of DNA strands during replication. This study documented the effectiveness of topoisomerase inhibitors, nitidine chloride (NC) and camptothecin (Cpt), on the growth of *Babesia* and *Theileria* parasites. The half maximal inhibitory concentrations (IC_{50}s) against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 1.01 ± 0.2, 5.34 ± 1.0, 0.11 ± 0.03, and 2.05 ± 0.4 μM for NC and 11.67 ± 1.6, 4.00 ± 1.0, 2.07 ± 0.6, and 0.33 ± 0.02 μM for Cpt, respectively. The viability experiment revealed that 4, 10, and 4 μM treatments of NC or 48, 8, and 8 μM treatments of Cpt were sufficient to stop the *in vitro* regrowth of *B. bovis*, *B. bigemina*, and *B. caballi*, respectively. However, *T. equi* regrew in all of the concentrations used. Moreover, increasing the concentration of NC and Cpt to 16 μM and 1.2 μM (8 × IC_{50}) did not eliminate *T. equi*. The micrographs of *B. bigemina* and *B. caballi* taken at 24 h and 72 h showed deformed merozoites and remnants of parasites within the red blood cell (RBC), respectively. The treatments of 25 mg/kg DA and 20 mg/kg NC administered intraperitoneally and 20 mg/kg NC given orally showed 93.7, 90.7, and 83.6% inhibition against *Babesia microti* (*B. microti*), respectively, compared to the untreated group on day 8. In summary, NC and Cpt were effective against *Babesia* and *Theileria* parasites *in vitro*. Moreover, 20 mg/kg NC administered intraperitoneally was as effective as 25 mg/kg DA against *B. microti* in mice and showed no toxic symptoms in mice. The results indicate that NC may, after further evaluations, prove to be an alternative drug against bovine and equine piroplasmoses.

1. Introduction

Species of the genus *Babesia* and *Theileria* cause bovine and equine piroplasmosis, which are associated with economic losses to the cattle and equine industries worldwide (Bock et al., 2004; Wise et al., 2014). The available drug options are limited to diminazene aceturate (Berenil®, Hoechst Ltd, Frankfurt, Germany) and imidocarb dipropionate (Imizol®, Schering-Plough, New Jersey, USA). Furthermore, diminazene aceturate (DA) has been withdrawn from the European market due to strong toxicity, and circumstantial reports have documented emerging resistance to the current remedies (Mosqueda et al., 2012). Therefore, the research to discover new drugs and drug targets is

Abbreviations: CI, Combination index value; Cpt, Camptothecin; DA, Diminazene aceturate; DDW, Double distilled water; iRBC, Infected red blood cells; NC, Nitidine chloride; NC-IP, Nitidine chloride intraperitoneally administered; NC-Oral, Nitidine chloride orally administered; NC-DA, Nitidine chloride-diminazene aceturate combination; Cpt-DA, Camptothecin-diminazene aceturate combination

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vital in addressing the current limitations by providing more drug options.

DNA topoisomerases are enzymes that play a vital role in solving topological errors ahead of the replication fork during DNA transcription (Binaschi et al., 1995). To perform this function, topoisomerases form covalent bonds with DNA strands, break the phosphate backbone to untangle or unwind the DNA strands, and then re-anneal the strands (Binaschi et al., 1995). Two types of topoisomerases are classified based on mode of action: type I topoisomerases are adenine triphosphate phosphate (ATP) independent and break only one of the double-helix strands of DNA, while type II topoisomerases are ATP dependent and break both of the helix strands of DNA (Binaschi et al., 1995; Pommier, 2006). Since topoisomerases play such a key role, they are an attractive drug target using topoisomerase inhibitors (Bouquet et al., 2012; Wang et al., 1993).

Camptothecin (Cpt) is one of the earliest topoisomerase I inhibitors discovered from *Camptotheca acuminata* during the screening of natural products for anticancer drugs in the 1960s (Pommier, 2006). Subsequent research on Cpt led to the development of two synthetic analogues, irinotecan and topotecan, that are used for cancer treatment (Mathijssen et al., 2002). Furthermore, a recent study showed that Cpt was effective against *Plasmodium falciparum* (Cortopassi et al., 2012). Nitidine chloride (NC) was discovered from *Toddalia asiatica* during the screening of natural compounds with antiplasmodial effects (Gakunju et al., 1995). Subsequent research documented several pharmacological properties. For example, NC modulates apoptosis-inducing pathways in cancer cells (Liao et al., 2013), possesses anti-inflammatory properties (Wang et al., 2012), and has anticatabolic effects in bone (Liu et al., 2016). In similarity to Cpt, NC interferes with the action of topoisomerase I enzymes (Wang et al., 1993).

In previous studies, topoisomerase inhibitors have shown effectiveness against parasites in the phylum Apicomplexa, including *Plasmodium* (Bouquet et al., 2012), *Toxoplasma* (Fichera et al., 1995), and *Cryptosporidium* (Woods et al., 1996). Since Apicomplexan parasites have commonalities in their metabolic pathways and genetic makeup (Shanmugasundram et al., 2013), we hypothesized that the efficacy shown by NC and Cpt against *P. falciparum* could be reproduced against *Babesia* and *Theileria* parasites. The present study assessed the effect of topoisomerase inhibitors NC and Cpt against the growth of *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi in vitro* and the efficacy of NC on *B. microti* in mice.

2. Materials and methods

2.1. Cultivation conditions

2.1.1. Parasites and mice

The Texas strain of *B. bovis*, the Argentine strain of *B. bigemina*, and the United States Department of Agriculture (USDA) strains of *B. caballi* and *T. equi* were used for the *in vitro* studies (Bork et al., 2004), and *B. microti* (Munich strain) was used for the *in vivo* studies (Lu et al., 2012). Female BALB/c mice (CLEA Japan Inc, Tokyo, Japan) housed under a pathogen-free environment with controlled temperature (22 °C) and humidity and a 12 h light/dark cycle were used for the cultivation of *B. microti* to perform *in vivo* studies in mice.

2.1.2. Chemicals and reagents

The NC, Cpt, and DA powders (Sigma-Aldrich, Tokyo, Japan) were prepared in dimethyl sulfoxide (DMSO) and double-distilled water (DDW), respectively, in stock solutions of 10 mM and stored at −30 °C. The 10,000 × SYBR Green 1 (SG1) nucleic acid stain (Lonza Rockland Inc, Rockland, USA) was stored at −30 °C, wrapped in aluminum foil paper for protection from direct light. A lysis buffer containing Tris (130 mM at pH 7.5), EDTA (10 mM), saponin (0.016% w/v), and Triton X − 100 (1.6% v/v) was prepared and stored at 4 °C.

2.1.3. In vitro cultivation

Purified bovine and equine red blood cells (RBCs) were used to maintain bovine parasites (*B. bovis* and *B. bigemina*) and equine parasites (*B. caballi* and *T. equi*), respectively. The cultivation was performed in a microaerophilic stationary phase culture system at 37 °C, 5% CO_2, 5% O_2, and 90% N_2 as previously described (Tuvshintulga et al., 2016). M199 (Sigma-Aldrich, Tokyo, Japan) supplemented with 40% cattle serum was used for cultivation of *B. bigemina* and *B. bovis*. M199 supplemented with 40% horse serum and 13.6 µg/ml hypoxanthine was used for the cultivation of *T. equi*. GIT (Sigma-Aldrich, Tokyo, Japan) supplemented with 40% horse serum was used to maintain the *B. caballi* culture. In all culture media, 60 µg/ml streptomycin and 0.15 µg/ml amphotericin-B were added to suppress bacterial and fungal growth, for avoiding contamination.

2.2. Growth inhibitory effects in vitro

The growth inhibitory effects of NC, Cpt, and DA against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were determined using a fluorescence assay as previously described (Guswanto et al., 2014). Briefly, the cultivation was performed in a total reaction volume of 100 µl that was loaded into a 96-well plate. The reaction mixture contained media at various concentrations plus 2.5% hematocrit for *B. bovis* and *B. bigemina* and 5% hematocrit for *B. caballi* and *T. equi,* at 1% parasitemia. The concentrations of NC, Cpt, and DA were 0.01, 0.1, 1, 10, and 100 µM. The positive control wells containing infected red blood cells (iRBCs) and the concentrations of DMSO (0.5%) as the diluent were included in the experiment. Wells with non-infected RBCs were used as a negative control. The experiment was conducted for 4 days without changing the media. On day 4, 100 µl of lysis buffer containing SG1 was directly added to each well and gently mixed by pipetting. The plate was wrapped in aluminum foil paper for protection from direct light and incubated for 6 h at room temperature. The relative fluorescence values were read at 485 and 518 nm excitation and emission wavelengths, respectively, using a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo Labsystems, Massachusetts, USA). The gain values were set to percentages after subtraction of the mean values of the negative control from those of the treated wells. The half maximal inhibitory concentration (IC_{50}) values were determined using the non-linear regression analysis (curve fit) in GraphPad Prism (GraphPad Software Inc., California, USA).

2.3. Determination of morphological changes and viability in vitro

The viability and morphological changes in drug-treated *Babesia* and *Theileria* parasites were observed using a microscopy assay as previously described (Tuvshintulga et al., 2017). With minor modifications, a 100 µl reaction volume containing 90 µl of medium at various drug concentrations and 10 µl of iRBCs normalized to 1% parasitemia were prepared and placed into a 96-well plate. The plate was incubated at 37 °C in a humidified multi-gas water-jacketed incubator. The 90 µl of medium was changed for 4 consecutive days and replaced with 90 µl of new medium containing the respective concentrations of NC and Cpt. The various concentrations of NC, Cpt, and DA used in this experiment were 0.5×, 1×, 2×, and 4× the IC_{50}. In the course of the 4 days of treatment, Giemsa-stained thin blood smears were prepared, and the parasitemia in 10,000 RBCs was monitored every 12 h. On day 5, 3 µl of RBCs from the treated and control wells was pipetted and added to 7 µl of fresh RBCs in a new 96-well plate. The cultivation was performed using drug-free medium that was replaced daily for the next 6 days. On day 10 (6 days after the last treatment), Giemsa-stained blood smears were prepared and observed under a light microscope. The viability of drug-treated parasites was recorded as positive (presence of parasites) or negative (absence of parasites). Each experiment was performed in triplicate in three separate trials. The morphological changes were observed under a light microscope, and