Research paper

Repurposing of commercially available anti-coccidials identifies diclazuril and decoquinate as potential therapeutic candidates against *Besnoitia besnoiti* infection

Alejandro Jiménez-Meléndez[a], Laura Rico-San Román[a], Andrew Hemphill[b], Vreni Balmer[b], Luis Miguel Ortega-Mora[a], Gema Álvarez-García[a,*]

[a] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040, Madrid, Spain
[b] Institute of Parasitology, Vetsuisse Faculty, University of Bern, Länggass-Strasse 122, CH-3012, Bern, Switzerland

ARTICLE INFO

ABSTRACT

Repurposing of currently marketed compounds with proven efficacy against apicomplexan parasites was used as an approach to define novel candidate therapeutics for bovine besnoitiosis. *Besnoitia besnoiti* tachyzoites grown in MARC-145 cells were exposed to different concentrations of toltrazuril, diclazuril, imidocarb, decoquinate, sulfadiazine and trimethoprim alone or in combination with sulfadiazine. Drugs were added either just prior to infection of MARC-145 cells (0 h post infection, hpi) or at 6 hpi. A primary evaluation of drug effects was done by direct immunofluorescence staining and counting. Potential effects on the host cells were assessed using a XTT kit for cell proliferation. Compounds displaying promising efficacy were selected for IC_{50} and IC_{99} determination by qPCR. In addition, the impact of drugs on the tachyzoite ultrastructure was assessed by TEM and long-term treatment assays were performed. Cytotoxicity assays confirmed that none of the compounds affected the host cells. Decoquinate and diclazuril displayed invasion inhibition rates of 90 and 83% at 0 h pi and 73 and 72% at 6 h pi, respectively. The remaining drugs showed lower efficacy and were not further studied. Decoquinate and diclazuril exhibited IC_{99} values of 100 nM and 29.9 μM, respectively. TEM showed that decoquinate primarily affected the parasite mitochondrium, whilst diclazuril interfered in cytokinesis of daughter zoites. The present study demonstrates the efficacy of diclazuril and decoquinate against *B. besnoiti in vitro* and further assessments of safety and efficacy of both drugs should be performed in the target species.

1. Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan protozoan belonging to the Toxoplasmatinae subfamily and closely related to *Neospora caninum* and *Toxoplasma gondii*. *B. besnoiti* causes bovine besnoitiosis, a debilitating disease of cattle characterized by non-specific clinical signs such as fever or oedemas at the acute stage and skin manifestations during the chronic stage that may end up with sterility in bulls (Gutiérrez-Expósito et al., 2017). In the absence of effective treatments or vaccines for disease control, the last 20 years have witnessed a steady increase in the number of infected herds, and the disease appeared in countries where it had not been described before. Thus, the European Food and Safety Authority (EFSA) has considered bovine besnoitiosis as re-emerging in Europe (European Food Safety Authority, 2010). Recent outbreaks have been described in Central Europe or even Ireland (Álvarez-García, 2016; Ryan et al., 2016).

Due to assumed similarities with other Toxoplasmatinae parasites, *B. besnoiti* is suspected to have a heteroxenous life cycle, but the definitive host is still elusive (Basso et al., 2011). In Europe, cattle act as the main intermediate host, where two asexual and infective stages of the parasite develop: tachyzoites, responsible for the acute stage of the disease, and bradyzoites, found inside tissue cysts and responsible for the characteristic skin lesions during the chronic stage.

Currently, there are no effective therapeutics for the treatment of bovine besnoitiosis. An effective drug should target the tachyzoite stage and affect the dissemination of the parasite into different organs during the acute disease stage, and should preferentially also impact on the tissue cysts that contain bradyzoites. In the past, the effects of a wide range of compounds were assessed in naturally infected bovines, as well as in experimentally infected rabbits and gerbils (Pols, 1960; Shkap

et al., 1985; Shkap et al., 1987). However, results have remained inconclusive due to fact that these assays were performed under variable experimental conditions, and there was a lack of well-established and reproducible *in vivo* models to study bovine besnoitiosis.

Previous *in vitro* studies demonstrated that thiazolides (Cortes et al., 2007), arylimidamides (Cortes et al., 2011), and bumped kinase inhibitors (BKIs) (Jiménez-Meléndez et al., 2017) exhibited promising *in vitro* activities against *B. besnoiti*. However, arylimidamides and thiazolides are not commercially available for ruminants in Europe and BKIs are "new generation drugs" in a pre-clinical stage of development (Van Voorhis et al., 2017). Thus, they may represent valuable therapeutical tools only in the long run. In contrast, repurposing of drugs with well characterized activities, and which are already on the market for other indications, might be a valuable strategy for a speedier implementation of novel treatments against besnoitiosis. The same approach has also been exploited for other closely related parasites such as *N. caninum* (Muller et al., 2015), *T. gondii* (Dittmar et al., 2016) or *Cryptosporidium parvum* (Bessoff et al. 2014). Thus, in this study decoquinate, diclazuril, toltrazuril, imidocarb, sulfadiazine and trimethoprim, were assessed for *in vitro* activity against *B. besnoiti*, as these drugs are commercialised in cattle for the treatment of relevant diseases caused by apicomplexan parasites.

Decoquinate is an anticoccidial quinolone initially developed for poultry in 1967 (Williams, 2006) and approved as an additive in feed to prevent intestinal coccidiosis in cattle and goats. Decoquinate affects the parasite mitochondrium and acts as a cytochrome *bc1* inhibitor, thus it impairs the transfer of electrons from ubiquinone to cytochrome C (Fry and Williams, 1984). The compound is also active against *N. caninum* tachyzoites (Lindsay et al., 1997) and also affects the proliferation of *T. gondii* (Lindsay et al., 1998). The coccidicides toltrazuril and diclazuril are triazinone derivatives and are effective against intracellular stages of *Eimeria* and *Isospora* spp. The exact mechanism of action is not well understood, but several studies have shown that these drugs affect enzymes of the respiratory chain, and also target other enzymes such as dihydrofolate reductase (DHFR) (Stock et al., 2017). Imidocarb is a dicationic diamidine of the carbanilide series of antiprotozoal compounds and currently the drug of choice for the treatment of bovine babesiosis caused by *Babesia* spp. (Vial and Gorenflot, 2006). The mode of action is uncertain, but it is supposed to interfere with the production and/or utilization of polyamines or the prevention of the entry of inositol into the erythrocytes. The antibiotics sulfadiazine and trimethoprim are used in cattle for the treatment of colibacillosis, metritis and pneumonia (Kaartinen et al., 1999), and they are also commonly applied for prophylaxis and treatment of toxoplasmosis in humans (Torre et al., 1998). They act synergistically by sequentially blocking dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), both of which are crucially involved in the folate biosynthesis pathway and essential for nucleoside biosynthesis and nucleic acid formation. Moreover, sulfadiazine can be used to treat coccidiosis (Daugschies and Najdrowski, 2005).

Thus, the objective of the present study was to evaluate the safety and efficacy of these six commercially available drugs against *B. besnoiti* tachyzoites *in vitro*.

2. Materials and methods

2.1. Parasite maintenance and cell cultures

The monkey kidney cell line MARC-145 and human foreskin fibroblasts (HFF), as well as tachyzoites from the *B. besnoiti* Spain1 (Bb Spain 1) isolate, were maintained according to previously published procedures (Jiménez-Meléndez et al., 2017). MARC-145 cell cultures were passaged twice a week, whilst HFF cultures only once a week. The *B. besnoiti* isolate used for all *in vitro* assays was tested negative to *Mycoplasma* spp. infection by PCR (Mycoplasma Gel Form Kit®, Biotools, Spain) following the manufacturer´s instructions and bovine viral

diarrhea virus (BVDV) by quantitative real-time PCR (qPCR) (Hoffmann et al., 2006). The fetal calf serum (FCS) used in all the experiments was previously checked for the absence of IgGs against *B. besnoiti*, *N. caninum* and *T. gondii* by IFAT (Fernández-García et al., 2009). For drug assays, tachyzoites were harvested three days post infection (dpi), when most of them were still intracellular, by recovering the infected cell monolayer with a cell scraper, followed by repeated passages through a 25-gauge needle at 4 °C and separation from cell debris on a PD-10 column (Frey et al., 2016). Tachyzoite viability was confirmed by trypan blue exclusion followed by counting in a Neubauer chamber. Purified viable tachyzoites were used to infect Marc-145 cell monolayers.

2.2. Cytotoxicity in MARC-145 cells

The potential toxicity of the compounds against MARC-145 cells was assessed by a XTT cell viability assay (Panreac-AppliChem, Barcelona, Spain). MARC-145 cells in the exponential phase of growth were seeded in 96-well flat-bottom plates at a density of 2×10^4 cells/well containing compounds at the maximum concentration employed in our assays and grown for 72 h at 37 °C in a 5% CO_2 humidified incubator. Afterwards, 50 µL of XTT reagent was added to each well and further incubated for 4 h. Fluorescence was measured at the respective excitation and emission wavelengths of 475 nm and 660 nm in a Biotek Multiplate Reader (Biotek, Winooski, VT, USA) and specific OD values were determined according to the instructions from the manufacturer. The compounds were tested in quadruplicate in three independent assays.

2.3. Primary drug assays on B. besnoiti-infected MARC-145 cells

In preliminary experiments, dose-finding studies were carried out with at least 4 concentrations for each drug, following the procedure described by Jiménez-Meléndez et al. (2017). Each concentration was tested in triplicate in two or more independent assays. Briefly, monolayers of MARC-145 cells (5×10^4 cells per well) were incubated in culture medium at 37 °C / 5% CO_2 and grown to confluence in 24-well plates. Treatments were initiated by adding the compounds to the cell cultures just prior to infection (0 h post infection, hpi) or 6 h after cells were infected (6 hpi). The drug concentrations employed in these experiments are outlined in Table 1, and were selected based on previous *in vitro* studies on *N. caninum*, *T. gondii* or other apicomplexan parasites (e.g *Babesia* spp., *Theileria* spp.). Depending on the compound, the solvents Dimethyl Sulfoxide or a mixture of NaOH/MetOH (Lindsay et al., 2013) were added to negative control wells at equal volumes (see Table 1). Cultures were infected with 10^3 purified tachyzoites of *B. besnoiti* Bb Spain-1 when the compounds were already present, or compounds were administered 6 hpi. In the latter case, infected monolayers were gently rinsed 3 times with Phosphate Buffered Saline (PBS) prior to the addition of the compounds in order to remove noninvaded tachyzoites. Drugs were kept in the medium of cell culture until immunofluorescence staining was performed at 72 hpi. Once the optimal concentrations for each compound were identified, the assays were repeated employing the same procedure under optimized conditions. In some experiments employing sulfadiazine and trimethoprim, alone or in combination, longer treatments of 6 days, involving at least two lytic cycles of the parasite, were carried out (Lindsay et al., 1994). Each condition was assessed in triplicate and all experiments were carried out in three independent assays.

2.4. Immunofluorescence staining

For immunofluorescence staining, supernatants of the cell cultures were discarded at 72 hpi, cells were washed 3 times with PBS and were fixed by the addition of ice-cold methanol for 10 min. After another wash in PBS, cells were permeabilized with 300 µl/ well of 0.2% Triton-

Table 1

Compounds tested in all *in vitro* assays and results obtained in the preliminary experiments carried out to determine the best concentrations for the subsequent drug screening.

Compound (Company)	Solvent	Drug Stock Solution	Drug Concentration used in assays			References[b]
			Previous trials (Effect observed in treated cell cultures)	Subsequent experiments	IC$_{50}$ and IC$_{99}$ determinations	
Decoquinate (Zoetis™)	NaOH 0.1N–MetOH 90%	2.4 mM	240, 200, 100, 50 nM (No lysis plaques, PV[a])	240 nM	240 nM – 0.24 nM	Lindsay et al. (2013)
Diclazuril (Elanco-Lilly™)	DMSO	10 mM	100 μM (Cytotoxicity) 70, 50 μM (No lysis plaques or PV) 30 μM (No lysis plaques, PV) 10 μM (PV)	30 μM	40 μM – 0.04 μM	Lindsay et al. (1995)
Toltrazuril (Bayer™)	DMSO	10 mM	100, 70, 50 μM (Cytotoxicity) 30 μM (No lysis plaques, PV) 10 μM (Lysis plaques and PV)	30 μM		Darius et al. (2004)
Imidocarb (MSD Animal Health™)	DMSO	10 mM	70, 50, 30, 10 μM (Lysis plaques and PV)	30 μM		Hines et al. (2015)
Sulfadiazine (CZV™)	DMSO	50 mM	200, 100, 70, 50 μM (Lysis plaques and PV)	100 μM		Lindsay et al. (1994)
Trimethoprim (CZV™)	DMSO	50 mM	200, 100, 70, 50 μM (Lysis plaques and PV)	100 μM		Lindsay et al. (1994)
Sulfadiazine + Trimethoprim (CZV™)	DMSO		100-100; 100-50; 100-30; 100-10 (Lysis plaques and PV)	100 μM – 10 μM		Lindsay et al. (1994)

[a] PV: parasitophorous vacuoles.

[b] Previous studies done with these compounds and other apicomplexan parasites in order to select appropriate doses to be tested.

X 100 in PBS for 30 min at 37 °C, followed by 3 additional washes with PBS. A primary polyclonal rabbit-anti tachyzoite Bb-Spain1 polyclonal antiserum (Gutiérrez-Expósito et al., 2013) was added at a dilution of 1:1000 in PBS and incubated for 1 h at 37 °C. After 3 additional washes with PBS, Alexa Fluor® 488 Goat Anti-Rabbit IgG (H + L), (Life technologies, Thermo Fisher Scientific, USA) were added per well at a dilution of 1:1000. The plates were incubated for 45 min at room temperature in the dark, and washed 3 times with PBS. In the final wash, DAPI stain was included to stain the nuclei. Finally, the plates were washed with distilled water and the total number of invasion events per well was counted using an inverted fluorescence microscope (Nikon eclipse TE200) at 200X magnification. Two categories of plaque forming tachyzoites were distinguished: parasitophorous vacuoles (PVs) and lysis plaques, as described by Frey et al. (2016).

2.5. IC$_{50}$ and IC$_{99}$ determination

Those compounds that showed the highest values of both parasite invasion and proliferation inhibition were selected for IC$_{50}$ and IC$_{99}$ determination (the effective concentrations to reduce proliferation by 50% or 99%, respectively). MARC-145 cells were grown to confluency in 24 well plates. Just prior to infection, drugs were added at final concentrations ranging between 40 μM and 4 nM for diclazuril and 240 nM and 0.24 nM for decoquinate. Bb-Spain 1 tachyzoites were added at a parasite: host cell ratio of 1:100 (10^3 tachyzoites per well). Control wells containing the drug solvents were also included in each culture plate. After 72 hpi, samples were collected using a lysis solution (100 μL PBS, proteinase K and AL Buffer) and stored at -80 °C until further DNA extraction according to the manufacturer´s instructions (DNeasy Blood and Tissue, Qiagen, Valencia, CA, USA). Each condition was assessed in triplicate and the experiments were carried out in three independent assays.

2.6. DNA extraction and quantitative real-time PCR (qPCR)

The harvested cell culture samples were incubated for 10 min at 56 °C, and DNA was purified using the spin column protocol for cultured cells according to the manufacturer's instructions contained in the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). DNA was eluted in 200 μl elution buffer. DNA content and purity of each sample

was measured by UV spectrometry using a Biotek Multiplate Reader (Biotek, Winooski, VT, USA).

The BbRT2 qPCR assay for the specific detection of *Besnoitia* spp. DNA from ungulates (i.e., *B. besnoiti, B. tarandi, B. caprae,* and *B. bennetti*) was performed according to Frey et al. (2016). Herein the SYBR Green system was used. Briefly, each 20 μl reaction contained 10 μl of Power SYBR Green master mix® (Applied Biosystems, Foster City, CA, USA), 0.5 μl of primer Bb3 (5′-CAA CAA GAG CAT CGC CTT C-3′; 20 μM), 0.5 μl of primer Bb 6 (5′-ATT AAC CAA TCC GTG ATA GCA G-3′; 20 μM), and 4 μl water. The qPCRs were run on a 7500 Fast Real-Time PCR System® (Applied Biosystems, Thermo Fisher Scientific, USA). 20–100 ng of DNA in a volume of 5 μl was added to each reaction. The DNA positive control was extracted from *B. besnoiti* tachyzoites cultured *in vitro*. The product of the DNA extraction process using water instead of cells was used as a negative control. In each qPCR, 10-fold serial dilutions of genomic DNA corresponding to 0.1–100,000 Bb-Spain1 tachyzoites were included. The cycling conditions were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence emission was measured during the 60 °C step. A dissociation stage was added at the end of each run, and the melting curves were analysed. BbRT2-PCR was run in duplicate for each sample. The threshold cycle values (Ct-values) obtained for positive samples in the BbRT2-PCR were also expressed as tachyzoites per reaction using the standard curve included in each run.

2.7. Studies on the impact of decoquinate and diclazuril on the viability of B. besnoiti tachyzoites

Tachyzoites from Bb-Spain 1 strain were grown as previously stated and decoquinate or diclazuril were added just prior to infection at the previously established IC$_{99}$ concentrations. Drugs were kept for 6, 24 or 48 h. Drug containing medium was discarded and fresh culture medium was added. Cell cultures were evaluated until 10 days post treatment (dpt), by daily visual inspection by light microscopy. Samples were collected at 1, 3 and 5 dpt for subsequent qPCR analysis. IFAT at 8 and 10 dpt were also performed to assess whether any tachyzoites were able to re-infect host cells (Winzer et al., 2015). Each assay was done in triplicate and at least three independent experiments were carried out.

2.8. Transmission electron microscopy

HFF cell cultures were maintained in T25 tissue culture flasks and were infected with 10^7 Bb-Spain1 tachyzoites (parasite: host cell ratio of 10:1). After allowing the tachyzoites to invade the host cells for 3 h, monolayers were washed three times with PBS and treated with decoquinate or diclazuril at the previously stated EC_{99} for each compound. Control flasks without drugs contained the corresponding amounts of either DMSO or NaOH/MetOH. Samples were processed for TEM analysis at different time points of treatment (1, 3 and 6 days) as described elsewhere (Müller et al., 2017).

2.9. Data analyses

Values for cytotoxicity of compounds are depicted in percentage (%) in relation to the respective vehicle, and were statistically evaluated using Student's-*t*- test using the raw data from the specific ODs calculated as explained in section 2.2.

To determine the percentage of inhibition of parasite growth, the invasion rates (IRs) were calculated by counting all the events per well, IRs were then related to the respective negative vehicle control to determine the relative growth (RG) of the parasite for each drug experiment. The percentage of inhibition was determined as follows:

% RG = (IR drug / IR vehicle) × 100

% Inhibition = 100 − %RG

For IC_{50} and IC_{99} calculations based on qPCR, the amount of DNA was quantified by spectrophotometry in each sample and adjusted. The RG in each drug concentration was determined relative to the vehicle control using the tachyzoite yield per ng of DNA. IC_{50} and IC_{99} values were calculated using the ED50 plus sheet for Microsoft Excel after a logarithmic transformation of the data.

Kruskal-Wallis test was performed to compare the efficacy of the 6 compounds against *B. besnoiti* in the drug screening, and a two-way ANOVA test followed by a Tukey post-test for multiple comparisons was employed to compare the different treatment durations. All statistical analyses were performed using the software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Cytotoxicity assessments in uninfected MARC-145 cells

At the concentrations used here, none of the compounds exhibited significant cytotoxicity when compared to their respective vehicle controls (DMSO or NaOH/MetOH) as shown by the results from the XTT assay (p > 0.05; *t*-test). Percentages of cytotoxicity of the screened compounds compared to the solvent-treated negative control wells were as follows: diclazuril: 3.2%; toltrazuril: 6%; imidocarb: 2.8%; decoquinate: 2.3%; sulfadiazine: 3%; trimethoprim: 3.1%; sulfadiazine + trimethoprim: 3.3%. Moreover, upon inspection by light microscopy, no alterations in the Marc-145 cells morphology were detected.

3.2. Primary drug assessments using immunofluorescence-based read-outs

The criteria employed to select the drug concentrations for the screening assays were the absence of cytotoxicity and the presence of an effect on *B. besnoiti* growth on the preliminary assays performed. In those assays, we found that toltrazuril was cytotoxic in the *in vitro* system employed for concentrations higher than 30 µM (50, 70 and 100 µM), leading to the detachment of the hosts cells (Table 1). In the initial drug treatments, initiated either during invasion (0 hpi) or at 6 hpi, decoquinate, diclazuril and toltrazuril were the most efficient drugs and inhibited parasite growth by more than 65% (Table 2). In general, the

efficacy was higher when compounds were administered concomitantly to infection at 0 h pi, except for imidocarb (Kruskal-Wallis test, p < 0.05).

Parasitic growth was markedly inhibited in wells treated with decoquinate, diclazuril and toltrazuril, since only PVs but not lysis plaques were found. In contrast, in cultures treated with the other compounds and in the control wells, distinct lysis plaques were clearly visible, indicating that parasites had completed the lytic cycle. Decoquinate and diclazuril were the most efficacious, and were consequently selected for further studies (Table 2). In contrast, sulfadiazine administered at 6 h pi was the least effective compound (19% of parasite growth inhibition, Kruskall-Wallis test, p < 0.01) (Table 2). When sulfadiazine or trimethoprim, alone or in combination, were kept in the cultures for up to 6 days, tachyzoites were still able to replicate and proliferate, as seen by the presence of confluent lysis plaques visualized by immunofluorescence, similar to non-treated control wells (data not shown).

As previously published for the isolate employed, 80% of the invasion events observed at 72 hpi consisted of lysis plaques in non-treated wells (Frey et al., 2016).

3.3. IC_{50} & IC_{99} determinations of decoquinate and diclazuril

For both, decoquinate and diclazuril, dose-dependent effects on parasite proliferation were observed (Kruskal Wallis, p < 0.01). When administered concomitantly to infection at concentrations higher than 0.24 nM, exposure of infected cells to decoquinate led to a significant reduction in parasite growth, and the IC_{50} was calculated to be 10 nM. For diclazuril, higher concentrations were needed: parasitic growth was markedly inhibited when the compound was administered at concentrations higher than 40 nM, and the IC_{50} was 135 nM (Fig.1). To reach 99% inhibition, 120 nM of decoquinate and 29.5 µM of diclazuril were required.

3.4. Decoquinate and diclazuril treatments do not act parasiticidal

Infected cultures were treated with decoquinate or diclazuril with concentrations corresponding to the IC_{99} for a maximum period of 48 h, and drugs were removed and the culture continued, this in order to study the long term effects of the treatments. Parasitic growth was hardly detected until 3 dpt, regardless which drug was used and how long the treatment had lasted. Subsequently, higher inhibition rates corresponded to longer treatments of up to 48 h (Fig. 2). Statistically significant differences between treated wells and their corresponding control wells were observed at 3 and at 5 dpt for any treatment durations (p < 0.001, Two-Way ANOVA), with a lowest parasitic load in treated wells. Differences were also found among the different treatment durations (6, 24 and 48 h) for both drugs at 5 dpt and higher inhibition rates corresponded to longer treatments (p < 0.001, Two-Way ANOVA) (Fig. 2).

Short treatments for 6 and 24 h with decoquinate were more effective since the tachyzoite yields obtained at 3 dpt in decoquinate treated wells were significantly lower than in diclazuril-treated wells. Further analysis by IF at 8 and 10 dpt showed that tachyzoites remained viable and were able to re-infect host cells even after treatments of 48 h with decoquinate. Lysis plaques were present at 10 dpt in all treated wells. However, in wells treated with diclazuril for 48 h, the parasitic load was clearly diminished both at 3 d pt and at 5 dpt, showing the lowest tachyzoite yield. Shorter treatments for up to 24 h were less effective and failed to inhibit parasite proliferation completely. This finding was also corroborated upon inspection in the light microscope and by IF, since lysis plaques were absent in wells treated with diclazuril for 48 h at 7 or 10 d pt, but present in wells treated for 6 h or 24 h at both post-infection time points (7 and 10 d pt) (data not shown).

Table 2
Percentage of inhibition of parasite growth in the drug screening when compounds were administered at 0 h post infection (hpi) or at 6 hpi.

	Drugs	Decoquinate	Diclazuril	Toltrazuril	Imidocarb	Sulfadiazine	Trimethoprim	SDZ + TM
Time of administration	**0 hpi**	90	83.2	82.3	40	43	41	50
	6 hpi	74	72,8	69	36	19	31	35

3.5. Ultrastructural alterations in B. besnoiti tachyzoites induced by decoquinate and diclazuril treatments

B besnoiti tachyzoites obtained from drug solvent treated controls exhibited the hallmarks of apicomplexan parasites and closely resembled *T. gondii* and *N. caninum* tachyzoites. A negative control culture treated with decoquinate solvent (NaOH/MetOH) is shown in Fig. 3A. Tachyzoites were located within a PV that was filled with a granular matrix, and the apically located secretory organelles such as micronemes, rhoptries, as well as dense granules were evident. In addition, tachyzoites contained one mitochondrium, of which only segments were seen on a given section plane, filled with a rather electron dense membranous matrix composed of cristae (Fig. 3A). Similar findings were obtained when tachyzoites were treated with diclazuril solvent (DMSO) as shown by Jiménez-Meléndez et al. (2017).

In *B. besnoiti* tachyzoites exposed to decoquinate for 24 h, no obvious structural alterations were evident, and they closely resembled their non-treated counterparts (data not shown). However, after 3 days of treatment, most notably the mitochondria were severely altered and lacking an electron dense matrix, and they were seemingly replaced by largely empty vacuoles that filled considerable space in the cytoplasm of these parasites (Fig. 3B–D). In addition, the cytoplasm was often filled with loose membrane residues. However, despite these alterations, after three days the parasites still retained their shape, were still within a parasitophorous vacuole (PV), but also already obviously dead parasites with distorted disorganized cytoplasm and vacuolization could be seen. After 6 days, mitochondria, were not discernible anymore, the cytoplasmic organization was completely lost and lipid droplets were formed (Fig. 3E). Also, there were parasites which were not enclosed by a parasitophorous vacuole membrane anymore. Only few seemingly still viable parasites could be found in these specimens.

In cultures treated with diclazuril, (Fig. 4), small but distinct effects related to the drug action appeared already at 24 h of treatment (Fig. 4A–C). These included a widening between the nuclear membrane and the cytoplasm of the tachyzoites. At 3 d of treatment, dividing parasites were visible that were still attached to the residual body (Fig. 4D. In addition, the gap around the nuclear periphery was more
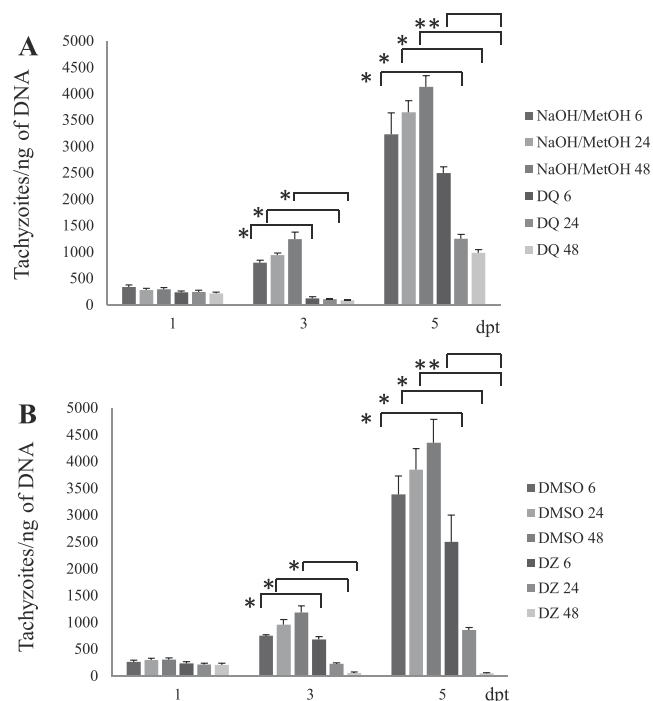

Fig. 2. Tachyzoite yield expressed as tachyzoites per ng of DNA from *Besnoitia besnoiti* infected MARC-145 cells, which were treated with decoquinate (A) or diclazuril (B) for 6, 24 or 48 h, and further cultured without drugs. Samples were collected at 1, 3 & 5 days post treatment (dpt).
*Statistically significant differences between treated wells and their corresponding control wells at 3 and at 5 dpt for any treatment durations (p < 0.001).
**Statistically significant differences among the different treatment durations (6, 24 and 48 h) for both drugs at 5 dpt (p < 0.001).

evident, and a compartmentalization took place, with occasional structures resembling amylopectin granules forming within the
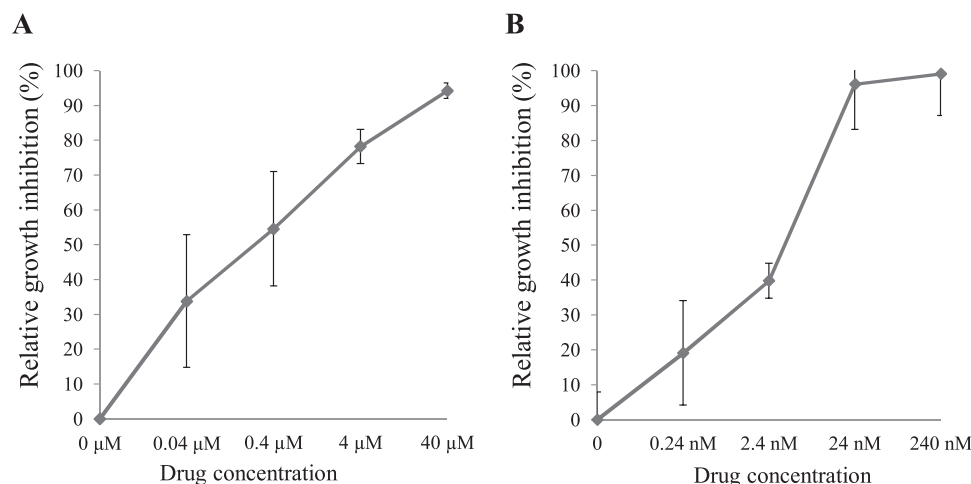

Fig. 1. Relative inhibition of *Besnoitia besnoiti* tachyzoite proliferation (in & with respect to the vehicle negative control). Decoquinate (A) and diclazuril (B) were administered at 0 h pi. Tachyzoite proliferation was assessed by qPCR.
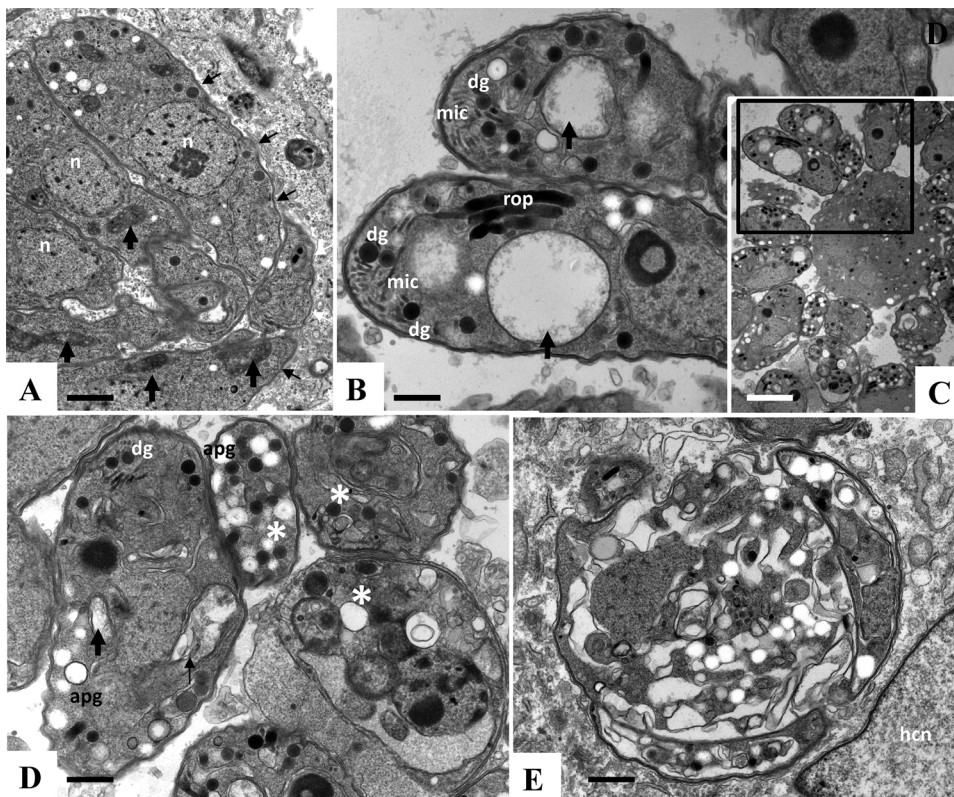
Fig. 3. Representative TEM images of *Besnoitia besnoiti* tachyzoites cultured in human foreskin fibroblast cells. (A) Tachyzoites treated with NaOH/MetOH, situated within a vacuole and surrounded by a parasitophorous vacuole membrane (depicted with thin blackarrows). Note the individual nuclei and the mitochondria with an electron dense matrix (thick vertical black arrows). (B–D) Tachyzoites treated with decoquinate during 3 days, residues of mitochondria lacking any discernible matriy are indicated by black arrows. (C) is a lower magnification overview of (B), the boxed area is enlarged in (B). Note the presence of dramatically altered parasites in (D), marked with an asterisk (*). (E) Image of a *B. besnoiti* tachyzoite exposed to decoquinate during 6 days, with severely altered structural features. Rop = rhoptries, dg = dense granules, mic = micronems, n = nucleus, apg = amylopectin granules. Scale bars: A = 0.5 μm; B = 0.3 μm; C = 1 μm; D = 0.3 μm; E = 0.4 μm.

cytoplasm of tachyzoites, Vacuolization also took place in the residual body. However, the PV and its membrane were still evident in many cases and the mitochondria appeared still largely unaffected. After 6 day, amylopectin granules became more prominent, and complexes with largely disorganized cytoplasm were formed (Fig. 4E).

4. Discussion

In this study, the safety and efficacy of a wide panel of commercially available compounds in Europe, namely toltrazuril, diclazuril, imidocarb, decoquinate, sulfadiazine and trimethoprim (alone or in combination with sulfadiazine) were assessed for activity against *B. besnoiti* tachyzoites for the first time. Some of these drugs had previously shown efficacy against other apicomplexan parasites and, in particular, against Toxoplasmatinae parasites, both *in vitro* (Hemphill et al., 2016) and *in vivo* (Sánchez-Sánchez et al., 2018). Two compounds, namely decoquinate and diclazuril, both commercialised for the treatment of various infections in cattle, inhibited *B. besnoiti* invasion and proliferation, with IC$_{50}$ values in the nanomolar range.

Inhibition rates of decoquinate and diclazuril in terms of inhibition of parasite invasion and proliferation reached values higher or close to 90%. The *in vitro* model in Marc-145 cells employed in the present work was previously used to assess the activities of a panel of bumped kinase inhibitors (BKIs), which target calcium dependent protein kinase 1, and similar results were obtained (Jiménez-Meléndez et al., 2017). In contrast, the low efficacy of imidocarb, sulfadiazine and trimethoprim (alone or in combination with sulfadiazine) against *B. besnoiti* infection may lead us to rule out their potential therapeutic activity. In addition, toltrazuril assays were aborted due to cytotoxicity exerted in the host cells.

Our results showed that decoquinate represents a safe compound in our *in vitro* model. Under the experimental settings employed, the efficacy of this compound was higher when it was administered at the time of infection, but it also interfered with proliferation in invaded tachyzoites. This result contrasts to previous studies carried out with *N.*

caninum tachyzoites, where decoquinate showed a minimum effect against extracellular zoites. It is known that decoquinate affects electron transport in mitochondria so that a feasible explanation to the findings observed in *N. caninum* could be the presence of less active mitochondria in extracellular tachyzoites (Lindsay et al., 1997). However, in *T. gondii* tachyzoites, it was demonstrated that decoquinate is able to affect oxygen consumption of extracellular tachyzoites (Pfefferkorn et al., 1993). Accordingly, decoquinate might be more active against those parasites with higher capacity to survive extracellularly, as shown for *B. besnoiti* (Frey et al., 2016). Interestingly, we obtained IC$_{50}$ values in the low nanomolar range (10 nM), in agreement with previous studies that have shown decoquinate to have *in vitro* activity against *T. gondii* tachyzoites with IC$_{50}$ of 0.005 μg/ml (12 nM) (Ricketts and Pfefferkorn, 1993). Although decoquinate is specifically designed to treat gastrointestinal coccidiosis in several species (e.g cattle, small ruminants and poultry) at concentrations around 0.5 mg/kg bw, it is well absorbed and reaches maximum plasma concentrations of 2 μM in milking cows (Leonardo et al., 2009). These concentrations are much higher than the IC$_{50}$ and IC$_{99}$ found *in vitro*. Accordingly, it may represent a valuable therapeutic tool to control acute clinical cases of bovine besnoitiosis as food additive at the recommended posology. Since remaining viable tachyzoites were able to resume proliferation once the treatment with the IC$_{99}$ was suspended, a parasitostatic effect is suggested for this compound for at least 48 h of treatment, as it has been described for hydroxiquinoxalines (Mehlhorn, 2008). This parasitostatic effect could be desirable since treated *Besnoitia* tachyzoites might be an antigenic stimulus and would allow the development of a strong immune response against the parasite, potentially preventing reinfection after recovering from the acute stage, as it has been postulated for other compounds such as BKIs (Winzer et al., 2015). In *N. caninum*, the parasiticidal or parasitostatic effect of this drug depends on the concentration employed. A coccidiocidal effect of decoquinate was observed against intracellular tachyzoites when administered at concentrations higher than 0.01 μg/mL (24 nM (Lindsay et al., 1997).

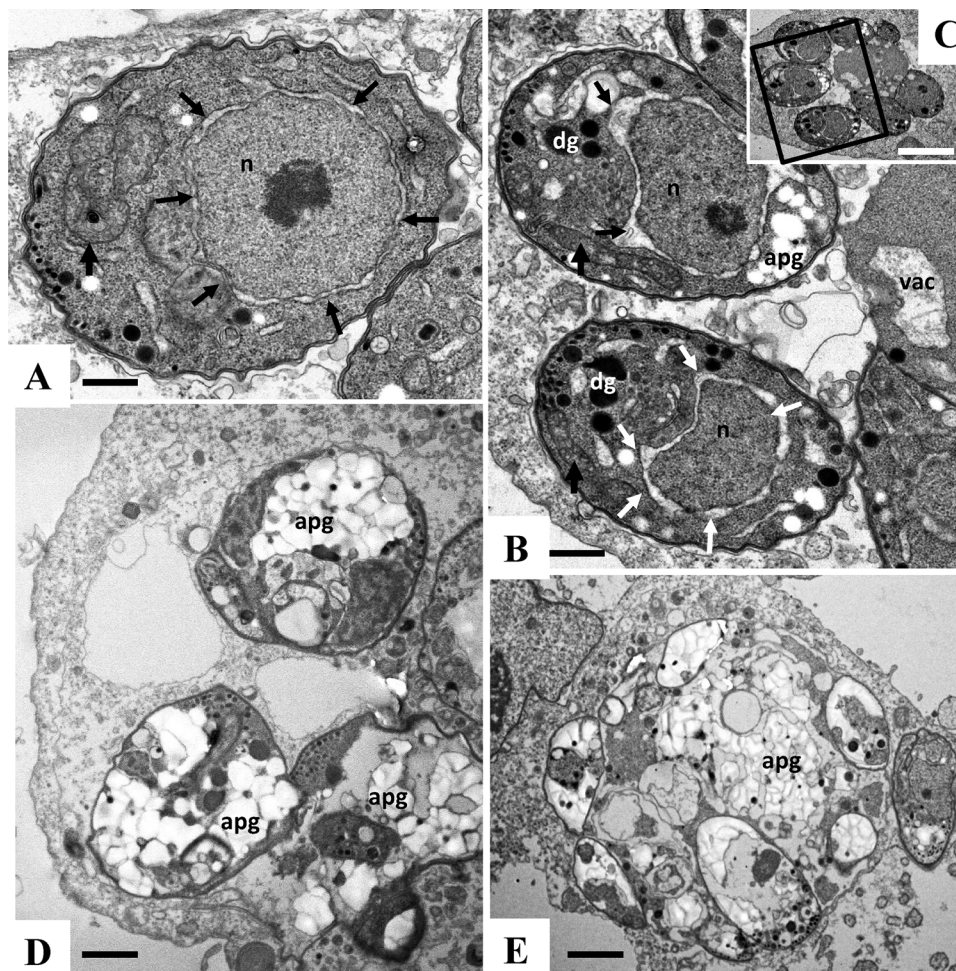Electron microscopy showed that the mitochondrium is, as

Fig. 4. Representative TEM images of *Besnoitia besnoiti* tachyzoites in human foreskin fibroblast cell cultures exposed to diclazuril after 24 h of treatments (A–C, the boxed area in C is shown at larger magnification in B); 3 days of treatment (D); 6 days of treatment (E). The vertical arrows indicate mitochondria, arrows surrounding nuclei (n) point towards the separation of the nuclear membrane and the cytoplasm. dg = dense granules, apg = amylopectin granules, vac shows a vacuole in the residual body. Scale bars: A = 0.3 μm; B = 0.4 μm; C = 1.1 μm; D = 0.4 μm; E = 0.8 μm.

expected, the main site of action of decoquinate. However, after 6 days of treatment, mitochondrial impairment led to more dramatic alterations and a general breakdown of the structural organization of the parasite, with deposits of amylopectin granules in the cytoplasm, all of this leading to a general loss of viability for most tachyzoites. This finding may indicate a transitional stage from tachyzoite to bradyzoite, as it has been described previously for tachyzoites from the RH strain of *T. gondii* treated with decoquinate (Lindsay et al., 1998). Thus, decoquinate could represent a stressing agent to induce the differentiation from *Besnoitia* tachyzoites to bradyzoites *in vitro*.

Regarding triazinone-derivative coccidiocidals, our results showed that diclazuril is safe at the highest concentrations used in the *in vitro* model employed and effective against *B. besnoiti* tachyzoites, inhibiting both parasite invasion and proliferation. Those results are in agreement with previous *in vitro* studies with *T. gondii* (Lindsay and Blagburn, 1994) and *N. caninum* tachyzoites (Lindsay et al., 1994). Indeed diclazuril inhibited *T. gondii* tachyzoite proliferation by 97% at a concentration of 0.005 μg/mL (12.2 nM). Remarkably, higher concentrations are needed for *B. besnoiti* since the IC$_{50}$ was 135 nM. In cattle, this compound is marketed against coccidiosis and the bioavailability of the compound is low, reaching maximum plasmatic concentrations of 95.8 nM (European Medicines Agency (EMEA), 2004). Thus, further pharmacokinetics studies assessing different dosages and possibly also formulations are needed to obtain a higher bioavailability of the compound in cattle. We also showed that treatments with the IC$_{99}$ for at

least 48 h are able to exert a parasiticidal effect on *B. besnoiti* tachyzoites, since lysis plaques were absent and the parasitic load was clearly diminished in those wells. These results are in agreement with TEM results, since after 6 days of treatment almost no viable parasites were present and a clear effect on the cytokinesis of daughter zoites, with the formation of multinucleated complexes was visualized. These findings are similar to those previously described for *T. gondii* tachyzoites treated with diclazuril, since an effect on endodiogeny together with the presence of multi-nucleated complexes were observed when treated at a concentration of 1 μg/mL (Lindsay et al., 1995). Opposite to our results, ultra-structural effects of diclazuril were not noted until 2 days after treatment whilst in *B. besnoiti* we have observed that the first effects appeared after 24 h of treatment. *In vivo* experiments regarding mice infected with tachyzoites from the RH strain of *T. gondii* have shown that this compound is able to prevent death of up to 80–100% of the infected animals after oral administration at 1.0 or 10 mg/kg on 1 day prior to infection and then daily for 10 days (Lindsay and Blagburn, 1994).

The other triazinone derivative studied in the present work, toltrazuril, was cytotoxic at concentrations up to 30 μM. This safety concern has been also described in other cell lines, such as HFF cells, showing that concentrations of 10 μg/mL (23 μM) diminished cell viability up to 92% (Qian et al., 2015). However, at the highest concentration employed here, a remarkable effect on parasitic growth inhibition was noted. This compound might present good activity against

B. besnoiti tachyzoites considering that it is well-absorbed in cattle and is rapidly metabolized to ponazuril (toltrazuril-sulfone), which is the major metabolite (Stock et al., 2017). Moreover, toltrazuril has been effective against *N. caninum* both in *in vitro* (Darius et al., 2004) and *in vivo* studies (Syed-Hussain et al., 2015; Strohbusch et al., 2009). Thus, before ruling out its therapeutic potential for bovine besnoitiosis, further experiments should be performed.

Imidocarb dipropionate was discarded in our experimental settings, since it failed to exert more than 30% parasite invasion and proliferation inhibition. No previous *in vitro* treatments with this compound against *T. gondii* or *N. caninum* have been reported. When similar series of compounds, diminazene aceturate and pentamidines were studied they were not effective against *B. besnoiti* in an *in vitro* model using epithelial-like Vero cells (Shkap et al., 1987). The lack of efficacy may be due to a less relevant myo-inositol synthesis pathway in *B. besnoiti*. Up to date, only glycolysis and glutamine byosinthesis metabolic routes have been reported in *B. besnoiti*-infected cells (Taubert et al., 2016). Besides, it has been postulated that the activity of imidocarb may resemble that of the trypanocidal and babesicidal berenil (diminazene aceturate) (Mehlhorn., 2008b), binding to DNA in the minor groove of the kinetoplast, which is absent in apicomplexan parasites.

Finally, regarding DHFR and DHFS inhibitors, alone or in combination, sulfadiazine failed to inhibit parasite growth by more than 50%, even when it was co-administered with trimethoprim at the time point of infection. Previous *in vitro* studies regarding Toxoplasmatinae parasites have shown variable results, since sulfadiazine is highly efficacious against *T. gondii* with IC_{50} in the range of 2.5 µg/mL (Sánchez-Sánchez et al., 2018) whilst little activity was exerted against *N. caninum* at concentrations up to 100 µg/ml (400 µM) and longer treatments were needed (Lindsay et al., 1994). This finding agrees with our results where low parasitic growth inhibition was observed when treatments lasted for 3 or 6 days. Besides, previous studies carried out in *Besnoitia* have also reported variable results. Sulfadiazine lacked efficacy upon *B. besnoiti* infection in gerbils (Shkap et al., 1987), whereas in other *Besnoitia* species, such as *B. darlingi,* treatment with sulfadiazine was able to diminish parasitic growth *in vitro* at concentrations higher than 4 µM in a 8 day-assay (50% growth inhibition) (Elsheikha and Mansfield, 2004). *In vivo* information regarding sulfonamide therapy in bovine besnoitiosis indicate that they are commonly used under field conditions to diminish the severity of clinical signs, but they usually fail to cure the infected cattle and recurrences are not rare, even if treatment is administered when clinical signs first appear (Jacquiet et al., 2010). The lack of efficacy of sulphadiazine observed *in vitro* may explain this *in vivo* variability. Trimethoprim also failed to exert a potent inhibition *in vitro*. However, a strong inhibition of *T. gondii* growth has been observed with an IC_{50} of 2.3 µg/ml, with striking morphological changes of the parasites (Sánchez-Sánchez et al., 2018). In addition, trimethoprim is also effective against *N. caninum* tachyzoites at 10 µg/ml (Lindsay et al., 1994).

In summary, the present study demonstrates proof of concept for the efficacy of decoquinate and diclazuril against *B. besnoiti in vitro,* and further assessments of safety and efficacy of both drugs should be performed in the target species. Concentrations of diclazuril needed to clear the parasite *in vitro* are higher than the ones reported in the literature for this compound. Thus, prior to attempt *in vivo* treatments, further studies regarding pharmacokinetic parameters, employing other administration routes or posologies, are needed in order to achieve higher bioavailability and raise maximum plasmatic concentrations. An ideal drug against *B. besnoiti* should allow the generation of a strong humoral immune response to avoid re-infections and preventing that new entries in the herd (specifically breeding bulls) get infected. Regarding this issue, the parasitostatic effect exerted by both compounds could favor the development of a humoral immune response.

References

Álvarez-García, G., 2016. From the mainland to Ireland - bovine besnoitiosis and its spread in Europe. Vet. Rec. 178, 605–607.

Basso, W., Schares, G., Gollnick, N.S., Rutten, M., Deplazes, P., 2011. Exploring the life cycle of *Besnoitia besnoiti* - experimental infection of putative definitive and intermediate host species. Vet. Parasitol. 178, 223–234.

Bessoff, K., Spangenberg, T., Foderaro, J.E., Jumani, R.S., Ward, G.E., Huston, C.D., 2014. Identification of *Cryptosporidium parvum* active chemical series by repurposing the open access malaria box. Antimicrob. Agents Chemother. 58, 2731–2739.

Cortes, H.C., Mueller, N., Esposito, M., Leitao, A., Naguleswaran, A., Hemphill, A., 2007. *In vitro* efficacy of nitro- and bromo-thiazolyl-salicylamide compounds (thiazolides) against *Besnoitia besnoiti* infection in Vero cells. Parasitology 134, 975–985.

Cortes, H.C., Muller, N., Boykin, D., Stephens, C.E., Hemphill, A., 2011. *In vitro* effects of arylimidamides against *Besnoitia besnoiti* infection in Vero cells. Parasitology 138, 583–592.

Darius, A.K., Mehlhorn, H., Heydorn, A.O., 2004. Effects of toltrazuril and ponazuril on the fine structure and multiplication of tachyzoites of the NC-1 strain of *Neospora caninum* (a synonym of *Hammondia heydorni*) in cell cultures. Parasitol. Res. 92, 453–458.

Daugschies, A., Najdrowski, M., 2005. Eimeriosis in cattle: current understanding. J. Vet. Med. B Infect. Dis. Vet. Public Health 52, 417–427.

Dittmar, A.J., Drozda, A.A., Blader, I.J., 2016. Drug repurposing screening identifies novel compounds that effectively inhibit *Toxoplasma gondii* growth. mSphere 1, e00042–15.

Elsheikha, H.M., Mansfield, L.S., 2004. Determination of the activity of sulfadiazine against *Besnoitia darlingi* tachyzoites in cultured cells. Parasitol. Res. 93, 423–426.

European Food Safety Authority, 2010. Scientific Statement on Bovine Besnoitiosis. Available from. http://www.efsa.europa.eu.

European Medicines Agency (EMEA), 2004. Diclazuril (Extension to All Ruminants and Porcine Species): Summary Report (2) - Committee for Veterinary Medicinal Products. Avalaible online 29th May 2018 from. . http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/document/document_detail.jsp?webContentId=WC500013730&mid=WC0b01ac058008d7ad.

Fernández-García, A., Risco-Castillo, V., Pedraza-Díaz, S., Aguado-Martínez, A., Álvarez-García, G., Gómez-Bautista, M., Collantes-Fernández, E., Ortega-Mora, L.M., 2009. First isolation of *Besnoitia besnoiti* from a chronically infected cow in Spain. J. Parasitol. 95, 474–476.

Frey, C.F., Regidor-Cerrillo, J., Marreros, N., García-Lunar, P., Gutiérrez-Expósito, D., Schares, G., Dubey, J.P., Gentile, A., Jacquiet, P., Shkap, V., Cortes, H., Ortega-Mora, L.M., Álvarez-García, G., 2016. *Besnoitia besnoiti* lytic cycle *in vitro* and differences in invasion and intracellular proliferation among isolates. Parasit. Vectors 9, 115.

Fry, M., Williams, R.B., 1984. Effects of decoquinate and clopidol on electron transport in mitochondria of *Eimeria tenella* (apicomplexa: Coccidia). Biochem. Pharmacol. 33, 229–240.

Gutiérrez-Expósito, D., Ortega-Mora, L.M., Marco, I., Boadella, M., Gortazar, C., San Miguel-Ayanz, J.M., García-Lunar, P., Lavin, S., Álvarez-García, G., 2013. First serosurvey of*Besnoitia* spp. infection in wild european ruminants in Spain. Vet. Parasitol. 197, 557–564.

Gutiérrez-Expósito, D., Ferre, I., Ortega-Mora, L.M., Álvarez-García, G., 2017. Advances in the diagnosis of bovine besnoitiosis: Current options and applications for control. Int. J. Parasitol. 47, 737–751.

Hemphill, A., Aguado-Martínez, A., Müller, J., 2016. Approaches for the vaccination and treatment of *Neospora caninum* infections in mice and ruminants models. Parasitology 143, 245–259.

Hines, S.A., Ramsay, J.D., Kappmeyer, L.S., Lau, A.O.T., Ojo, K.K., Van Voorhis, W.C., Knowles, D.P., Mealey, R.H., 2015. *Theileria equi* isolates vary in susceptibility to imidocarb dipropionate but demonstrate uniform *in vitro* susceptibility to a bumped kinase inhibitor. Parasites Vectors 8, 33.

Hoffmann, B., Depner, K., Schirrmeier, H., Beer, M., 2006. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J. Virol. Methods 136, 200–209.

Jacquiet, P., Liénard, E., Franc, M., 2010. Bovine besnoitiosis: Epidemiological and clinical aspects. Vet. Parasitol. 174, 30–36.

Jiménez-Meléndez, A., Ojo, K.K., Wallace, A.M., Smith, T.R., Hemphill, A., Balmer, V., Regidor-Cerrillo, J., Ortega-Mora, L.M., Hehl, A.B., Fan, E., Maly, D.J., Van Voorhis, W.C., Alvarez-Garcia, G., 2017. *In vitro* efficacy of bumped kinase inhibitors against *Besnoitia besnoiti* tachyzoites. Int. J. Parasitol. 47, 811–821.

Kaartinen, L., Löhönen, K., Wiese, B., Franklin, A., Pyörälä, S., 1999. Pharmacokinetics of

sulphadiazine-trimethoprim in lactating dairy cows. Acta Vet. Scand. 40, 271–278.

Leonardo, Q., Rosiles, R., Bautista, J., González-Monsón, N., Sumano, H., 2009. Oral pharmacokinetics and milk residues of decoquinate in milking cows. J. Vet. Pharmacol. Ther. 32, 403–406.

Lindsay, D.S., Blagburn, B.L., 1994. Activity of diclazuril against *Toxoplasma gondii* in cultured cells and mice. Am. J. Vet. Res. 55, 530–533.

Lindsay, D.S., Rippey, N.S., Cole, R.A., Parsons, L.C., Dubey, J.P., Tidwell, R.R., Blagburn, B.L., 1994. Examination of the activities of 43 chemotherapeutic agents against *Neospora caninum* tachyzoites in cultured cells. Am. J. Vet. Res. 55, 976–981.

Lindsay, D.S., Rippey, N.S., Toivio-Kinnucan, M.A., Blagburn, B.L., 1995. Ultrastructural effects of diclazuril against *Toxoplasma gondii* and investigation of a diclazuril-resistant mutant. J. Parasitol. 81, 459–466.

Lindsay, D.S., Butler, J.M., Blagburn, B.L., 1997. Efficacy of decoquinate against N*eospora caninum* tachyzoites in cell cultures. Vet. Parasitol. 68, 35–40.

Lindsay, D., Toivio-Kinnucan, M., Blagburn, B., 1998. Decoquinate induces tissue cyst formation by the RH strain of Toxoplasma gondii. Vet. Parasitol. 77, 75–81.

Lindsay, D.S., Nazir, M.M., Maqbool, A., Ellison, S.P., Strobl, J.S., 2013. Efficacy of decoquinate against *Sarcocystis neurona* in cell cultures. Vet. Parasitol. 196, 21–23.

Mehlhorn, H. (Ed.), 2008. Encyclopedia of Parasitology: A-M 280 Springer, Germany 381 pp.

Muller, J., Aguado-Martinez, A., Manser, V., Balmer, V., Winzer, P., Ritler, D., Hostettler, I., Arranz-Solis, D., Ortega-Mora, L., Hemphill, A., 2015. Buparvaquone is active against *Neospora caninum in vitro* and in experimentally infected mice. Int. J. Parasitol. Drugs Drug Resist. 5, 16–25.

Müller, J., Aguado-Martínez, A., Balmer, V., Maly, D.J., Fan, E., Ortega-Mora, L., Ojo, K.K., Van Voorhis, W.C., Hemphill, A., 2017. Two novel calcium-dependent kinase 1-inhibitors interfere with vertical transmission in mice infected with *Neospora caninum* tachyzoites. Antimicrob. Agents Chemother. AAC 61 02324-16.

Pfefferkorn, E., Borotz, S.E., Nothnagel, R.F., 1993. Mutants of *Toxoplasma gondii* resistant to atovaquone (566C80) or decoquinate. J. Parasitol. 559–564.

Pols, J.W., 1960. Studies on bovine besnoitiosis with special reference to the aetiology. Onderstepoort J. Vet. Res. 28, 265–356.

Qian, W., Wang, H., Shan, D., Li, B., Liu, J., Liu, Q., 2015. Activity of several kinds of drugs against Neospora caninum. Parasitol. Int. 64, 597–602.

Ricketts, A.P., Pfefferkorn, E.R., 1993. *Toxoplasma gondii*: Susceptibility and development of resistance to anticoccidial drugs *in vitro*. Antimicrob. Agents Chemother. 37, 2358–2363.

Ryan, E.G., Lee, A., Carty, C., O'Shaughnessy, J., Kelly, P., Cassidy, J.P., Sheehan, M., Johnson, A., de Waal, T., 2016. Bovine besnoitiosis (*Besnoitia besnoiti*) in an irish dairy herd. Vet. Rec. 178, 608.

Sánchez-Sánchez, R., Vázquez, P., Ferre, I., Ortega-Mora, L.M., 2018. Treatment of toxoplasmosis and neosporosis in farm ruminants: state of knowledge and future trends. Curr. Top. Med. Chem Accepted.

Shkap, V., De Waal, D.T., Potgieter, F.T., 1985. Chemotherapy of experimental *Besnoitia besnoiti* infection in rabbits. Onderstepoort J. Vet. Res. 52, 289.

Shkap, V., Pipano, E., Ungar-Waron, H., 1987. *Besnoitia besnoiti*: Chemotherapeutic trials *in vivo* and in vitro. Rev. Elev. Med. Vet. Pays Trop. 40, 259–264.

Stock, M., Elazab, S., Hsu, W., 2017. Review of triazine antiprotozoal drugs used in veterinary medicine. J. Vet. Pharmacol. Ther. 41, 184–194.

Strohbusch, M., Muller, N., Hemphill, A., Krebber, R., Greif, G., Gottstein, B., 2009. Toltrazuril treatment of congenitally acquired *Neospora caninum* infection in newborn mice. Parasitol. Res. 104, 1335–1343.

Syed-Hussain, S.S., Howe, L., Pomroy, W.E., West, D.M., Hardcastle, M., Williamson, N.B., 2015. Study on the use of toltrazuril to eliminate *Neospora caninum* in congenitally infected lambs born from experimentally infected ewes. Vet. Parasitol. 210, 141–144.

Taubert, A., Hermosilla, C., Silva, L., Wieck, A., Failing, K., Mazurek, S., 2016. Metabolic signatures of *Besnoitia besnoiti*-infected endothelial host cells and blockage of key metabolic pathways indicate high glycolytic and glutaminolytic needs of the parasite. Parasitol. Res. 115, 2023–2034.

Torre, D., Casari, S., Speranza, F., Donisi, A., Gregis, G., Poggio, A., Ranieri, S., Orani, A., Angarano, G., Chiodo, F., Fiori, G., Carosi, G., 1998. Randomized trial of trimethoprim-sulfamethoxazole versus pyrimethamine-sulfadiazine for therapy of toxoplasmic encephalitis in patients with AIDS. italian collaborative study group. Antimicrob. Agents Chemother. 42, 1346–1349.

Van Voorhis, W.C., Doggett, J.S., Parsons, M., Hulverson, M.A., Choi, R., Arnold, S., Riggs, M.W., Hemphill, A., Howe, D.K., Mealey, R.H., 2017. Extended-spectrum antiprotozoal bumped kinase inhibitors: a review. Exp. Parasitol. 180, 71–83.

Vial, H.J., Gorenflot, A., 2006. Chemotherapy against babesiosis. Vet. Parasitol. 138, 147–160.

Williams, R., 2006. Tracing the emergence of drug-resistance in coccidia (*Eimeria* spp.) of commercial broiler flocks medicated with decoquinate for the first time in the United Kingdom. Vet. Parasitol. 135, 1–14.

Winzer, P., Muller, J., Aguado-Martinez, A., Rahman, M., Balmer, V., Manser, V., Ortega-Mora, L.M., Ojo, K.K., Fan, E., Maly, D.J., Van Voorhis, W.C., Hemphill, A., 2015. *In vitro* and *in vivo* effects of the bumped kinase inhibitor 1294 in the related cyst-forming apicomplexans *Toxoplasma gondii* and *Neospora caninum*. Antimicrob. Agents Chemother. 59, 6361–6374.