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A quantitative reverse-transcriptase PCR assay for the assessment of drug activities against intracellular *Theileria annulata* schizonts

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ABSTRACT

Intracellular schizonts of the apicomplexans *Theileria annulata* and *Theileria parva* immortalize bovine leucocytes thereby causing fatal immunoproliferative diseases. Buparvaquone, a hydroxynaphthoquinone related to parvaquone, is the only drug available against *Theileria*. The drug is only effective at the onset of infection and emerging resistance underlines the need for identifying alternative compounds. Current drug assays employ monitoring of proliferation of infected cells, with apoptosis of the infected host cell as a read-out, but it is often unclear whether active compounds directly impair the viability of the parasite or primarily induce host cell death. We here report on the development of a quantitative reverse transcriptase real time PCR method based on two *Theileria* genes, *tasp* and *tap104*, which are both expressed in schizonts. Upon *in vitro* treatment of *T. annulata* infected bovine monocytes with buparvaquone, TaSP and Tap104 mRNA expression levels significantly decreased in relation to host cell actin already within 4 h of drug exposure, while significant differences in host cell proliferation were detectable only after 48–72 h. TEM revealed marked alterations of the schizont ultrastructure already after 2 h of buparvaquone treatment, while the host cell remained unaffected. Expression of TaSP and Tap104 proteins showed a marked decrease only after 24 h. Therefore, the analysis of expression levels of mRNA coding for TaSP and Tap104 allows to directly measuring impairment of parasite viability. We subsequently applied this method using a series of compounds affecting different targets in other apicomplexan parasites, and show that monitoring of TaSP- and Tap104 mRNA levels constitutes a suitable tool for anti-theilerial drug development.

1. Introduction

Apicomplexan parasites are responsible for a variety of diseases in humans, pets and/or farm animals, and are thus of considerable medical and economic importance. Those most relevant for farm animals are *Babesia*, *Besnoitia*, *Cryptosporidium*, *Eimeria*, *Neospora*, *Sarcocystis*, *Theileria*, and *Toxoplasma*, causing diseases of great socio-economic impact worldwide. Treatment options for many of these diseases are limited, and include either culling of infected livestock, prevention of infection by vaccination, and/or chemotherapy (Müller and Hemphill, 2013a).

Theileria parasitize white blood cells. The most important species are *Theileria parva* and *Theileria annulata*, which cause an acute and usually fatal lymphoproliferative disease in cattle (Morrison and McKeever, 2006). Following transmission by ticks, *T. parva* sporozoites invade lymphocytes, and *T. annulata* infect monocytes, macrophages, dendritic cells and B cells, and, within a few days, develop into schizonts. Development of schizonts causes host cells to undergo proliferation (Dobbelaere et al., 1988). This leads to expansion of the infected cell population and dissemination via the lymphoid system. Cattle in some endemic areas exhibit a degree of innate resistance with low mortality, while cattle introduced from outside succumb to the infection rapidly (Boulter and Hall, 1999).

An important feature of *Theileria* infections is that the host cells are 'immortalize' upon infection and resume proliferation (Dobbelaere and Heussler, 1999). Thus, continuous cultures of *T. annulata* could be established by infecting peripheral blood mononuclear cells (PBMCs) with sporozoites (Preston et al., 1998). *T. parva* schizont-infected lymphoblastoid cultures were

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established from infected animals by employing feeder layer cells and by infecting PBMC with *T. parva* sporozoites (Shkap and Pipano, 2000). These *in vitro* systems are the most important tools to prepare sufficient amounts of the parasite for genome sequencing (Pain et al., 2005) and to study the developmental processes such as invasion of host cells by sporozoites (Shaw, 2003), interactions with the host cell (Heussler and Stanway, 2008) with a focus on the mechanisms of immortalization of the host cell by the parasite (Heussler et al., 2002).

To control bovine theileriosis, three strategies are currently employed: (i) control of the ticks by acaricides, which is expensive, causes environmental damage, and leads to resistance development; (ii) vaccination strategies based on either infection-and-treatment method or attenuated live vaccines, which have been extensively reviewed especially with regard to their risks (McKeever and Morrison, 1998; McKeever, 2007, 2009); and (iii) chemotherapy, which currently represents the most promising anti-theilerial strategy.

Buparvaquone, a hydroxynaphthoquinone related to parvaquone, is the only drug available against *Theileria*, and is a potent selective inhibitor of mitochondrial electron transport, specifically at Complex III (b–c1 complex), in *Theileria*, but also *Plasmodium*, *Eimeria* and *Toxoplasma* (Hudson et al., 1985; Fry and Pudney, 1992). Cattle infected with *T. annulata* or *T. parva* are cured within a few days by a single dose injection (McHardy and Morgan, 1985). The compound has to be administrated, however, during the early stage of infection to avoid the destruction of the immune system that occurs during the advanced stages of the disease. Moreover, first cases of resistance against buparvaquone have been reported (Mhadhbi et al., 2010; Sharifiyazdi et al., 2012). The mode of action and the mechanisms of resistance formation are still unknown. Upon killing the *Theileria* schizont, the infected cell loses its transformed phenotype and rapidly succumbs to apoptosis, making it difficult to distinguish between host cell-mediated effects and the direct action of a given compound upon the parasite (Küenzi et al., 2003).

There is an urgent need for new drugs to treat bovine theileriosis. Before setting up a suitable screening strategy, however, it is paramount to be able to distinguish whether a drug acts primarily against the parasite, or whether it mainly affects the proliferative host cell. The former represents the ideal chemotherapeutic scenario, since the inactivation of the parasite will restore apoptosis of the host cell. In the latter case, chances are high that the drug will affect a host cell target, and adverse side effects are more likely to occur.

In this study we present a quantitative reverse transcriptase real time PCR protocol that allows rapid and reliable monitoring of direct effects upon the *Theileria* metabolism by comparing the mRNA levels of two major schizont-surface proteins, namely TaSP and Tap104 (Witschi et al., 2013; Woods et al., 2013), to host mRNA levels in buparvaquone treated *T. annulata* infected macrophages. Results were confirmed by showing that buparvaquone directly affects the structural integrity of the parasite but not host cell, within few hours of treatment. We subsequently applied this method using a series of compounds affecting different targets in other apicomplexan parasites.

2. Materials and methods

2.1. Tissue culture media, biochemicals, and drugs

If not otherwise stated, all tissue culture media were purchased from Gibco-BRL (Zürich, Switzerland), and biochemical reagents were from Sigma (St. Louis, MO). Kits for molecular biology were purchased from Qiagen (Hilden, Germany). Buparvaquone (McHardy and Morgan, 1985) was provided by Cross Vetpharm Group Limited (Dublin, Ireland), and was kept as 1.5 mM stock solution in dimethyl sulfoxide (DMSO) at −20 °C. Tetracycline (Sato and Wilson, 2005), clindamycin (Fichera and Roos, 1997), trans-iodo-boranyl-chalcone (TIBC) (Hayashida et al., 2013) and ciprofloxacin (Lizundia et al., 2009) were purchased from Sigma–Aldrich (Buchs, Switzerland). All compounds were kept as 100 mM stock solutions in dimethyl sulfoxide (DMSO) at −20 °C, except ciprofloxacin which was stored in water (pH 4.5) at −20 °C. The dicationic compounds DB745 and DB750 (Schorer et al., 2012), the artemisinine derivatives artemiside and artemisone (Dunay et al., 2009), and the thiazolide nitazoxanide (Esposito et al., 2007b) were stored as above.

2.2. Culture of bovine macrophages infected with T. annulata

T. annulata infected bovine macrophages (TaC12 cells) were cultured as described (von Schubert et al., 2010). For drug treatment experiments, TaC12 cells were seeded into 75 cm^2 culture flasks, treated with the compound or respective amounts of DMSO as a control and harvested at various time points. Each experimental treatment was carried out in quadruplicate (4 different flasks), and these experiments were repeated at least twice to ensure reproducibility. Cells were detached by removing the medium and resuspending the cell layer in 1 ml of PBS containing 1 mM EDTA. Then, the number of viable cells was counted using a 0.4% trypan blue solution, cells were pelleted by centrifugation (300*g*, 5 min, 4 °C), immediately frozen in liquid nitrogen and stored at −80 °C for subsequent DNA, RNA and protein purification.

2.3. Processing of samples for quantitative PCR

Simultaneous DNA, RNA, and protein purification was performed using a Qiagen Allprep DNA/RNA/protein kit according to the standard protocol suitable for cell cultures provided by the manufacturer. DNA was eluted in 100 µl elution buffer from the kit and diluted 20 times in water prior to analysis by quantitative PCR.

RNA was purified including a DNase I digestion (to remove residual genomic DNA) according to the instructions provided by the manufacturer, eluted with 40 µL of RNase-free water, quantified by measuring the absorption at 260 nm, and stored at −80 °C.

2.4. Quantitative real-time PCR

Issuing from previously published results (Ros-García et al., 2012), real-time PCR on DNA was performed using the primers Ta18S-F and Ta18S-R (Table 1). As external standards, samples containing between 10 and 10^4 copies of the plasmid containing the 18S-fragment were included. For this purpose, the 18S fragment was amplified from *T. annulata* genomic DNA and cloned into the vector pCRBluntII (Invitrogen, Carlsbad, Ca) according to the manufacturer's instructions. Synthesis of cDNA for quantitative RT-PCR was performed with 2 µg of RNA using the Qiagen Omniscript™ kit with random primers according to the manufacturer's

Table 1
Overview of primers used in this study.

Gene	Accession-no.	Primer	Sequence
Ta18S	KF429795.1	Ta18S_F	GACCTTAACCTGCTAAATAGG
		Ta18S_R	CAGGCCTCTCGGCCAAGG
Tap104	TA08425	Tap104_F	TCATAGGTCTACAGAACTGGA
		Tap104_R	TTTAGGTGGTTCTGGACCCT
TaSP	TA17315	TaSP_F	AGCAGCCCCTTGTCATGGG
		TaSP_R	TAATAGCTTTTGCACGGAGGA
Actin	NM_001100	α-AC1	GAGACCACCTACAACAGCATCATG
		α-AC2	CACCTTGATCTTCATGGTGCTGGG