Research Article

In vitro treatment of *Besnoitia besnoiti* with the naphto-quinone buparvaquone results in marked inhibition of tachyzoite proliferation, mitochondrial alterations and rapid adaptation of tachyzoites to increased drug concentrations

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Abstract

We here assessed the *in vitro* efficacy of the naptho-quinone buparvaquone (BPQ) against *Besnoitia besnoiti* tachyzoites *in vitro*. BPQ is currently licensed for the treatment of theileriosis in cattle in many countries, but not in the EU. In 4-day treatment assays, BPQ massively impaired tachyzoite proliferation with an IC_{50} of 10 ± 3 nM, and virtually complete inhibition was obtained in the presence of nM BPQ. Exposure to $1\,\mu$M BPQ leads to ultrastructural changes affecting initially the mitochondrial matrix and the cristae. After 96 h, most parasites were largely distorted, filled with cytoplasmic amylopectin granules and vacuoles containing components of unknown composition. Host cell mitochondria did not appear to be notably affected by the drug. However, upon prolonged exposure (14–16 days) to increased BPQ concentrations, *B. besnoiti* tachyzoites exhibited the capacity to adapt, and they resumed proliferation at dosages of up to $10\,\mu$M BPQ, albeit at a lower rate. These BPQ-adapted parasites maintained this lower susceptibility to BPQ treatment after freeze–thawing, and inspection by the transmission electron microscopy revealed that they underwent proliferation in the absence of structurally intact mitochondria.

Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan parasite, belonging to the family Sarcocystidae and sub-family Toxoplasmatinae (Cortes *et al.*, 2014). The genus *Besnoitia* is comprised 10 species. *Besnoitia besnoiti* is closely related to *Neospora caninum*, which causes abortion and stillbirth in cattle and neuromuscular disease in dogs, and to *Toxoplasma gondii*, which causes disease not only in animals, but also in humans. In contrast to these two species, for which canids and felids serve as definitive hosts, the life cycle, and the definitive host of *B. besnoiti* has not been elucidated (Oryan *et al.*, 2014). Cattle and wild bovids act as intermediate hosts (Alvarez-Garcia *et al.*, 2013). Upon infection, *B. besnoiti* tachyzoites proliferate in endothelial cells of blood vessels. Subsequently, the formation of tissue cysts in different tissues takes place. During the acute phase of infection, clinical signs such as hyperthermia, weight loss, nasal and ocular discharge, and fibrinoid necrotic vascular lesions can result in death of infected animals (Basso *et al.*, 2011; Lesser *et al.*, 2012; Alvarez-Garcia *et al.*, 2013; Dubey *et al.*, 2013). In the chronic phase scleroderma, scars, nodules on udders and progressive thickening and wrinkling of the skin occur, and infection of male genital organs may result in sterility (Frey *et al.*, 2013). One form of transmission of bovine besnoitiosis occurs mechanically by haematophagous insects (Bigalke, 1968; Lienard *et al.*, 2011), through mating or medical devices, without any sexual cycle involved (Cortes *et al.*, 2014; Alvarez-Garcia *et al.*, 2013). Besnoitiosis has been noted as an emerging disease in Southern Europe (Waap *et al.*, 2014) and in tropical and subtropical areas of Africa, Asia including the Middle East and South America (Jacquiet *et al.*, 2010; Alvarez-Garcia *et al.*, 2013; Uzeda *et al.*, 2014). More recent cases have shown that bovine besnoitosis has spread from Portugal and France into Italy, Switzerland, Germany and it has also emerged in Central-Eastern Europe, including Hungary (Hornok *et al.*, 2014), Belgium and Ireland (Vanhoudt *et al.*, 2015; Ryan *et al.*, 2016).

There is no chemotherapy available (Cortes *et al.*, 2014). *Besnoitia* tachyzoites proliferate and cause disease in experimentally infected gerbils and rabbits, but neither in mice nor rats (Shkap *et al.*, 1987a). Oxytetracycline has prevented the death of infected gerbils, but only when administered simultaneously with the parasites (Shkap *et al.*, 1987b). Formalin, pentamidines, sulphonamides, trimethoprim, pyrimethamine and oxytetracycline have been evaluated as potential treatment options in experimentally infected rabbits (Pols, 1960; Shkap *et al.*, 1985; Shkap *et al.*, 1987a). Only limited numbers of animals were used, and assessments were mainly based on clinical features and histopathology, thus, no definitive conclusions can be drawn from these studies. Formalin, sulphametazine, toltrazuril and oxytetracycline have been assessed as potential treatments in cattle (reviewed in Cortes *et al.*, 2014).

The repurposing of established drugs may be a suitable strategy to discover novel compounds against besnoitiosis (Sateriale *et al.*, 2014). *In vitro*, *Besnoitia* tachyzoites are easily

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cultured, as they proliferate in a variety of host cells of different mammalian origin, including the two monkey kidney cell lines Vero and MARC-145, and in human foreskin fibroblasts (HFF) among others (Cortes *et al.*, 2006; Frey *et al.*, 2016). More detailed investigations employing *in vitro* cultures have shown that thiazolides (Cortes *et al.*, 2007a), new-generation pentamidine derivates and arylimidamides (Cortes *et al.*, 2011), and calcium-dependent protein kinase class 1 (CDPK1) inhibitors such as BKI-1294 and related compounds (Jimenez-Melendez *et al.*, 2017) inhibited proliferation of *Besnoitia* tachyzoites *in vitro*.

Buparvaquone (BPQ) is another interesting candidate for drug repurposing (McHardy and Morgan, 1985). The drug is commercially available in different areas of the world with different names and formulations (McDougall *et al.*, 2016). However, none of these commercial products are available in the EU.

In *Theileria*-endemic regions BPQ is the drug of choice to be used against bovine theileriosis. Against *Theileria parva*-infected cells, BPQ displays an EC_{50} of 0.0003 mg L^{-1}, it has a plasma half-life of at least 7 days, and a low toxicity (LD_{50} in rats >8000 mg kg^{-1}). Besides an outstanding activity against *T. parva* and *T. annulata* (Hashemi-Fesharki, 1991; Hostettler *et al.*, 2014), BPQ is also active against other protozoan parasites including *Leishmania* spp. (Croft *et al.*, 1992; da Costa-Silva *et al.*, 2017), *Theileria equi* (formerly named *Babesia equi*) (Zaugg and Lane, 1992), and against *N. caninum* and *T. gondii*, not only *in vitro* but also in both non-pregnant and pregnant mouse models (Müller *et al.*, 2015, 2016, 2017b). Experiments in pregnant mice did not only demonstrate the outstanding efficacy of BPQ treatment in preventing vertical transmission by these parasites but also documented the safety of the compound upon application during pregnancy. As BPQ is already licensed for use in cattle, although not in the EU, we studied the effects of the drug against *B. besnoiti* tachyzoites, and demonstrate that, despite a low IC_{50} in short-term treatment assays, these parasites adapted to increased drug concentrations within few days, and resistant parasites exhibited distinct ultrastructural characteristics, including a lack of discernible mitochondria.

Material and methods

Tissue culture media, biochemicals and drugs

If not stated otherwise, 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). BPQ was provided by Cross Vetpharm Group Limited (Dublin, Ireland), and was kept as a 10 mM stock solution in dimethyl sulphoxide (DMSO) at −20 °C.

Host cell cultivation and maintenance of B. besnoiti tachyzoites

HFF were maintained in DMEM (Dulbecco's modified Eagle medium), and Vero cells were cultured in the RPMI-1640 medium, both with phenol red supplemented with 10% heat-inactivated and sterile filtrated fetal calf serum, 50 U of penicillin mL^{-1}, and 50 μg streptomycin mL^{-1} (=culture medium). Cultures were maintained at 37 °C and 5% CO_2 in tissue culture flasks (Sarstedt, Nürnbrecht, Germany), and were passaged at least once a week. Tachyzoites of *B. besnoiti* (Bb1Evora, kindly provided by Dr Helder Cortes, the University of Evora, Portugal) were maintained by serial passages in Vero cells as previously described (Cortes *et al.*, 2007a). Tachyzoites were harvested by removing infected cell layers with a rubber cell scraper, followed by repeated passages through a 25-gauge needle

at 4 °C to liberate parasites. Tachyzoites were separated from cell debris by Sephadex-G25 chromatography and were immediately used for infection experiments (Hemphill *et al.*, 1996).

Determination of BPQ efficacy

HFF were grown in six-well plates until a confluent monolayer was formed. Just prior to infection, BPQ was added at concentrations of 30, 60, 120 and 240 nM. The bumped kinase inhibitor BKI 1294 (Jimenez-Melendez *et al.*, 2017; Van Voorhis *et al.*, 2017) was added as an inhibition control at 5 μM. Controls received the corresponding amounts of DMSO. HFF were then infected with 5×10^4 freshly purified *B. besnoitia* tachyzoites, in a total volume of 5 mL. After 4 days, cells were collected with a cell scraper, centrifuged, washed once in PBS and the pellet was stored at −20 °C prior to quantification of parasite proliferation. Assays were carried out in quadruplicates and each experiment was repeated at least twice. DNA purification was performed employing the DNeasy Blood & Tissue Kit (Qiagen, Basel, Switzerland) according to the standard protocol suitable for animal cells. *Besnoitia besnoiti* parasite load was determined by real-time PCR as previously described (Cortes *et al.*, 2007b). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 culture-derived tachyzoites included in each run.

Visualization of BPQ-induced changes by transmission electron microscopy (TEM)

HFF were grown to confluence in T-25-flasks and were infected with *B. besnoiti* (10^6 tachyzoites per flask). After 48 h, cultures were supplemented with 1 μM BPQ, or the corresponding amount of DMSO as a control. At 24, 48 and 96 h of drug treatments monolayers were washed with 100 mM sodium cacodylate buffer (pH 7.3) and fixed with cacodylate buffer containing 2.5% glutaraldehyde for 10 min (Alaeddine *et al.*, 2013). Cells were collected using a rubber cell scraper and centrifuged for 10 min at 1200 rpm at 20 °C. The supernatant was removed, and infected cells were fixed further in glutaraldehyde/cacodylate at 4 °C overnight. Post-fixation in 2% OsO_4, dehydration, embedding in Epon 820 epoxy resin and cutting of ultrathin sections was done as previously described (Guionaud *et al.*, 2010; Alaeddine *et al.*, 2013). Specimens were viewed on a CM12 TEM operating at 80 kV.

Adaptation of B. besnoiti tachyzoites to increased BPQ concentrations

HFF were grown to confluence in T25 tissue culture flasks and were infected with 10^6 *B. besnoitii* tachyzoites. After 24 h, the medium of the flasks was substituted by fresh culture medium, or medium containing 100 nM, 500 nM or 1 μM of BPQ as indicated in Table 1. Flasks were daily checked microscopically for the occurrence of parasite growth. Patches of HFF monolayers destroyed as a consequence of tachyzoite egress were considered as proof of growth. After 9 days, parasites cultured in 500 nM or 1 μM of BPQ were exposed to higher drug concentrations (5, 7.5 and 10 μM, respectively) for another 9 days. During these time periods, the flasks were again microscopically checked daily for signs of re-emerging parasite replication and parasitophorous vacuole formation. Stabilates of *B. besnoiti* cultures adapted to 5 and 10 μM (named R_5 or R_10, respectively) were prepared. As controls, stabilates of parasites that had not been exposed to BPQ were also prepared. All samples were stored at −196 °C for at least 1 month prior to further assessments.

Table 1. Treatment of *Besnoitia besnoiti* infected HFF with BPQ does not act parasiticial and tachyzoites adapt readily to increased BPQ concentrations

Initial continuous BPQ concentration (nM) for 9 days	Time point when host cell lysis observed during treatment (dpi)	Increased continuous BPQ concentration (nM)	Time point when tachyzoite proliferation was observed (dpi)
0	3		
100	9		–
500	9	5000	16[a]
1000	9	7500	16
		10 000	14[a]

Confluent HFF grown in T-25 tissue culture flasks were infected with 10^6 *B. besnoiti* tachyzoites. After 2 h non-invaded parasites were removed and medium with or without 100, 500 and 1000 nM BPQ was added. Cultures were maintained during a maximal timespan of 9 days in the presence of the drug, during which parasites resumed proliferation at all concentrations. Those parasites treated with 500 and 1000 nM BPQ were exposed to increased BPQ concentration for another 9 days, but proliferation in those cultures was also observed at the time points indicated.
dpi, days post-infection.
[a]Indicates cultures of which stabilates were made that were re-introduced into the culture.

Assessments of BPQ-adapted tachyzoites

Drug adapted as well as non-adapted stabilates were thawed and cultured on Vero cells without drug pressure for up to 2 weeks (3–4 passages). Subsequently, they were harvested and 10^4 tachyzoites were seeded onto HFF monolayer grown in six-well plates. Just prior to infection, BPQ was added at concentrations, of either 0, 100, 625 or 5000 nM, and specimens were cultured at 37 °C and 5% CO_2 for 4 days. Subsequently, the parasite load was determined by real-time PCR as described above. To visualize the ultrastructure of drug-adapted *B. besnoiti* tachyzoites, stabilates grown in Vero cells for 2 weeks were fixed and processed for TEM as described above, and specimens were viewed on a Phillips 400 TEM operating at 80 kV.

Statistics

IC$_{50}$ values were calculated after the logit-log-transformation of the relative growth (RG; control = 1) according to the formula $\ln[(RG/(1-RG)] = a \times \ln(\text{drug concentration}) + b$ and subsequent regression analysis by the corresponding software tool contained in the Excel software package (Microsoft, Seattle, WA, USA). Student's *t*-test was used to assess the growth of BPQ-adapted and treated *B. besnoiti* strains with respect to the non-adapted wild-type strain exposed to 100 nM BPQ.

Results

In short-term treatment assays, BPQ severely interferes in *B. besnoiti* tachyzoite proliferation

To establish the *in vitro* efficacy assays, HFF monolayers were infected with *B. besnoiti* tachyzoites and the proliferation of parasites was monitored by quantitative PCR over time. As a control for inhibition of infection, we added the CDPK1-inhibitor BKI-1294 (5 μM) to some wells prior to infection. The other wells received only DMSO as a solvent control. In the solvent control samples, a proliferation of tachyzoites could be measured after 2 days post-infection (p.i.), reaching the exponential phase after 4 days. In the cells treated with BKI-1294, no proliferation was visible (Fig. 1A). Since after 4 days, the infected monolayers showed first lesions and the numbers of extracellular parasites started to
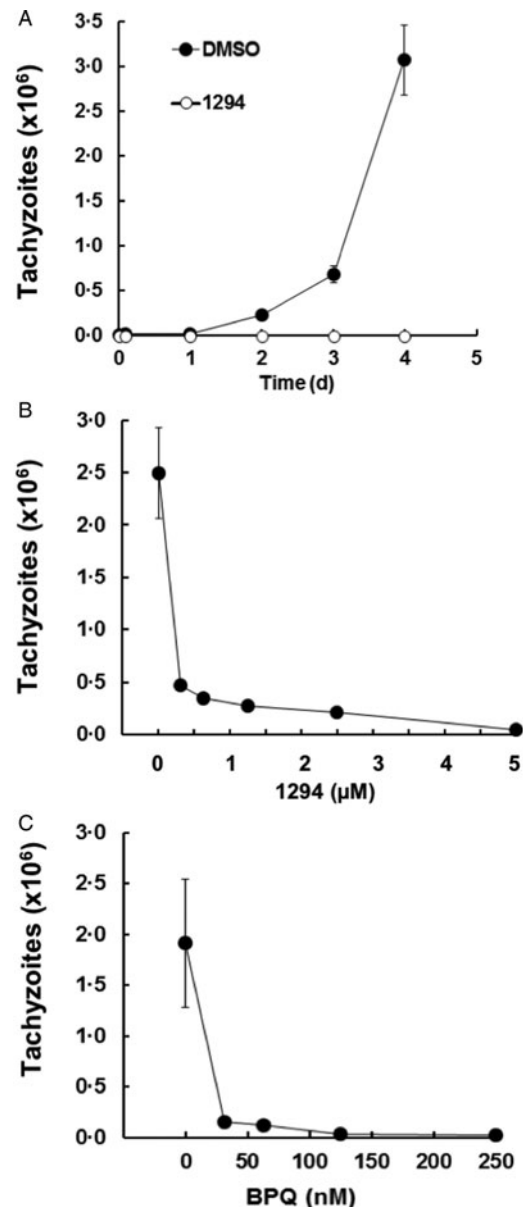

Fig. 1. Effects of BKI-1294 (1294) and BPQ on the intracellular proliferation of *Besnoitia besnoitii* tachyzoites in human HFF. HFF were grown to confluence in six-well tissue culture plates, supplemented with BKI-1294, BPQ or with DMSO as a solvent control and were immediately infected with *B. besnoitii* tachyzoites (2×10^4 per well). (A) Cultures treated with 5 μM BKI-1294 and harvested after 1–4 days. (B) Cultures were treated with a concentration series of BKI-1294 and harvested after 4 days. (C) Cultures were exposed to a concentration series of BPQ and harvested after 4 days. *Besnoitia besnoitii* tachyzoites were quantified by qPCR. Mean ± S.E. values correspond to four replicates.

increase, the endpoint of our efficacy tests was set at 4 days p.i. In these tests, BKI-1294 inhibited the proliferation of *Besnoitia* tachyzoites with an IC$_{50}$ of 0.12 ± 0.03 μM, and nearly complete inhibition was achieved at 5 μM (Fig. 1B). The IC$_{50}$ of BPQ was much lower, namely 10 ± 3 nM, virtually complete inhibition was obtained with 125 nM BPQ, and the IC$_{99}$ of BPQ was 250 nM (Fig. 1C).

BPQ-treatment induces severe ultrastructural changes in *B. besnoiti* tachyzoites

Besnoitia besnotia-infected HFF were exposed to 1 μM BPQ for 24, 48 and 96 h, or were maintained in the absence of BPQ, but in the presence of the corresponding amount of DMSO,
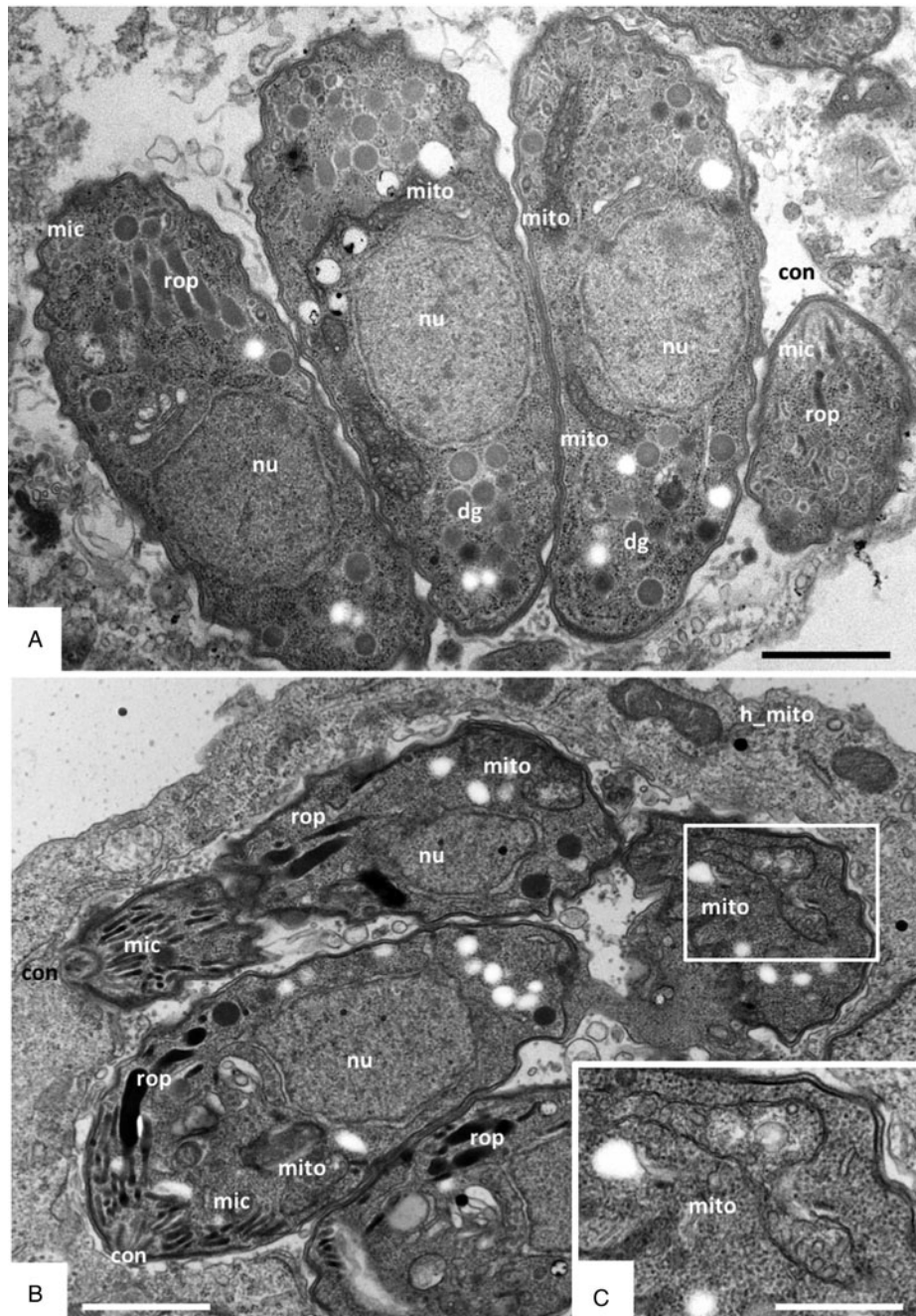
Fig. 2. Ultrastructural changes in *Besnoitia besnoiti* tachyzoites induced by BPQ treatment. Infected HFF were fixed and processed for TEM on day 3 post-infection and treatment with DMSO but no drug (A), or after 24 h of 1 μM BPQ treatment (B, C). Panel (A) shows a parasitophorous vacuole containing several tachyzoites located within the HFF cytoplasm. Note that mitochondria (mito) contain an electron-dense matrix and clearly discernible cristae. Panels (B, C) show infected HFF exposed to 1 μM BPQ for 24 h. The boxed area in (B) is enlarged in (C). Note the changes in the mitochondrial matrix (mito) occurring with drug exposure (B, C), while the host cell mitochondria (h_mito) appear unaffected; nu, parasite nuclei; mic, micronemes; rop, rhoptries; dg, dense granules; con, conoid. Bars in (A) and (B) = 0.6 μM, in (C) = 0.32 μM.

and processed for TEM. Tachyzoites, cultured in the absence of BPQ (Fig. 2A) exhibited the typical features of apicomplexan parasites, including the apical conoid and a set of secretory organelles named micronemes and rhoptries at the anterior end, and dense granules localized apically as well as at the posterior part. A prominent feature of these parasites is the mitochondrion, parts of which are seen on a single section, with a relatively electron-dense matrix and cristae-like structures clearly visible in untreated parasites (Fig. 2A). Exposure to 1 μM BPQ for 24 h (Fig. 2B, C) did not lead to severe changes in parasite ultrastructure, but some alterations, most notably in some of the mitochondria, became already evident, including a less electron dense matrix and slight disturbances in cristae structure (Fig. 2C). Changes became more profound after 48 h of BPQ treatment (Fig. 3A, B), where discernible mitochondria were largely absent, and the mitochondrial matrix was basically replaced by an amorphous substance. At this stage, rhoptries, dense granules and micronemes were still visible, and parasites kept residing within a parasitophorous vacuole. Upon treatment for 96 h (Fig. 3C, D), tachyzoites were still trapped within the parasitophorous vacuole but appeared largely distorted, the cytoplasm became continuously filled with amylopectin granules and other vacuoles containing components of the unknown composition, and nuclei appeared fragmented. However, the conoid remained still intact, with adjacent micronemes and dense granules in place, but rhoptries were not discernible anymore. Overall, BPQ treatment induced considerable damage, initially targeting the mitochondria, and subsequently exposure to this compound led to severe alterations in tachyzoites. In contrast, host cell mitochondria did not appear to be notably affected by the drug.

Upon long-term treatment, BPQ does not act parasiticidal and B. besnoiti tachyzoites readily adapt to increased BPQ concentrations

To investigate whether BPQ would also affect an established infection and whether this drug acted parasiticidal or parasitostatic, infected HFF monolayers were treated with 0.1, 0.5 or 1 μM
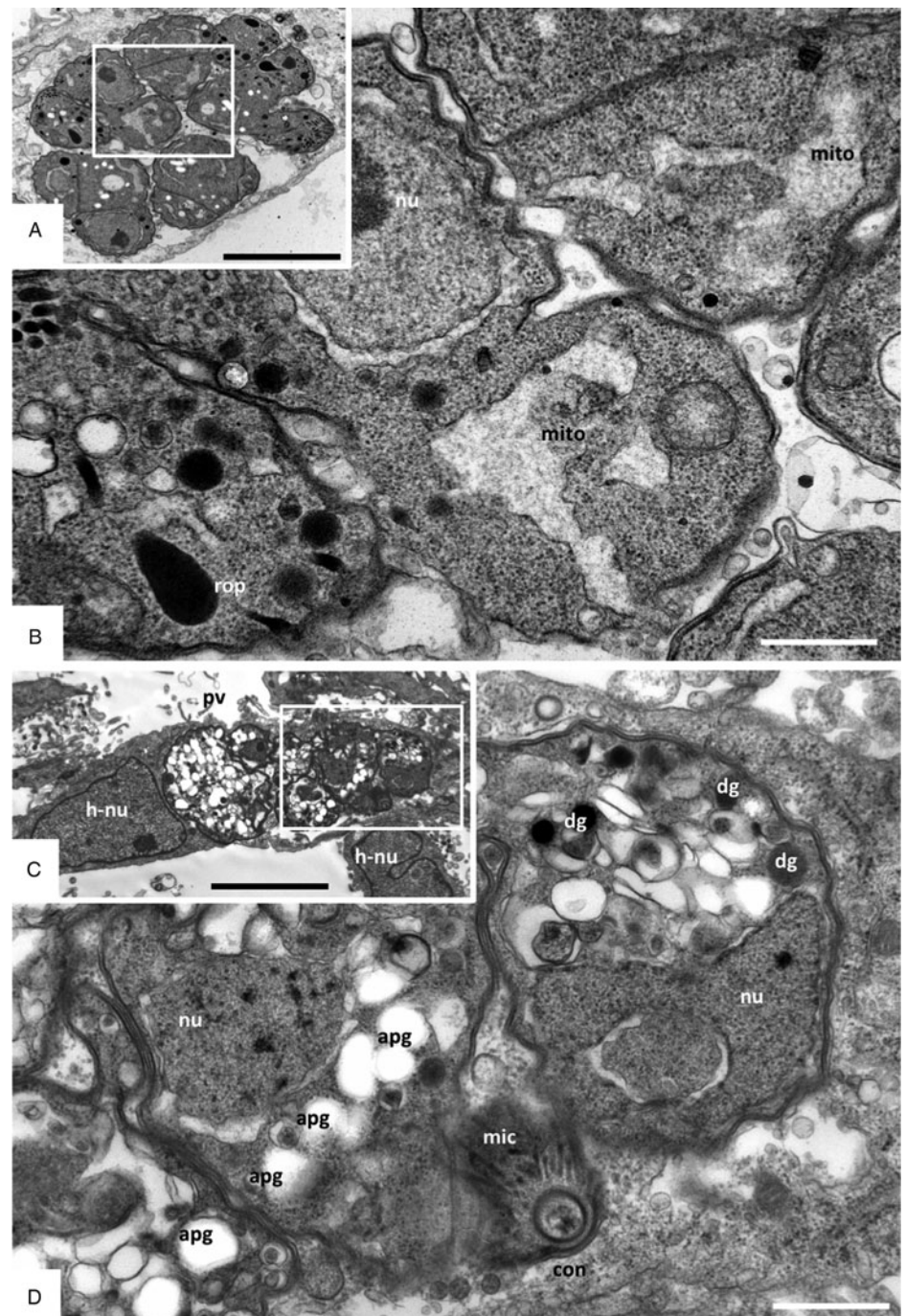
Fig. 3. Ultrastructural changes in *Besnoitia besnoiti* tachyzoites visualized after 2 and 4 days of BPQ treatment. Panels (A, B) show alterations induced after 48 h, (C, D) after 96 h. The boxed areas in (A) and (C) are enlarged in (B) and (D), respectively. Note the profound BPQ-induced changes in tachyzoite mitochondria in (B), and parasites still located within a parasitophorous vacuole (pv) in (C), including large numbers of amylopectin granules (apg); nu, parasite nuclei; mic, micronemes; rop, rhoptries; con, conoid. Bars in (A) = 1.8 μm; in (B) = 0.35 μm; (C) = 2.2 μm; in (D) = 0.3 μm.

BPQ at 24 h p.i., and cultures were inspected daily by light microscopy. Initially, BPQ stopped intracellular proliferation when added 24 h p.i. However, when treatments were continued for a period of 9 days, the initial inhibitory effects got lost, and parasites started to resume growth in the presence of BPQ. The concentrations of BPQ were subsequently increased to 5, 7.5 and 10 μM (see Table 1). At these concentrations, the proliferation of parasites was still noted after 2 weeks of drug exposure. Higher concentrations of BPQ were not sustained by the host cells. Thus, upon prolonged exposure, *B. besnoiti* tachyzoites exhibit a remarkable adaptive potential, rendering them largely tolerant to massively increased concentrations of BPQ.

To see whether this adaptation was permanent or occurred only transiently, tachyzoites adapted to 5 and 10 μM of BPQ were transformed into stabilates, stored at −196 °C for 1 month, and were regrown in Vero cells without drug pressure for up to 2 weeks. Subsequently, they were harvested and subjected to

drug efficacy tests on HFF as described. Non-resistant tachyzoites were processed identically, and these control *Besnoitia* were strongly susceptible to treatment with 100 nM BPQ (Fig. 4). However, *Besnoitia* that were adapted to 5 μM or 10 μM BPQ (R_5 or R_10; i.e. adapted to 5 or 10 μM) had maintained their strongly decreased susceptibility and resumed proliferation even at 5 μM BPQ, namely to 28% of the control value for R_5 and to 58% of the control value for R_10 (Fig. 5). These effects were statistically significant (Student's *t*-test, $P < 0.01$).

Visualization of these adapted *Besnoitia* tachyzoites by TEM (Fig. 4) showed that they, similar to non-adapted tachyzoites, were localized within a parasitophorous vacuole, underwent endodyogeny and contained micronemes, rhoptries and dense granules similar to non-adapted tachyzoites (Fig. 2A). However, a prominent feature of these BPQ adapted parasites was the apparent lack of an organized mitochondrial matrix. In some cases, the matrix was completely lacking, and mitochondria were filled with
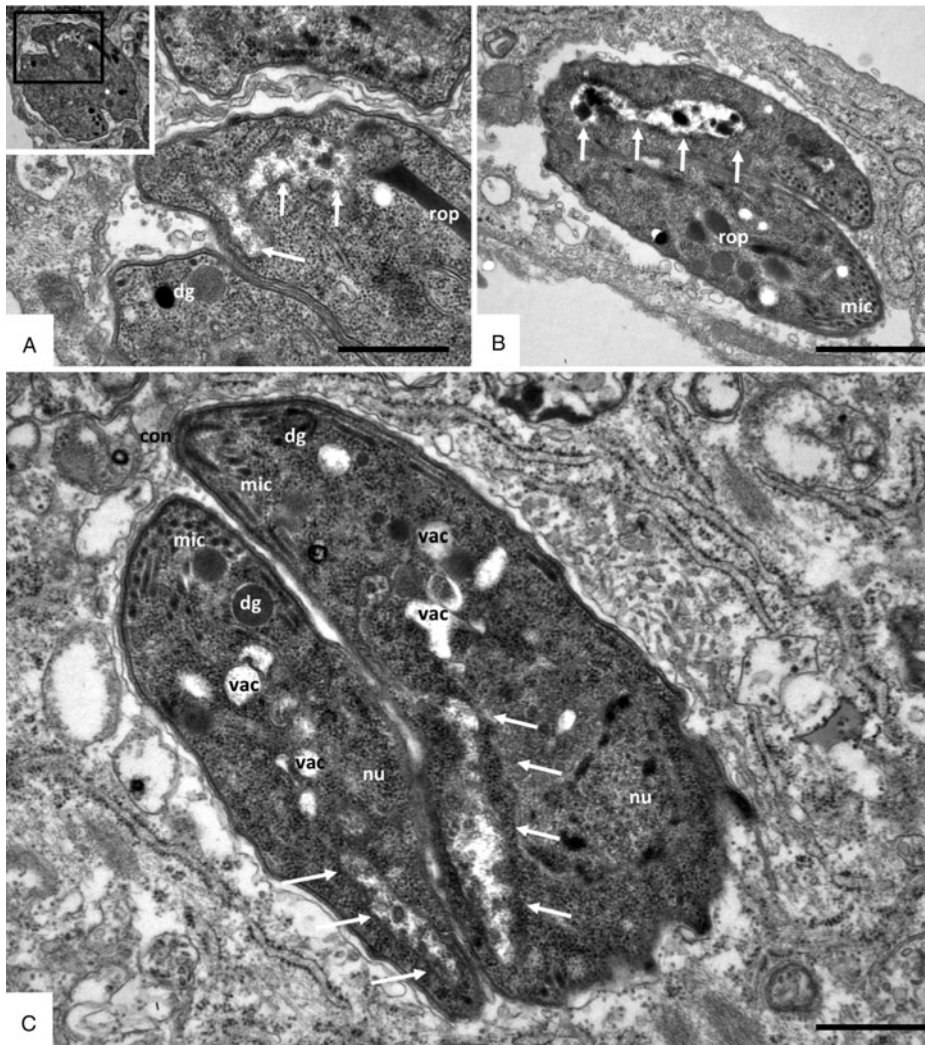
Fig. 4. Ultrastructure of BPQ-adapted *Besnoitia besnoiti* strains grown in HFF after stabilate preparation and re-introduction into culture for 2 weeks. Panels (A, B) show R_5 and (C) shows the R_10 strain. The insert in (A) is a low magnification view, and the respective boxed area is shown enlarged. Parasites appear structurally normal, except for the absence of a visible mitochondrial matrix [indicated by arrows in (A), (B) and (C)]. nu, parasite nucleus; dg, dense granules; mic, micronemes; rop, rhoptries; con, conoid; vac indicates cytoplasmic vacuoles. Bars in (A) = 0.66 μM; (B) = 1 μM; (C) = 0.6 μM.

amorphous electron-dense material of unknown composition (Fig. 4A, B). In other instances, the matrix was partially present, most notably in the periphery of the mitochondria, but no matrix was discernible in the central part of the mitochondrium (Fig. 4C). Tachyzoites that had adapted to increased BPQ concentrations also exhibited frequent vacuolization.

Discussion

The present study shows that in 4-day treatment assays BPQ is highly efficacious against *B. besnoiti* tachyzoites with IC$_{50}$ values in the nanomolar range. Thus, *in vitro*, BPQ is more effective in terms of proliferation inhibition than thiazolides (Cortes *et al.*, 2007*a*) and arylimidamides (Cortes *et al.*, 2011), two classes of compounds investigated in previous screens. BPQ was also more effective than a range of BKIs recently studied (Jimenez-Melendez *et al.*, 2017), including the previously characterized BKI-1294. The IC$_{50}$ for BKI-1294 against *B. besnoitia* obtained in this study (0.12 ± 0.03 μM) was in the same range but slightly higher than the one (0.045 μM) reported by Jimenez-Melendez *et al.* (2017). Such differences can be attributed to the different isolates, and also different host cells, that were used, and most likely also culture conditions that vary between the laboratories. BKI-1294 impairs the activity of CDPK1, which is an essential kinase involved in host cell invasion and egress of apicomplexan parasites (Lourido *et al.*, 2010). In *T. gondii*, *N. caninum* and *B. besnoiti*, BKI-1294 treatment of already intracellular parasites leads to the formation of multinucleated complexes (Ojo *et al.*,

2014; Winzer *et al.*, 2015; Jimenez-Melendez *et al.*, 2017). Thus, besides interference in invasion and egress, BKI-1294 also impairs processes involved in the completion of the cell cycle and formation of daughter zoites. In contrast, the major processes involved in the mechanism of action of BPQ are not those associated with host cell invasion, but BPQ rapidly affects the structural integrity of intracellular parasites and thus interferes in intracellular proliferation. After 24 h of treatment, the primary indications of interference that are noted are rather subtle changes in the matrix of the mitochondrium of *B. besnoitia* tachyzoites. Other alterations that were visualized at later time points, such as increased cytoplasmic vacuolization, fragmentation of the nucleus and a general build-up of amylopectin granules in the cytoplasm of these parasites, have also been described after treatments with nitazoxanide and pentamidine derivatives (Cortes *et al.*, 2007*a*; Cortes *et al.*, 2011), and also during treatment with bumped kinase inhibitors recently reported by Jimenez-Melendez *et al.* (2017). Thus, these features are most likely associated with general physiological stress and upcoming cell death rather than being specific indicators of the mechanism of action of BPQ. The rapid action of BPQ in good agreement with previous findings on *Theileria* where gene expression of the intracellular parasite decreases already after 2 h of BPQ treatment and increased vacuolization of the parasite cytoplasm is seen already after 6 h (Hostettler *et al.*, 2014). There is, however, a marked difference in *N. caninum*, which is closely related to *B. besnoiti*, where ultrastructural changes upon treatment with 1 μM BPQ were reported to be clearly visible only after 3 days of treatment (Müller *et al.*, 2015). In 4-day
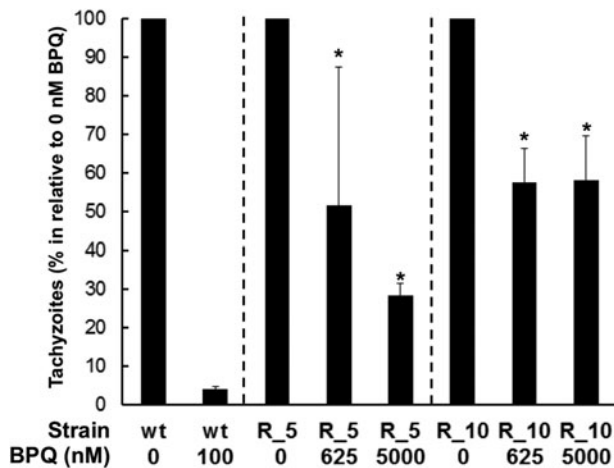
Fig. 5. Effects of BPQ on the intracellular proliferation of normal and BPQ-adapted *Besnoitia besnoiti* tachyzoites in HFF. HFF monolayer were infected with either normal (wt = *B. besnoitii* never exposed to BPQ) or BPQ-adapted tachyzoites [R_5 and R_10 are two strains that still underwent proliferation in the presence of 5 and 10 μM BPQ (see Table 1)]. BPQ was added at 0, 625 or 5000 nM, and proliferation tachyzoites were quantitatively assessed by qPCR after 4 days. Mean ± S.E. values correspond to four replicates. Asterisks indicate highly significant growth ($P < 0.01$) in relation to the control treated with 100 nM BPQ.

proliferation assays, BPQ inhibited *N. caninum* tachyzoite replication in a similar manner as *B. besnoiti* ($IC_{50} = 4.9$ nM; MIC = 100 nM). In the long term, tachyzoites also adapted and resumed proliferation in the presence of 100 nM BPQ after 20 days. However, for *N. caninum* parasiticidal activity was noted after 9 days of culture with 0.5 μM or 6 days with 1 μM BPQ.

Treatment of *B. besnoiti*-infected host cells with increasing concentrations of BPQ starting at 100 nM and going up to 1000 nM leads to a rapid adaptation of these parasites with unique features, showing that BPQ, while being highly effective in the short term (i.e. within the first few days of treatment), does not act parasiticidal, even after several days of continuous treatment at concentrations of up to 10 μM. In two independent experiments, we have selected *B. besnoiti* strains proliferating in the presence of 5 μM BPQ, thus at a concentration nearly three orders of magnitude above the IC_{50}. Tachyzoites of these strains maintained their decreased susceptibility to BPQ even after freeze–thawing of stabilates and re-introduction into culture for 2 weeks. When inspected by TEM, we found that these parasites exhibited the overall normal features of apicomplexan parasites, with the exception of distinct structural alterations in the mitochondrium, which was lacking an intact matrix, partially devoid of content or filled with electron dense inclusions of unknown nature. At this point, it is not clear whether the mitochondrium in these parasites is functional or not. Clearly, these structural alterations do not automatically imply that all mitochondrial activities are impaired, thus the question of how these parasites can overcome this obvious structural defect needs to be investigated in the future.

Previous studies in *Theileria* showed that point mutations of the cytochrome *b*-gene are associated with resistance to BPQ (Sharifiyazdi *et al.*, 2012; Mhadhbi *et al.*, 2015). The cytochrome-bc1-complex (respiratory Complex III) transfers electrons from coenzyme Q to cytochrome *c* (Mitchell, 1975). BPQ could block this transfer, thereby causing the relocation of electrons to other biomolecules generating free radicals and reactive oxygen species (ROS) detrimental to the parasite. By point mutations on the target or – like in this case – by the disappearance of structurally intact mitochondria, the parasite could overcome this effect of the drug. A plethora of studies with yeast has revealed the role of

mitochondria in ROS-dependent cell death [see Pereira *et al.* (2008) and references therein]. In one case, the deleterious effects of ROS production are counteracted by the induction of petite mutants lacking mitochondria (Granot *et al.*, 2003).

The cytochrome-bc1-complex may not be the only target for BPQ, as shown more recently by the identification of a secreted prolyl-isomerase of *T. annulata* as another potential target (Marsolier *et al.*, 2015). Inhibition of this enzyme would, however, cause rather delayed effects, as seen previously for *N. caninum*, due to the accumulation of misfolded effector proteins whereas inhibition of the respiratory chain is in good agreement with the more rapid effects observed in *Besnoitia* and with the high adaptation potential by the absence of functional mitochondria. Since *Besnoitia* tachyzoites exhibiting strongly altered mitochondria are fully viable (albeit with a slower proliferation rate), their energy metabolism must be entirely depending on glycolysis. Moreover, the intermediate metabolites provided by intact mitochondria must be scavenged from the host cell. Certainly, more research is needed to elucidate this mechanism that leads to this marked tolerance to increased drug concentrations.

Our observations could potentially discourage the repurposing of BPQ for the treatment of besnoitiosis in cattle. However, more recently other compounds such as several BKIs were shown to exhibit excellent efficacy *in vivo* in both pregnant and non-pregnant mice infected with *N. caninum* (Ojo *et al.*, 2014; Winzer *et al.*, 2015; Müller *et al.*, 2017*a*), and BPQ was also shown to be highly effective in a pregnant mouse model for congenital toxoplasmosis, clearly limiting cerebral infection and disease in dams and in offspring mice (Müller *et al.*, 2017*b*). Thus, BPQ could be potentially useful for the treatment of besnoitiosis when applied in combination with another highly effective compound that exerts another mechanism of action.

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