Veterinary Parasitology 197 (2013) 527–533

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

The *in vitro* interactions and *in vivo* efficacy of atovaquone and proguanil against *Babesia gibsoni* infection in dogs

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ARTICLE INFO

Article history:
Received 21 January 2013
Received in revised form 17 May 2013
Accepted 3 June 2013

Keywords:
Babesia gibsoni
Malarone®
Atovaquone
Proguanil

ABSTRACT

In vitro interactions between atovaquone (ATV) and proguanil (PG) against *Babesia gibsoni* and the clinical efficacy of this combination therapy using Malarone® which is the anti-malarial drug containing ATV and PG were evaluated. This combination showed synergism against uncloned wild-type and ATV-resistant *B. gibsoni in vitro* examinations using a modified fixed ratio method. Administration of Malarone® to experimentally *B. gibsoni* infected two dogs in chronic stage and three dogs in acute stage resulted in decrease in parasitemia, and clinical improvements were observed. However, all dogs showed relapse of parasitic infection with a single-nucleotide polymorphism in the cytchrome *b* gene (M121I). Some side effects were confirmed: self-limiting vomiting in two dogs and hyperphosphatasia in another dog. Mild increases in the levels of alanine aminotransferase were confirmed in two dogs. This is the first study to evaluate the interactions *in vitro* and the clinical efficacy of ATV and PG against canine *B. gibsoni* infection in dogs.

1. Introduction

Babesia gibsoni infect the red blood cells of dogs and induce severe hemolytic anemia, which is often accompanied by fever, jaundice, hemoglobinuria, and an enlarged spleen (Groves and Dennis, 1972; Farwell et al., 1982). *B. gibsoni* is not zoonotic parasite, but two genetically related Babesia-like piroplasms have been identified from human. WA1 which was identified from a patient in Washington State (Quike et al., 1993), and unnamed Babesia-like organism in northern California in USA (Persing et al., 1995) were closely related to *B. gibsoni*. The patients infected the later parasite initially showed influenza-like symptoms characteristic of early infection with *Babesia microti* (Persing et al., 1995). The canine infection of *B. gibsoni* has been reported to be endemic in Asia, Africa, Europe, North America (Kjemtrump et al., 2000), and Australia (Muhlnickel et al., 2002). Although the incidence of canine *B. gibsoni* infection has been increasing worldwide, the definitive treatment strategy for it has not been established. Various therapeutic modalities, including diminazene aceturate, antibiotics, or their combination therapies have been described (Boozer and Macintire, 2003; Wulansari et al., 2003; Suzuki et al., 2007; Lin et al., 2012).

Atovaquone (ATV) is a well-tolerated, metabolically stable, and effective agent with broad-spectrum anti-parasite activity (Baggish and Hill, 2002). ATV monotherapy was effective for acute canine *B. gibsoni* infection, but it resulted in relapse. And also it coursed the emergence of drug-resistant variants with some single-nucleotide polymorphisms (SNPs) in the cytochrome *b* gene (cyt *b*), which is presumed to be an ATV binding site (Matsuu et al., 2004, 2006). In a subsequent study, the emergence of ATV resistance was suspected to be due to the selective multiplication of *B. gibsoni* M121I variants (Iguchi et al., 2012). Combination therapy with ATV and

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http://dx.doi.org/10.1016/j.vetpar.2013.06.006

azithromycin (AZM) against canine *B. gibsoni* infection has been reported (Birkenheuer et al., 2004). However, this therapeutic modality could not completely eliminate parasites in acute *B. gibsoni* infection and an ATV-resistant parasite possessing M121I in *cyt b* (Jefferies et al., 2007; Sakuma et al., 2009). Other therapeutic modalities combined with ATV rather than AZM will be necessary to obtain a satisfactory anti-babesia effect. However, to the authors' knowledge, there are no *in vitro* studies evaluating the interaction of drugs with ATV against *B. gibsoni*.

Proguanil (PG) is a highly protein-bound molecule that has been used in combination with ATV for the treatment of *Plasmodium* spp. infections because of their synergistic interaction (Baggish and Hill, 2002; Fivelman et al., 2004). A tablet formula that combines ATV and PG, Malarone®, is available for treatment of human malaria. In our previous *in vitro* study, PG was effective against ATV-resistant *B. gibsoni* with M121I in the same way as wild-type (WT) *B. gibsoni* (Iguchi et al., 2012). Nevertheless, the interaction between ATV and PG and the therapeutic efficacy of Malarone® against canine *B. gibsoni* infection has not been examined.

In the present study, we analyzed the interaction of ATV and PG against two *in vitro* culture strains of *B. gibsoni* (WT and ATV-resistant strain with M121I in *cyt b*). Subsequently, we evaluated the efficacy of ATV and PG against *B. gibsoni* in acute and chronic stage of experimentally infected dogs. We assessed whether Malarone® can inhibit the recurrence and emergence of resistance against ATV or not.

2. Materials and methods

2.1. Interaction of AVT and PG against B. gibsoni in vitro

The interaction of ATV and PG against the uncloned WT *B. gibsoni* and ATV-resistant *B. gibsoni* was examined. The parasites were isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan, in 2004. They were maintained in an *in vitro* culture at our laboratory as WT *B. gibsoni* (Matsuu et al., 2008). ATV-resistant *B. gibsoni* were developed by exposure to ATV for 144 h (Iguchi et al., 2012). The parasites demonstrated low sensitivity against ATV and *cyt b* of that had a SNP at nt363 (G to T), which resulted in the substitution of methionine with isoleucine (M121I). Cultures of these parasites were carried out as reported previously. In brief, 200 μL of packed infected RBCs were dispensed into 1800 μL of culture medium to obtain a 10% packed cell volume (PCV) in each well of a 12-well plate. Each parasite was incubated at 37 °C in a humidified atmosphere containing 5% CO_2 (Iguchi et al., 2012).

In vitro drug interactions were assessed using a modified fixed ratio isobologram method (Fivelman et al., 2004). ATV and PG were obtained from Wako Chemicals (Osaka, Japan), and a stock solution was prepared with dimethyl sulfoxide (DMSO). After a preliminary experiment against WT and ATV-resistant *B. gibsoni*, the 50% inhibitory concentration (IC_{50}) was calculated (Matsuu et al., 2008). ATV and PG were six-point twofold diluted, and the IC_{50} fell near the midpoint of a dilution series. That is, ATV was diluted as 1.6, 0.8, 0.4, 0.2, 0.1, and 0 μM against WT *B. gibsoni*, 3.2, 1.6, 0.8, 0.4, 0.2, and 0 μM, against ATV-resistant *B. gibsoni*, and 100, 50, 25, 12.5, 6.25, and 0 μM of PG against both types of *B. gibsoni*. Combination solution for WT *B. gibsoni* were prepared in fixed ratio solutions at ratio of 0.6:0, 0.8:6.25, 0.4:12.5, 0.2:25, 0.1:50 and 0:100 of ATV and PG (μM). Combination solution for ATV-resistant *B. gibsoni* of that were prepared at ratio of 3.2:0, 1.6:6.25, 0.8:12.5, 0.4:25, 0.2:50 and 0:100 of ATV and PG (μM). Each combination solution was serially diluted five times in twofold dilutions (Fivelman et al., 2004). Combination solution for 16:6.4 μM of ATV and PG was also prepared and this was serially diluted in fivefold dilution to assess interactions of ATV and PG at the ratio of 2.5:1 as same as Malarone®. The final concentration of DMSO was adjusted to 0.05%. Packed infected RBCs (20 μL) were dispensed into 180 μL of culture medium with each concentration of drug to obtain a 10% PCV in each well of a 96-well plate. The parasites were incubated at 5% CO_2 and 37 °C for 6 days. Each day, half of the medium was replaced with fresh medium containing ATV and PG. The rate of growth inhibition of the parasitized erythrocytes from each of the wells was calculated 6 days from the blood smear (Matsuu et al., 2008).

Assessment of drug interaction is based on calculation of the sum of the fractional inhibitory concentrations (ΣFICs) at the given IC_{50} by the formula

$$\frac{IC_{50}\text{ of ATV in the mixture}}{IC_{50}\text{ of ATV alone}} + \frac{IC_{50}\text{ of PG in the mixture}}{IC_{50}\text{ of PG alone}}.$$

ΣFICs < 1 denote synergism, ΣFICs ≧ 1 and <2 denote additive interaction, ΣFICs ≧ 2 and <4 denote slight antagonism, and ΣFICs ≧ 4 denote marked antagonism (Gupta et al., 2002).

2.2. Efficacy of Malarone® against experimentally infected dogs

Malarone® (GlaxoSmithKline, Buckinghamshire, UK), which is a medication containing 250 mg ATV and 100 mg PG, was used as the test compound. Before starting the infectious experiments, Malarone® were administered to 4 healthy dogs (2 Beagles and 2 American Cocker Spaniels) for 10 days (17–25 mg/kg ATV and 7–10 mg/kg PG) to assess the incidence of side effects. General conditions were checked every day and blood samples were collected pre-treatment, during treatment (on day 8), and post treatment (on day 14) for complete blood count (CBC) (Cell Alpha, Nihon Kohden, Tokyo, Japan) and biochemical analyses; alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (Cre), total protein (TP), albumin/globlin ratio (A/G), and total bilirubin (T-bil) (DRI-CHEM 3500i, Fuji Film, Tokyo, Japan).

The experimental infections were undertaken at Kitasato University and the Animal Care and Ethics Committee of the university approved the use of animals. Five beagles (dogs A–E; two females and three males, 2–7 years old) were obtained from Oriental Yeast Company (Tokyo, Japan) and maintained in an air controlled and isolated room to prevent another infection for two weeks before the study starting. All dogs were maintained in separate cages and fed a commercial dog food according to the