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

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

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Summary

We here assessed the *in vitro* efficacy of the naptho-quinone buparvaquone (BPQ) against *B. besnoiti* tachyzoites *in vitro*. BPQ is currently licensed for the treatment of theileriosis in cattle. 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 125 nM BPQ. Exposure to 1 µM BPQ lead 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 µM BPQ, albeit at a lower rate. These BPQ-adapted parasites maintained this lower susceptibility to BPQ treatment after freeze-thawing, and inspection by TEM revealed that they underwent proliferation in the absence of structurally intact mitochondria.

KEY FINDINGS

- In short term in vitro assays (4 days), the napthoquinone buparvaquone inhibits proliferation of *Besnoitia besnoiti* tachyzoites (IC50 = 10 nM)
- Ultrastructural investigations indicate that the mitochondrion is the primary target of the drug
- *B besnoiti* rapidly adapts to increased BPQ concentrations, and maintains proliferation at a concentration of up to 10 µM.
- Resistance / adaptation is maintained by freeze-thawing of strains adapted to 5 and 10 µM BPQ
- BPQ-adapted proliferating strains exhibit mitochondria that are structurally severely impaired.

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 of 10 species. *B. 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, formation of tissue cysts takes place, mainly in mesenchymal host cells in the mucous membranes, the genital tract and superficial skin layers. During the acute phase of infection, clinical symptoms are hyperthermia, weight loss, nasal and ocular discharge, and fibrinoid necrotic vascular lesions causing congestion and infarcts. Moreover, nephritic syndromes leading to hyperproteinuria, hypoalbuminaemia and mild

leukocytosis may cause the death of infected animals (Dubey *et al.*, 2013). Hyperthermia could also lead to abortion (Basso *et al.*, 2011; Lesser *et al.*, 2012; Alvarez-Garcia *et al.*, 2013). The chronic phase of bovine besnoitiosis is characterized by scleroderma, scars, nodules on udders and progressive thickening and wrinkling of the skin. Infection of male genital organs may result in sterility (Frey *et al.*, 2013). One form of transmission of bovine besnoitiosis occurs mechanically from cattle to cattle, by hematophagous insects (Lienard *et al.*, 2011) such as horse flies and the stable fly *Stomoxys calcitrans* (Bigalke, 1968*)*, without any sexual cycle involved. Other ways of transmission are from cow to cow, via the process of mating, or through medical devices (Frey *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 (Jacquiet *et al.*, 2010), and South America (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).

There is no chemotherapy available for the treatment of affected animals (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). Oxytetracyline has prevented death of infected gerbils, but only when administered simultaneously with the parasites (Shkap *et al.*, 1987c). 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.*, 1987b). However, only a limited number of animals was used and these studies were carried out under highly variable experimental conditions. The fact that assessments were mainly based on clinical features and histopathology

did not allow definitive conclusions. 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 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), commercially available as Butalex™, is another interesting candidate for drug repurposing (McHardy & Morgan, 1985). 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, it has a plasma half-life of at least 7 days, and a low toxicity (LD_{50} in rats >8000mg/kg). Besides an outstanding activity against *T. parva and T. annulata* (Hashemi-Fesharki, 1991; Hemphill & Croft, 1997; Hostettler *et al.*, 2014), BPQ is also active against other protozoan parasites including *Leishmania spp*. (Croft *et al.*, 1992), *Theileria equi (*formerly named *Babesia equi*) (Zaugg & 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; Müller *et al.*, 2016; Müller *et al.*, 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, 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 sulfoxide (DMSO) at -20°C.

Host cell cultivation and maintenance of B. besnoiti tachyzoites.

Human foreskin fibroblasts (HFF) were maintained in Dulbecco's Modified Eagle Medium (DMEM), and Vero cells were cultured in RPMI-1640 medium, both with phenol red supplemented with 10% heat inactivated and sterile filtrated fetal calf serum (FCS), 50 U of penicillin/ml, and 50 µg streptomycin/ml (= 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, 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 6-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 concentrations ranging 0 to 5 µM. Controls received the corresponding amounts of DMSO. HFF were then infected with 5 x 10^4 freshly purified *B. besnoitia* tachyzoites, in a total volume of 5 ml. At selected time points as indicated below, 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. DNA purification was performed employing the DNeasy Blood & Tissue Kit (Qiagen, Basel, Switzerland) according to the standard protocol suitable for animal cells. *B. 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 24 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. Postfixation 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 80kV.

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 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 one month prior to further assessments.

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. Subsequently they were harvested and 10^4 tachyzoites were seeded onto HFF monolayer grown in 6 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 80kV.

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 x ln(drug concentration) + b and subsequent regression analysis by the corresponding software tool contained in the Excel software package (Microsoft, Seattle, WA, USA).

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 d post-infection (p.i.), reaching the exponential phase after 4 days. In the cells treated with BKI-1294, no proliferation was visible (Fig. 1 A). Since after 4 days, the infected monolayers showed first lesions and the numbers of extracellular parasites started to 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, and virtually complete inhibition was obtained with 125 nM BPQ (Fig. 1C).

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

B. besnotia infected HFF were exposed to 1 µM BPQ for 24, 48 and 96 h, or were maintained in the absence of BPQ, and processed for TEM. Tachyzoites, cultured in the absence of BPQ (Fig. 2) 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 (Fig. 1). 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. 2 B). Exposure to 1 µM BPQ for 24 h (Fig. 3A, B) 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. 3B). Changes became more profound after 48 h of BPQ treatment (Fig 3C, D), 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 discernible, and parasites kept residing within a parasitophorous vacuole. Upon treatment for 96 h (Fig. 4), 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 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 BPQ at 24 h post infection (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. At these concentrations, proliferation of parasites was still noted after two 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 one 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. 5). However, *Besnoitia* that were adapted to 5 µM or 10 µM BPQ (R_5 or R_10; i. e. resistant at 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).

Visualization of these adapted *Besnoitia* tachyzoites by TEM (Fig. 6) 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. 2). 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 amorphous electron-dense material of unknown composition (Fig. 6 A, 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. 6 C). Tachyzoites that had adapted to increased BPQ concentrations also exhibited frequent vacuolization.

DISCUSSION

The present study shows that in four-day treatment assays BPQ 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.*, 2007a) 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. 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.*, 2007a; Cortes *et al.*, 2011), and are most likely associated to 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

298 clearly visible only after 3 days of treatment (Müller *et al.*, 2015). In four-day proliferation
299 assays, BPQ inhibited *N. caninum* tachyzoite replication in a similar manner as *B. besnoiti* (IC_{50}
300 = 4.9 nM; MIC = 100 nM). In the long term, tachyzoites also adapted and resumed proliferation
301 in the presence of 100 nM BPQ after 20 days. However, for *N. caninum* parasiticidal activity was
302 noted after 9 days of culture with 0.5 µM or 6 days with 1 µM BPQ.

303 Treatment of *B. besnoiti*-infected host cells with increasing concentrations of BPQ
304 starting at 100nM and going up to 1000 nM leads to a rapid adaptation of these parasites with
305 unique features, showing that BPQ, while being highly effective in the short term (i.e. within the
306 first few days of treatment), does not act parasiticidal, even after several days of continuous
307 treatment at concentrations of up to 10 µM. In two independent experiments, we have selected *B.*
308 *besnoiti* strains proliferating in the presence of 5 µM BPQ, thus at a concentration nearly 3 orders
309 of magnitude above the IC_{50}. Tachyzoites of these strains maintained their decreased
310 susceptibility to BPQ even after freeze-thawing of stabilates and re-introduction into culture for 2
311 weeks. When inspected by TEM, we found that these parasites exhibited the overall normal
312 features of apicomplexan parasites, with the exception of distinct structural alterations in the
313 mitochondrium, which was lacking an intact matrix, partially devoid of content or filled with
314 electron dense inclusions of unknown nature. At this point, it is not clear whether the
315 mitochondrium in these parasites is functional or not. Clearly, these structural alterations do not
316 automatically imply that all mitochondrial activities are impaired, thus the question how these
317 parasites can overcome this obvious structural defect needs to be investigated in the future.

318 Previous studies in *Theileria* showed that point mutations of the cytochrome b-gene are
319 associated with resistance to BPQ (Sharifiyazdi *et al.*, 2012; Mhadhbi *et al.*, 2015). The
320 cytochrome-bc1-complex (respiratory Complex III) transfers electrons from coenzyme Q to
321 cytochrome c (Mitchell, 1975). BPQ could block this transfer, thereby causing the relocation of

322 electrons to other biomolecules generating free radicals and reactive oxygen species (ROS)
323 detrimental to the parasite. By point mutations on the target or – like in this case – by
324 disappearance of structurally intact mitochondria, the parasite could overcome this effect of the
325 drug. A plethora of studies with yeast has revealed the role of mitochondria in ROS dependent
326 cell death (see (Pereira *et al.*, 2008) and refs therein). In one case, the deleterious effects of ROS
327 production are countered by the induction of petite mutants lacking mitochondria (Granot *et al.*,
328 2003).

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

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

could be potentially useful for treatment of besnoitiosis when applied in combination with another highly effective compound that exerts another mechanism of action.

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REQUIRED STATEMENTS

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Conflicts of interest

None

Ethical standards

Not applicable

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FIGURE LEGENDS

Fig. 1. Effects of BKI-1294 (1294) and BPQ on intracellular proliferation of *Besnoitia besnoitii* tachyzoites in human foreskin fibroblasts (HFF). HFF were grown to confluence in 6-well tissue culture plates, supplemented with BKI-1294, BPQ, or with dimethyl sulphoxide (DMSO) as a solvent control and were immediately infected with *B. besnoitii* tachyzoites ($2x10^4$ 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. *B. besnoitii* tachyzoites were quantified by qPCR. Mean ± SE values correspond to four replicates.

Fig. 2. Ultrastructure of *B. besnoiti* tachyzoites. Infected HFF were fixed and processed for TEM on day 3 post-infection and treatment with DMSO but no drug. (A) Low magnification view of several tachyzoites located within the HFF cytoplasm, the boxed area in (A) is shown enlarged in (B). (C) Parasitophorous vacuole within the HFF cytoplasm containing proliferating tachyzoites. Note in (B) and (C) that mitochondria (mito) contain an electron dense matrix and clearly discernible cristae; nu = parasite nuclei; mic = micronemes; rop = rhoptries; dg = dense granules. Bars in (A) = 1.2 µm; in (B) = 0.26 µm; in (C) = 0.6 µm

Fig. 3. Ultrastructural changes in *B. besnoiti* tachyzoites visualized during the first 2 days of BPQ treatment. Infected HFF were exposed to 1 µM BPQ for 24 h (A, B) or 48 h (C, D). The boxed areas in (A) and (C) are enlarged in (B) and (C), respectively. Note the progressive changes in the mitochondrial matrix (mito) occurring with increasing time of drug exposure (B, C), while the host cell mitochondria (h_mito) appear unaffected (A). nu = parasite nuclei; mic =

546 micronemes; rop = rhoptries; con = conoid. Bars in (A) = 0.6 µm; in (B) = 0.32 µm; in (C) = 2.2
547 µm; in (D) = 0.35 µm.
548
549 **Fig. 4. Ultrastructural alterations in *B. besnoiti* tachyzoites seen after 4 days of treatment**
550 **with BPQ.** The boxed areas in (A) and (C) are enlarged in (B) and (C), respectively. Note the
551 profound BPQ-induced changes in tachyzoites, which are located within a parasitophorous
552 vacuole (pv), including large numbers of amylopectin granules (apg) in (B), and the
553 fragmentation of the cytoplasm in (D). nu = parasite nucleus, H-nu = host cell nucleus; dg =
554 dense granules; con = conoid; mic = micronemes. Bars in (A) = 1.5 µm; in (B) = 0.3 µm; in (C) =
555 1.5 µm; in (D) = 0.3 µm.
556
557 **Fig. 5. Effects of BPQ on intracellular proliferation of normal and BPQ-adapted *B. besnoiti***
558 **tachyzoites in HFF.** HFF monolayer were infected with either normal (wt = *B. besnoitii* never
559 exposed to BPQ) or BPQ-adapted tachyzoites (R_5 and R_10 are two strains that still underwent
560 proliferation in the presence of 5 and 10 µM BPQ (see Table 1)). BPQ was added at 0, 625 or
561 5000 nM, and proliferation tachyzoites was quantitatively assessed by qPCR after 4 days. Mean ±
562 SE values correspond to four replicates.
563
564 **Fig. 6. Ultrastructure of BPQ-adapted *B. besnoiti* strains grown in HFF after stabilate**
565 **preparation and re-introduction into culture for 2 weeks**. (A and B) show R_5 and (C) shows
566 the R_10 strain. The insert in (A) is a low magnification view, and the respective boxed area is
567 shown enlarged. Parasites appear structurally normal, except for the absence of a visible
568 mitochondrial matrix (indicated by arrows in (A), (B) and (C)). nu = parasite nucleus; dg = dense

569 granules; mic = micronemes; rop = rhoptries; con = conoid; vac indicates cytoplasmic vacuoles.

570 Bars in (A) = 0.66 μm; (B) = 1 μm; (C) = 0.6 μm.

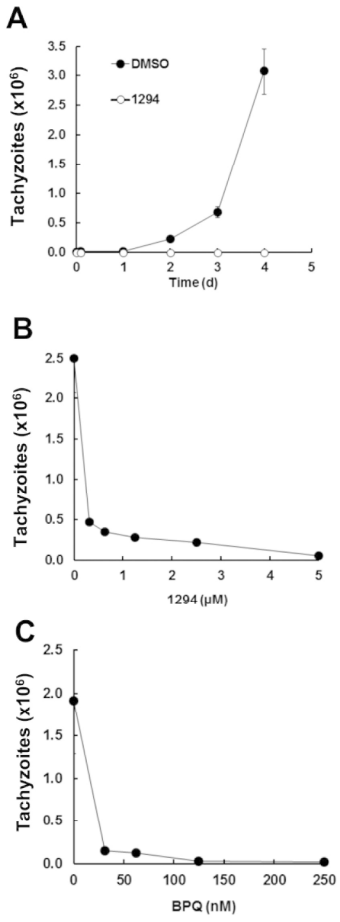

Fig.1

Figure 1

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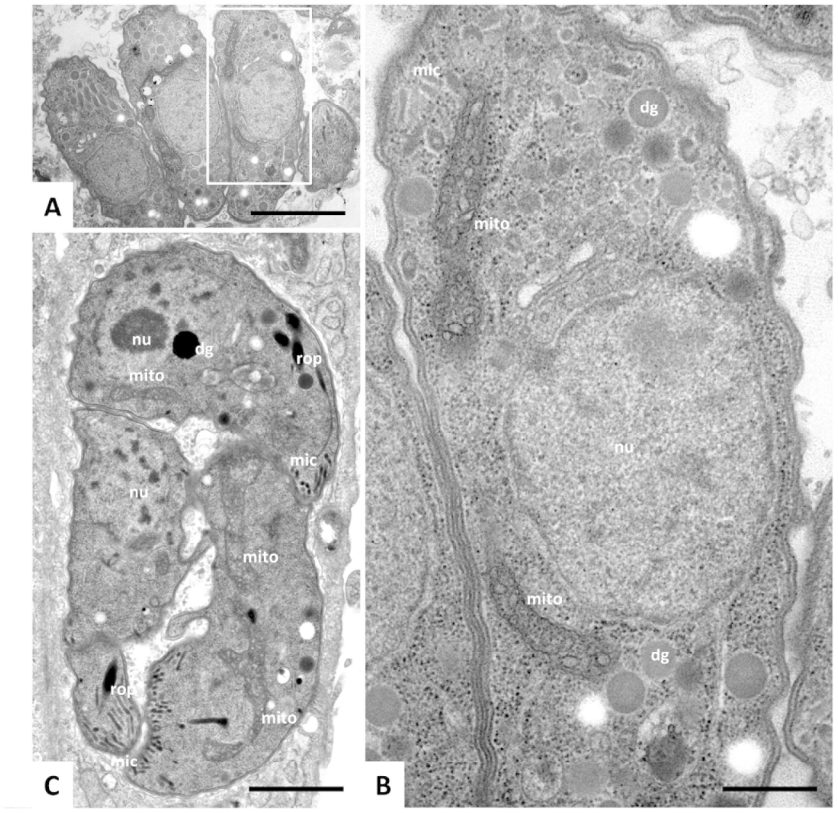

Fig.2

Figure 2

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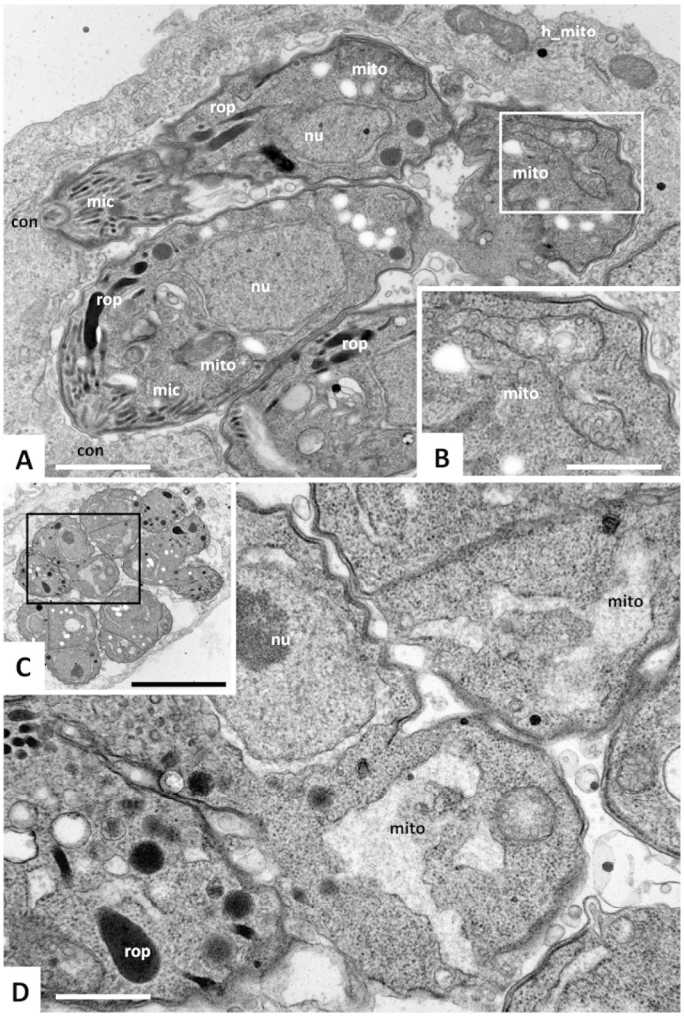

Fig.3

Figure 3

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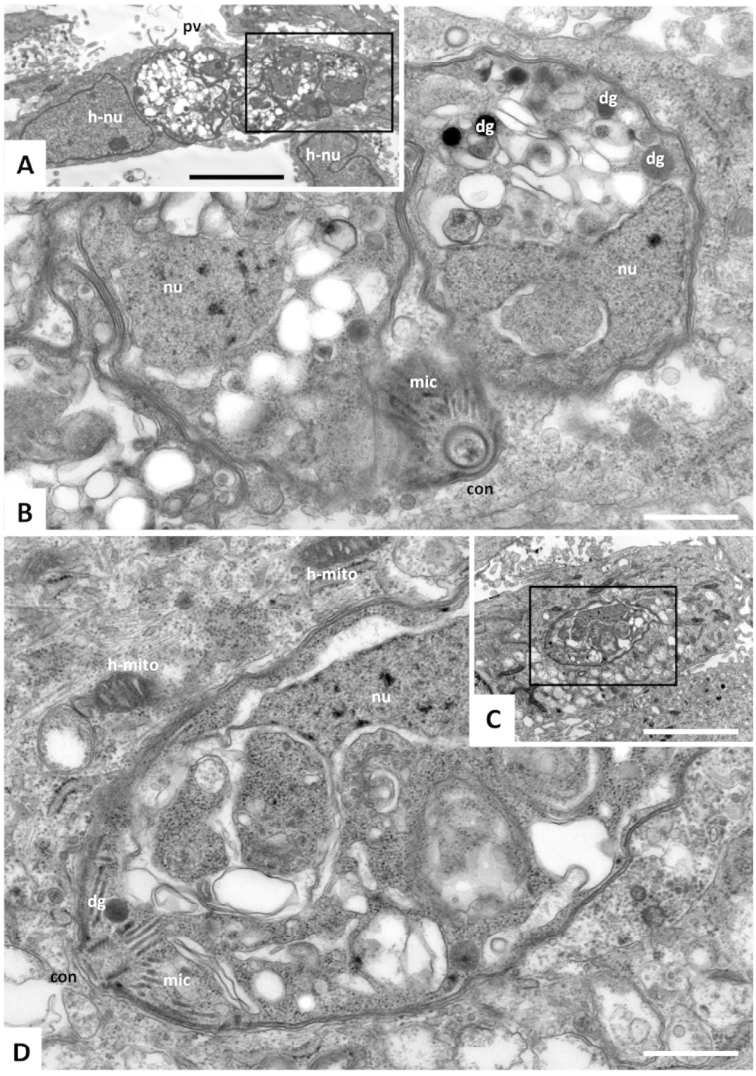

Fig.4

Figure 3

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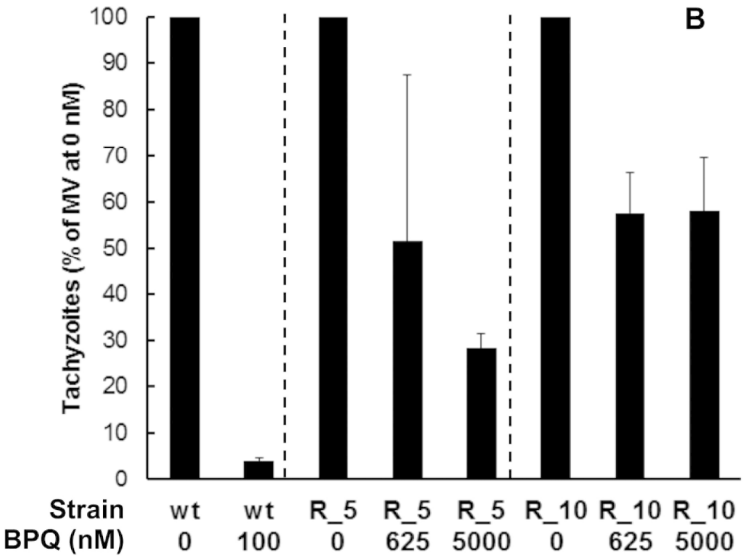

Fig.5

Figure 5

190x254mm (300 x 300 DPI)

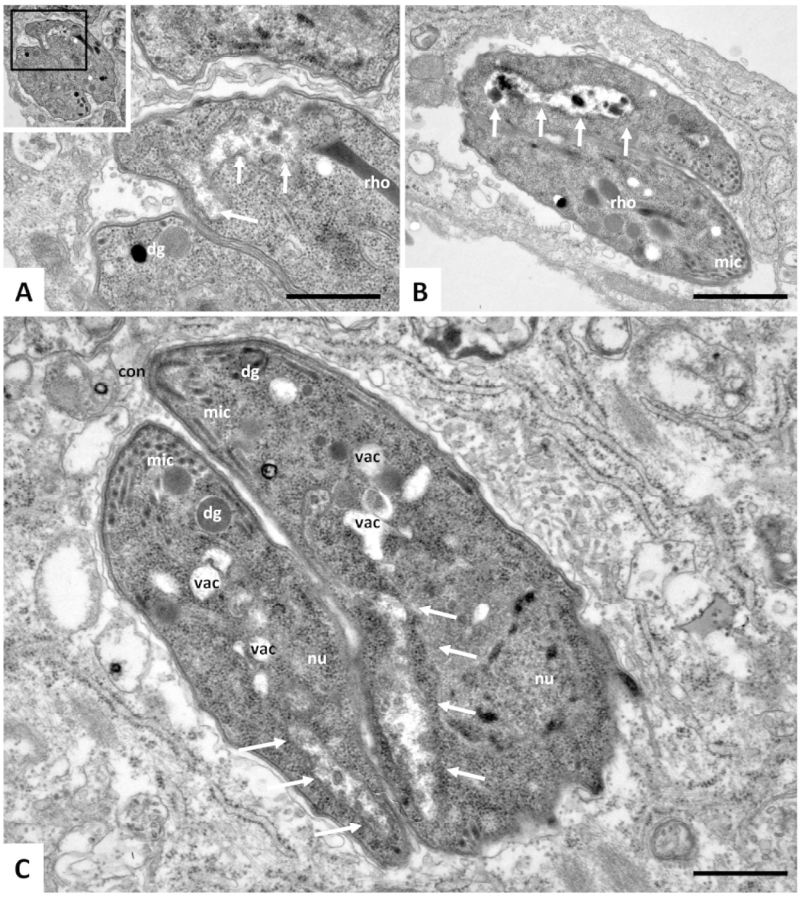

Fig.6

Fig. 6

190x254mm (300 x 300 DPI)