The adaptive potential of a survival artist: characterization of the *in vitro* interactions of *Toxoplasma gondii* tachyzoites with di-cationic compounds in human fibroblast cell cultures

CHRISTIAN KROPF[1], KARIM DEBACHE[1], CHRISTOPH RAMPA[1], FABIENNE BARNA[1], MICHELLE SCHORER[1], CHAD E. STEPHENS[2], MOHAMED A. ISMAIL[3,4], DAVID W. BOYKIN[3] *and* ANDREW HEMPHILL[1]*

[1] *Institute of Parasitology, Vetsuisse Faculty, University of Berne, Switzerland*
[2] *Department of Chemistry and Physics, Augusta State University, Augusta, Georgia 30904-2200, USA*
[3] *Department of Chemistry, Georgia State University, PO Box 4098, Atlanta Georgia, USA*
[4] *Department of Chemistry, College of Science, King Faisal University, PO Box, Hofuf 31982, Saudi Arabia*

(*Received 30 June 2011; revised 29 August 2011; accepted 5 September 2011; first published online 19 October 2011*)

SUMMARY

The impact of di-cationic pentamidine-analogues against *Toxoplama gondii* (Rh- and Me49-background) was investigated. The 72 h-growth assays showed that the arylimidamide DB750 inhibited the proliferation of tachyzoites of *T. gondii Rh* and *T. gondii Me49* with an IC_{50} of 0·11 and 0·13 μM, respectively. Pre-incubation of fibroblast monolayers with 1 μM DB750 for 12 h and subsequent culture in the absence of the drug also resulted in a pronounced inhibiton of parasite proliferation. However, upon 5–6 days of drug exposure, *T. gondii* tachyzoites adapted to the compound and resumed proliferation up to a concentration of 1·2 μM. Out of a set of 32 di-cationic compounds screened for *in vitro* activity against *T. gondii*, the arylimidamide DB745, exhibiting an IC_{50} of 0·03 μM and favourable selective toxicity was chosen for further studies. DB745 also inhibited the proliferation of DB750-adapted *T. gondii* (IC_{50} = 0·07 μM). In contrast to DB750, DB745 also had a profound negative impact on extracellular non-adapted *T. gondii* tachyzoites, but not on DB750-adapted *T. gondii*. Adaptation of *T. gondii* to DB745 (up to a concentration of 0·46 μM) was much more difficult to achieve and feasible only over a period of 110 days. In cultures infected with DB750-adapted *T. gondii* seemingly intact parasites could occasionally be detected by TEM. This illustrates the astonishing capacity of *T. gondii* tachyzoites to adapt to environmental changes, at least under *in vitro* conditions, and suggests that DB745 could be an interesting drug candidate for further assessments in appropriate *in vivo* models.

Key words: *Toxoplasma gondii*, di-cationic pentamidine analogues, arylimidamides, proliferation, tachyzoites, *in vitro* models.

INTRODUCTION

The obligatory intracellular apicomplexan parasite *Toxoplasma gondii* is one of the most successful parasites on this planet. It infects members of *Felidae* as definitive hosts, and has a wide range of intermediate hosts, including warm-blooded animals and humans (Tenter *et al*. 2000; Dabritz and Conrads, 2010). Approximately one third of the human population is infected with *T. gondii* worldwide, but despite the high prevalence, in the vast majority of cases disease is benign and asymptomatic (Barratt *et al*. 2010). However, clinical problems can arise in 2 situations. First, in individuals with an immature immune system or in immune-compromised patients infection can lead to toxoplasmic encephalitis (TE) or even death. TE occurs as a result of the reactivation of brain cysts that encapsulate slowly proliferating bradyzoites, which then undergo bradyzoite-to-tachyzoite stage conversion, and is common in chronically infected patients, which receive immunosuppressive therapy or suffer from human immunodeficiency virus (HIV) infection (Tenter *et al*. 2000; Barratt *et al*. 2010; Henriquez *et al*. 2010). Secondly, congenital toxoplasmosis poses a major health risk upon primary maternal infection during pregnancy, leading to placental or fetal infection (or both), which often results in abortion or damage of the fetal tissues (Feldman *et al*. 2010). In addition, *T. gondii* is also a highly significant economic and veterinary medical concern in the livestock industry, and as such represents an important zoonosis (Dubey, 2009).

Toxoplasmosis begins generally mildly or is asymptomatic, with an acute phase in which tachyzoites invade the cells, divide rapidly, and disseminate throughout the host. With the onset of the host immune response, the proliferative tachyzoite stage is replaced by the quiescent bradyzoites, which form tissue cysts predominantly located in the central

* Corresponding author: Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland. Tel: +41 31 631 2384. Fax: +41 31 631 2477. E-mail: andrew.hemphill@vetsuisse.unibe.ch

Parasitology (2012), **139**, 208–220.
doi:10.1017/S0031182011001776

nervous system (CNS) and muscle tissue, where they do not cause apparent immunopathology and can persist for many years, up to a lifetime (Carruthers and Suzuki, 2007; Laliberté and Carruthers, 2008). The chemotherapeutical options for the treatment of toxoplasmosis are limited. A synergistic combination therapy comprised of sulfadiazine and pyrimethamine, targeting the synthesis and the reduction of folic acid in tachyzoites, has been shown to be the most efficient option (Montoya and Liesenfeld, 2004). However, adverse reactions can occur, which include haematological toxicity and hypersensitivity. Another option employs clindamycin-pyrimethamine combination therapy, but this treatment is also prone to side effects (Pereira-Chioccola *et al*. 2009). The major problem in the treatment of *T. gondii* infection is the non-effectiveness of compounds against bradyzoites that are enclosed in tissue cysts (Barratt *et al*. 2010).

Pentamidine and its analogues represent a class of broad-spectrum antimicrobial compounds, with activities against a wide range of intracellular and extracellular protozoan parasites (Soeiro *et al*. 2005; Wilson *et al*. 2008; Buckner and Navabi, 2010). Since its discovery, pentamidine has been successfully applied to treat African trypanosomiasis, leishmaniasis, and malaria in humans, and the pentamidine-derivative diminazene aceturate is commonly used for trypanosome chemotherapy in livestock (Werbovetz, 2006). More recently, novel analogues, known as arylimidamides, with a more favourable pharmacokinetic profile, improved bioavailability and lower host toxicity were shown to be effective against *Leishmania donovani* and *Trypanosoma cruzi in vitro* and *in vivo* (Wang *et al*. 2010; Batista *et al*. 2010*a*).

The *in vitro* efficacy of pentamidine and some pentamidine analogues against *T. gondii* has been demonstrated previously (Lindsay *et al*. 1991), and proliferation inhibitory properties were reported at concentrations of around 10 μg/ml. More recently, Leepin *et al*. (2008) demonstrated the *in vitro* efficacy of a set of arylimidamides (DB750, DB766, DB786, DB811, formerly named reversed diamidines), which inhibited the proliferation of *T. gondii* and the closely related *Neospora caninum* with IC_{50}s at submicromolar concentrations ranging between 0·16 and 0·66 μM (=0·14–0·5 μg/ml), indicating the potential of these di-cationic compounds, especially DB750 and DB786, for chemotherapeutical purposes. DB811 was recently reported to inhibit the proliferation of the related apicomplexan *Besnoitia besnoiti* in Vero cells at an IC_{50} of 0·08 μM (Cortes *et al*. 2011).

Here, we further explore the *in vitro* characteristics of DB750 in relation to *T. gondii*. We demonstrate that, despite low IC_{50} in 3-day growth assays, *Toxoplasma* tachyzoites could readily adapt to this compound during culture within a few days, while this was not possible for the closely related *N. caninum*. Screening of additonal di-cationic compounds for anti-*Toxoplasma* activity lead to the identification of DB745, which inhibited the proliferation of *T. gondii* tachyzoites at an IC_{50} of 0·03 μM. Also, the effects of DB745 and DB750 were evaluated by transmission electron microscopy analysis of DB750-adapted and non-adapted *T. gondii* parasites.

MATERIALS AND METHODS

Culture media, buffers and reagents

Unless otherwise stated, all tissue-culture media were purchased from Gibco-BRL (Zurich, Switzerland) and biochemical reagents were from Sigma (St Louis, MO, USA). The di-cationic compounds used in this study were synthesized at the Department of Chemistry and Center for Biotechnology and Drug Design, Georgia State University, USA. They were kept as a dry powder or as stock solutions of 1 mg/ml in dimethyl sulfoxide and were stored at −20 °C.

Cell culture and parasite purification

Vero cells were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 50 U of penicillin/ml, and 50 μg of streptomycin/ml at 37 °C with 5% CO_2 in tissue-culture flasks and were trypsinized 3 times a week. Human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified Eagles's medium (DMEM) with 10% FCS, 50 U of penicillin/ml, and 50 μg of streptomycin/ml at 37 °C with 5% CO_2 in tissue-culture flasks. Cultures were trypsinized once a week. *Toxoplasma gondii Me49, T. gondii Rh, T. gondii-β-gal*, (a transgenic strain with Rh-background expressing β-galactosidase; McFadden *et al*. 1997) were cultured in Vero cells (Scheidegger *et al*. 2005). Intracellular parasites were harvested by trypsinization of infected Vero cells, followed by repeated passages through a 25-gauge needle at 4 °C, and separation from cell debris on a Sephadex-G25 column as described previously (Hemphill, 1996). Purified tachyzoites were used to infect HFF monolayers as described below.

In vitro *drug treatment assays employing T. gondii Me49 and DB750-adapted* T. gondii*_DB750 by quantitative real time PCR*

HFF cells were grown to confluency in 24-well tissue-culture plates, either adherent to glass coverslips or directly on the plastic surface. Each well was infected with 5×10^4 cell culture-derived and freshly purified *T. gondii* tachyzoites re-suspended in DMEM containing 5% FCS, 50 U of penicillin/ml, and 50 μg of streptomycin/ml (=full medium). Following incubation for 1 h at 37 °C in a 5% CO_2 athmosphere, unbound parasites were removed by

Table 1. IC_{50} determination of selected compounds against different *Toxoplasma gondii* strains, and assessment of HFF toxicity

(IC50 s are given in μM +/− S.D. Three strains were evaluated: *T.g. β-gal* is a transgenic strain (Rh-background) that expresses β-galactosidase constitutively. *T.g. Me49* is a cyst-forming strain, *T.g._DB750* was generated by culture of *T.g. Me49* in the presence of 1·2 μM DB750. (–) indicates not tested.)

Compound-name	IC_{50} (μM) *T.g. β-gal* colorimetric assay	IC_{50} (μM) *T.g. Me49* qPCR	IC_{50} (μM) *T.g._DB750* qPCR	HFF toxicity (μM) light microscopy
DB750	0·11 +/− 0·006**	0·13 +/− 0·021**	0·48 +/− 0·03	> 2*
DB745	0·03 +/− 0·002	0·03 +/− 0·016	0·07 +/− 0·08	> 2*
DB702	0·15 +/− 0·007	(—)	(—)	> 1*
DB1282	0·23 +/− 0·016	(—)	(—)	> 0·5*
DB1127	(—)	(—)	(—)	< 0·5*

* Values are given for exposure of HFF to drugs for 2 × 3 days at 37 °C, 5% CO_2, and induction of 50% or greater cell death.
** Values are in a similar range to that previously determined for *T. gondii Rh* (0·16 μM) by real time PCR (Leepin *et al*. 2008).

washing in DMEM, and 1 ml of full medium was added, containing the compounds at concentrations as indicated in the individual experiments. In some experiments, DB750 (1 μM) was added to parasites and/or host cells already prior to infection, as indicated below. For a list of some of these compounds refer to Table 1. Each experiment included controls such as (i) parasite-infected HFF in full medium containing respective concentrations of the DMSO-solvent and (ii) uninfected HFF monolayers in drug-containing full medium to assess selective toxicity. The cultures were maintained at 37 °C in a 5% CO_2 atmosphere during different periods of time as indicated below, and were inspected daily by light microscopy. Samples for quantitative real time PCR analysis were taken by removal of the medium and addition of a mixture of 200 μl of phosphate-buffered saline (PBS), 180 μl of lysis buffer and 20 μl of proteinase K (DNAeasy Kit, QIAGEN, Basel, Switzerland). Samples were stored at −20 °C until further analysis was carried out. The quantification of parasites was done by qPCR according to previously described protocols (Costa *et al*. 2000; Scheidegger *et al*. 2005). As external standards, samples containing the DNA from 0, 10, 100, and 1000 T. *gondii* tachyzoites were included. The parasite numbers in the experimental samples were deduced by interpolation from the standard curve.

In vitro *drug treatment assays employing* T. gondii β-gal

These assays were done essentially as previously described (Müller *et al*. 2009). HFF monolayers were grown to confluency in 96-well flat-bottommed microtitre plates. For each assay, 8 wells were used. Uninfected control-HFF received 200 μl of full medium, all other monolayers were infected with 100 μl of full medium containing 2×10^3 freshly purified *T. gondii β-gal* tachyzoites as above. After 1 h, drug- and solvent-control samples were added, and plates were incubated for 72 h at 37 °C/5% CO_2. In some experiments, DB750 or DB745 (1 μM) were added already prior to infection, either to the host cells or to the parasites, as indicated below. Subsequently, plates were centrifuged at 80 ***g*** for 5 min at 20 °C, the medium was removed, and wells were washed once with 200 μl of PBS and centrifuged again. Each well received 90 μl of PBS containing 0·05% Triton-X-100 and 10 μl of the enzyme substrate (5 mM chlorophenylred-β-galactoside in PBS), and the absorption was read at various time-points. As determined by McFadden *et al*. (1997), the initial velocity (ΔA570/min) was proportional to the number of tachyzoites down to 50 per well.

Determination of IC_{50} values

IC_{50} values of selected compounds (see Table 1) were determined in cultures treated for 72 h with drugs at different concentrations ranging from 0 to 2 μM. IC_{50} values were calculated after the logit-log-transformation of the relative growth (RG; control = 1) according to the formula $\ln[(RG/(1\text{-}RG)] = a \times \ln(\text{drug concentration}) + b$ and subsequent regression analysis by the corresponding software tool contained in the Excel software package (Microsoft, Seattle, WA, USA).

Assessment of host cell toxicity

HFF were seeded into 24-well tissue-culture plates and were grown to confluency. Medium was removed and 2 ml containing the compounds of interest (DB702, DB745, DB1127 and DB1282) were added at concentrations between 0·5, 1, 2, and 3 μM. Controls received corresponding amounts of DMSO-solvent. After 3 days of culture, the medium was replaced with fresh medium containing the same amounts of the drugs, and culture was repeated for another 3 days. Monolayers were inspected microscopically on a daily basis and, at day 6, cell death was

assessed by trypsinization, staining of cells with Trypan blue, and counting the live and the dead cells in a Neubauer chamber.

Determination of the effects of DB745 treatment on extracellular T. gondii β-gal

T. gondii β-gal tachyzoites (2×10^5 parasites in 1 ml) were re-suspended in pre-warmed (37 °C) or pre-cooled (4 °C) full medium, and were incubated at the respective temperature for 30 min. Subsequently, tubes were complemented with 1 μM DB745, or the respective concentration of solvent (DMSO), and were further incubated for either 2 h or 4 h at 4 °C and 37 °C, respectively. Subsequently, the tachyzoites were centrifuged at 300 ***g*** for 10 min at 4 °C, and were re-suspended in 1 ml of full medium. Then 100 μl of the suspension was used per well of a 96-well plate to infect a HFF monolayer grown to confluency, and 8 wells were used for 1 experimental assay. The 96-well plates were placed at 37 °C in 5% CO_2 for 72 h. Assessment of tachyzoite numbers was done by detection of β-galactosidase activity as described above (McFadden *et al*. 1997; Müller *et al*. 2009).

In vitro *adaptation of* T. gondii Me49 *to increased concentrations of DB750 and DB745*

Confluent HFF monolayers were infected with *T. gondii Me49* in small tissue-culture flasks and were exposed to a stepwise increase in drug concentration over time, starting with 0·05 μM for DB750 and 0·02 μM for DB745. The drug concentration was elevated stepwise every 3–4 days by 0·05 and 0·02 μM, respectively, with daily evaluation by light microscopy. The drugs reached maximum concentrations of 1·2 μM for DB750 and 0·46 μM for DB745. Every 6–9 days, parasites were passaged onto fresh HFF by trypsinization of infected cultures, washing them in PBS, and adding them to fresh monolayers previously grown overnight. For both compounds a further increase in drug concentration had to be terminated due to host cell toxicity as judged by microscopical assessment.

Proliferation of non-adapted T. gondii *tachyzoites and DB750-adapted* T. gondii_DB750 *in the presence and absence of DB750*

Confluent HFF monolayers, grown in 24-well tissue-culture plates, were infected with 10^3 freshly purified non-adapted *T. gondii* or DB750-adapted *T. gondii_DB750* in full medium for 1 h at 37 °C/5% CO_2. Subsequently wells received DB750 to a final concentration of 1·2 μM, or the appropriate amount of solvent (DMSO) alone, and were further incubated at 37 °C/5% CO_2 . Sample collection was done at 12 h, 36 h, 60 h, 84 h, 108 h, 134 h and 158 h following initiation of drug treatment. For this, the supernatants were removed, wells were trypsinized, and cells were collected by centrifugation at 340 ***g*** for 10 min at 4 °C. DNA was purified and samples were processed for quantitative real-time PCR, both as described above.

Differential immunofluorescence staining of extracellular and intracellular T. gondii Me49 *tachyzoites*

The protocol previously applied for *N. caninum* (Hemphill *et al*. 1996; Naguleswaran *et al*. 2003) was used. In short, non-adapted *T. gondii Me49* and *T. gondii_DB750* were resuspended in full medium to 5×10^7 parasites/ml. Parasites were treated or not with 1 μM DB745 for 1 h at 37 °C. Subsequently, 100 μl of parasite suspensions were allowed to settle onto HFF cell monolayers grown on poly-L-lysine (100 μg/ml) coated glass coverslips in 24-well tissue-culture plates at 37 °C and 5% CO_2 for 40 min. Subsequently, coverslips were rinsed in PBS and were placed into fixation buffer containing PBS/3% paraformaldehyde/0·05% glutaraldehyde for 10 min at 22 °C. The coverslips were washed extensively in PBS and were incubated in PBS containing 1% bovine serum albumin (BSA) and 50 mM glycine (blocking buffer) for 30 min. The first antibody layer (polyclonal rabbit anti-*T. gondii* antiserum; Scheidegger *et al*. 2005) was applied at a dilution of 1:500 in blocking buffer for 25 min, followed by 3 washes in PBS, and the secondary antibody (goat anti-rabbit-Texas red (Becton Dickinson Immunocytometry Systems)) at a 1:200 dilution for 25 min. After washing in PBS (3 times × 5 min), cells were permeabilized by placing the coverslips into pre-cooled methanol and acetone (× 20 °C) for 5 min each. After rehydration in PBS, coverslips were placed into blocking buffer, and were labelled with the same polyclonal rabbit anti-*Toxoplasma gondii* antiserum (1:500), and with a goat anti-rabbit-FITC (Becton Dickinson Immunocytometry System) at a 1:200 dilution. Specimens were then washed extensively (1 min in 2 × concentrated PBS for 1 min, followed by additional rinses 5 × 5 min in PBS). Finally, coverslips were mounted onto glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, USA). Results were obtained by inspection of 20 randomized chosen fields (with at least 15 host cells) at 40 times magnification on a Nikon ECLIPSE 80i fluorescence microscope using all 3 channels. By counting the FITC-labelled parasites the overall number of *T. gondii* tachyzoites was determined. The number of parasites located on the surface of the HFF cells was determined by counting the Texas red immunolabelled tachyzoites. Finally, the number of intracellular parasites was calculated.

Transmission electron microscopy (TEM)

HFF monolayers grown in 25 cm^2 tissue-culture flasks were infected with *T. gondii Me49* or with *T.gondii_DB750*. At 2 days post-infection, treatment with DB750 or DB745 were initiated, both at 1 μM. Samples were collected 24 h later, by removing the medium, washing the monolayers twice with 100 mM sodium cacodylate buffer (pH 7·2), and fixation in 100 mM sodium cacodylate buffer, pH 7·3, containing 2·5% glutaraldehyde for 2 h. Then specimens were washed twice with 100 mM cacodylate buffer, scraped off with a rubber policeman, and centrifuged at 100 ***g*** for 10 min at 4 °C. Post-fixation was done in cacodylate buffer containing 2% OsO_4 at 22 °C. Subsequently, specimens were washed in water pre-stained in 1% uranyl acetate in water for 30 min, followed by an extensive wash with water. The samples were dehydrated in a graded series of ethanol (30, 50, 70, 90, and 100%), and were embedded in Epon 820 epoxy resin as described by Leepin *et al*. (2008). The resin was polymerized at 65 °C for 24 h. Ultrathin sections (~80 nm) were cut on a Reichert and Jung ultramicrotome and were loaded onto 300-mesh copper grids (Plano GmbH, Marburg, Germany), and stained with uranyl acetate and lead citrate (Hemphill *et al*. 2004). Grids were viewed on a Philips 400 transmission electron microscope (TEM) operating at 80 kV.

RESULTS

In vitro *activity of DB750 against* T. gondii Rh *and* T. gondii Me49

As determined by real time PCR quantification, DB750 was previously shown to be active against *T. gondii Rh* (Rh-strain) tachyzoites, with an IC_{50} of 0·16 μM (Leepin *et al*. 2008). In order to investigate whether *T. gondii Me49* (Me49-strain) tachyzoites exhibit a similar susceptibility, infected HFF monolayers were subjected to increasing concentrations of DB750 (0–2 μM) for a period of 72 h. Real time PCR showed that DB750 inhibited growth of *T. gondii Me49* with an IC_{50} of 0·13 μM, which was in the same range, as previously reported for *T. gondii Rh* tachyzoites (Leepin *et al*. 2008).

To further characterize the effects of DB750 in *T. gondii* tachyzoites, and to facilitate screening for other potentially interesting compounds in a more cost-effective manner, a *T. gondii* transgenic strain (*T. gondii β-gal*; Rh-background) was employed that constitutively expressed β-galactosidase (McFadden *et al*. 1997). DB750 inhibited proliferation of *T. gondii β-gal* with an IC_{50} of 0·11 μM (see Table 1). Thus, *T. gondii Rh, T. gondii β-gal,* and *T. gondii Me49* exhibited similar susceptibility to the action of DB750.

Further investigations were carried out employing *T. gondii β-gal* (Fig. 1). As expected, culture of

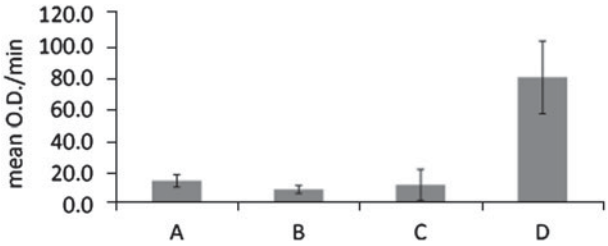


Fig. 1. Effects of DB750 against *Toxoplasma gondii β-gal* employing 72 h *in vitro*-growth assays. (A) *T. gondii* tachyzoites were allowed to infect HFF monolayers and 1 μM DB750 was added 1 h later. (B) DB750 was added already during the invasion phase. (C) HFF monolayers were cultured for 12 h in medium containing 1 μM DB750, washed extensively with medium, and freshly isolated *T. gondii* tachyzoites were added subsequently. (D) Freshly purified *T. gondii* tachyzoites were re-suspended in the medium supernatant obtained from (C) and added to HFF monolayers. Note the inhibition of parasite growth in (A), (B) and (C), and extensive parasite proliferation in (D). Shown is 1 of 3 experiments with essentially identical outcome.

T. gondii β-gal tachyzoites in HFF for 72 h in the presence of 1 μM DB750 did not result in tachyzoite proliferation, regardless of whether the drug was added 1 h after infection or during the invasion phase (see Fig. 1A, B). As previously observed for *N. caninum* (Leepin *et al*. 2008), incubation of uninfected HFF monolayers with 1 μM DB750 for 12 h prior to infection, and subsequent infection with *T. gondii* tachyzoites and culture for 72 h, also did not result in any parasite growth (see Fig. 1C). In order to investigate whether the activity of DB750 was maintained in the supernatant of HFF monolayers after 12 h of treatment, freshly purified *T. gondii* tachyzoites were suspended in the medium supernatant originating from HFF cultures treated for 12 h, and were used for infection and culture on fresh HFF monolayers for 72 h. *T. gondii* tachyzoites readily proliferated in that pre-used medium (Fig. 1D).

T. gondii *tachyzoites can readily adapt to the effects of DB750 treatment* in vitro

In order to establish whether *T. gondii* tachyzoites would be able to adapt, or possibly acquire resistance, to DB750, we cultured *T. gondii Me49* tachyzoites in HFF monolayers, by increasing the drug concentration stepwise every 2–3 days, starting at 0·05 μM and ending at 1·2 μM. Upon exposure to concentrations of more than 1·2 μM DB750 for more than 10 days, HFF monolayers exhibited aberrant morphological alterations, which indicated toxic effects upon longer-term exposure (data not shown). Nevertheless, a DB750-adapted *T. gondii* strain with Me49 background (*T. gondii_DB750*) was generated which still readily proliferated at 1·2 μM drug concentration. The ability of *T. gondii_DB750* to resist elevated drug concentrations was retained after freezing of

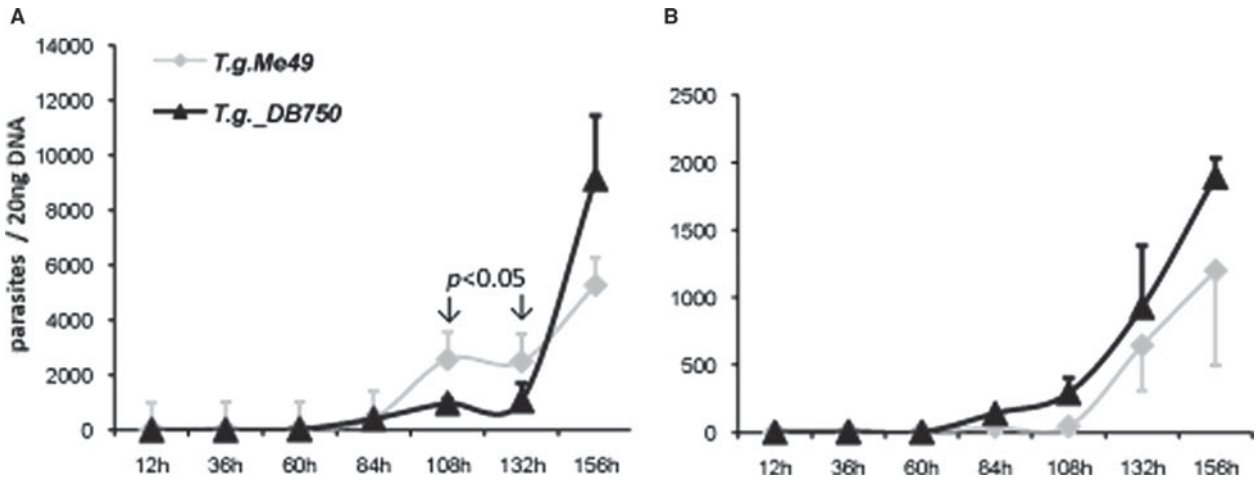


Fig. 2. Proliferation kinetics of *Toxoplasma gondii Me49* and DB750-adapted *T. gondii_DB750* in HFF monolayers. (A) Growth curves in the absence of DB750 and (B) in the presence of 1·2 μM DB750. Parasite quantification was performed by quantitative real time PCR at different time-points as indicated in the x-axis over a period of 156 h. Shown is 1 of 2 experiments with essentially identical outcome. *P* values <0·05 indicate significant differences in parasite numbers of the two strains when cultured in the absence of DB750.

infected cells in liquid nitrogen using a medium composed of FCS/10% DMSO, short-term storage in liquid nitrogen, and subsequent thawing and culture in DB750-containing medium. The proliferation of *T. gondii_DB750* in the presence of different concentrations (0–2 μM) of DB750 was assessed in 72 h-growth assays in HFF, and yielded an IC_{50} of 0·48 μM, which was almost 4 times higher than that for the non-adapted strain (0·13 μM; see Table 1).

Subsequently, the proliferation-kinetics of non-adapted *T. gondii Me49* and *T. gondii-DB750* within a time frame of 156 h were comparatively assessed by real time PCR in the presence and absence of 1·2 μM DB750, respectively (Fig. 2). Within the first 84 h of culture in the absence and presence of DB750 both *T. gondii* strains exhibited similar growth kinetics. Subsequently, in the absence of DB750, the non-adapted *T. gondii Me49* tachyzoites proliferated at a significantly higher rate ($P<0·05$) compared to *T. gondii_DB750*, until the time-point 156 h post-infection, at which the numbers of *T. gondii_DB750* increased dramatically (Fig. 2A). In the presence of 1·2 μM DB750 (Fig. 2B), the DB750-adapted strain was proliferating slightly more rapidly from 84 h post-infection onwards until the end of the assay. However, surprisingly, a substantial proliferation of non-adapted *T. gondii Me49* tachyzoites could be observed from 84 h post-infection onwards, and the parasite numbers of the two strains were not significantly different at any time-point. Thus, DB750 did not exhibit parasiticidal activity against non-adapted *T. gondii Me49* tachyzoites when applied at high (1·2 μM) concentration. Conversely, *T. gondii Me49* tachyzoites rapidly adapted to the action of the compound within the short time frame of 5–6 days of treatment. The same potential to readily adapt to DB750 treatment was observed for *T. gondii β-gal* tachyzoites (data not shown).

In vitro *screening of di-cationic pentamidine derivatives against* T. gondii *tachyzoites leads to the identification of DB745*

T. gondii β-gal tachyzoites were used for the screening of a series of selected di-cationic pentamidine derivatives, including 10 di-arylimidamides (DB666, DB667, DB702, DB710, DB745, DB780, DB786, DB865, DB891, or DB930), 6 diamidines (DB1282, DB1341, DB1362, DB1407, DB1450 or DB1479), 15 newly generated mono-arylimidamides (DB1980, DB1996, DB1997, DB2001, DB2002, DB2006, DB2007, DB2018, DB2036, DB2045, DB2048, DB2074, DB2079, DB2081, or DB2083), and the di-guanidino analogue DB1127, all at an initial concentration of 1 μM. Evaluation of β-galactosidase activity after 72 h of exposure to these drugs revealed that the mono-arylimidamides tested did not show any adverse effects on *T. gondii* proliferation nor on host cells, but proliferation-inhibitory effects were imposed by the di-arylimidamides DB702 and DB745, the di-guanidino-analogue DB1127, and the di-amidine DB1282 (see Table 1).

Assessment of host cell toxicity by light microscopical inspection of uninfected HFF monolayers exposed to increasing concentrations of the drugs (0, 0·5, 1, 2 or 3 μM) twice/day for 3 days revealed that DB1282 induced over 50% cell death already at 1 μM, and DB1127 lead to 80% HFF death at a concentration of 0·5 μM. Fifty % of viable HFF compared to untreated samples were found in cultures treated with 2 μM DB702 (Table 1). For DB745, treatments with 0·5–2 μM did not result in adverse reactions in HFF monolayers, while at 3 μM a 50% reduction in viable HFF was observed. Thus, DB745, with an IC_{50} for *T. gondii β-gal* of 0·03 μM, and favourable selective toxicity was selected for further study (see Table 1).

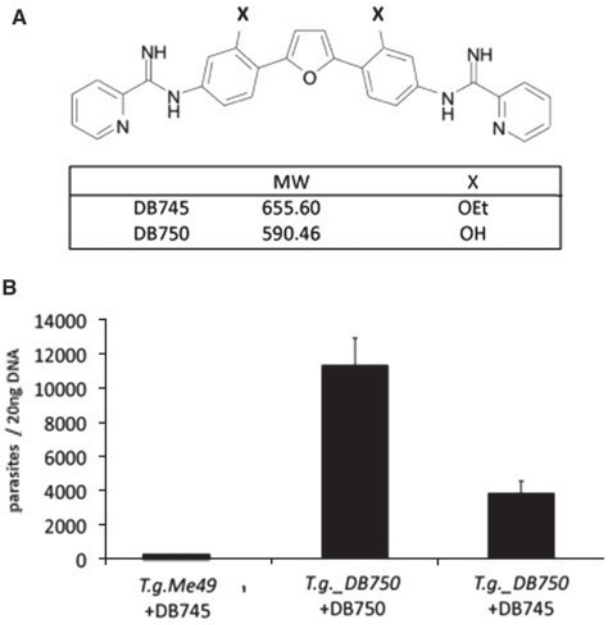
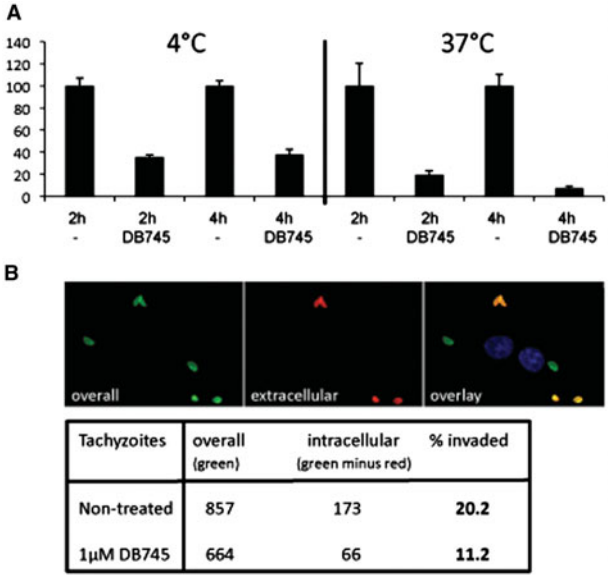


Fig. 3. Properties of DB750 and DB745. (A) Structures and molecular weights of DB750 and DB745. (B) Proliferation inhibitory effects of DB750 and DB745 over a period of 72 h. Parasite quantification was done by quantitative real time PCR. In (B) the proliferation of *Toxoplasma gondii Me49* over a period of 72 h was completely inhibited by the presence of 1 μM DB745 (left). *T. gondii_DB750* proliferation was not inhibited by the presence of 1·2 μM DB750 (middle), but significantly impaired by the presence of 1 μM DB745 (right). Shown is 1 of 2 experiments with essentially identical outcome.



Tachyzoites	overall (green)	intracellular (green minus red)	% invaded
Non-treated	857	173	20.2
1µM DB745	664	66	11.2

Fig. 4. Effects of DB745 on the infectivity of extracellular *Toxoplasma gondii* tachyzoites. (A) *T. gondii β-gal* tachyzoites were incubated in the presence or absence of 1 μM DB745 for 2 h or 4 h, either at 4 °C or at 37 °C. They were then used for infection and proliferation in HFF monolayers for a period of 72 h prior to quantification of β-galactosidase activity. Results in terms of parasite proliferation are presented as a percentage in relation to treatments without drug (given as 100% β-galactosidase activity). The assays were done in quadruplicate. (B) Assessment of DB745-mediated inhibition of invasion of *T. gondii Me49* tachyzoites by immunofluorescent distinction of intracellular and extracellular parasites. Results were obtained by differential staining and subsequently counting the parasites in 20 randomly chosen fields at 40 times magnification. Extracellularly located parasites fluoresce with Texas-red and the overall number of parasites was determined by FITC-staining. Note the reduction of invasive parasites after incubation with 1 μM DB745 for 1 h. Shown are the results of 1 of 2 experiments with essentially identical outcome.

Characterization of the effects of DB745 on proliferation and host cell invasion of non-adapted (T. gondii Me49) *and DB750-adapted* (T. gondii_DB750) *tachyzoites*

DB750 and DB745 exhibit a similar molecular structure (Fig. 3A). They differ in molecular weight (590·46 and 655·60, respectively), and in contrast to DB750, DB745 possesses an ethyl-moiety on each of the 1′-positions of the aryl rings. The proliferation of non-adapted *T. gondii Me49* and *T. gondii_DB750* in the presence of 1 μM DB745 was assessed in 72 h growth-assays, and compared with *T. gondii_DB750* tachyzoites treated with 1·2 μM DB750 (Fig. 3B). As expected, DB745 completely blocked the proliferation of non-adapted *T. gondii Me49* tachyzoites (IC_{50} = 0·03 μM; see Table 1). The DB750-adapted *T. gondii_DB750* tachyzoites readily underwent proliferation in the presence of 1·2 μM DB750 (IC_{50} = 0·48 μM; see Table 1), but were significantly impaired in the presence of DB745 (IC_{50} of 0·07 μM; see Table 1). However, the inhibition of proliferation was significantly less pronounced as for non-adapted *T. gondii Me49*.

In order to investigate whether DB745 also had an impact on extracellular *T. gondii* tachyzoites, *T. gondii β-gal* tachyzoites were separated from their host cells and exposed to DB745 (1 μM) for periods of 2 h and 4 h, either at 4 °C or at 37 °C (Fig. 4A). Subsequently, the drugs were removed, and parasites were added to HFF monolayers, and proliferation of tachyzoites was assessed after 72 h. Upon incubation of isolated tachyzoites at 4 °C, DB745 already had a profound impact on the infectivity of the parasites, yielding a reduction of tachyzoite numbers of approximately 60% after both 2 and 4 h exposure-time to the drug. At 37 °C, the impact of DB745 on parasite infectivity was even more pronounced, yielding a reduction in parasite numbers of 80% after 2 h, and over 90% upon 4 h of extracellular exposure to DB745 (Fig. 4A). This suggested that DB745 impaired the viability of extracellular parasites in a time- and temperature-dependent manner.

The effect of DB745 on host cell invasion was assessed using *T. gondii Me49* and employing immunofluorescence microscopy and applying a

differential staining procedure that allowed distinction of intracellular and extracellular parasites (Hemphill *et al*. 1996; Naguleswaran *et al*. 2003). Following exposure of non-adapted *T. gondii Me49* tachyzoites to 1 μM DB745 for 1 h, parasites were washed and allowed to interact with HFF monolayers for 1 h (Fig. 4B). As a control, non-treated parasites were subjected to the same procedure without compound. Of 857 non-treated tachyzoites, 173 (20·2%) were located within host cells. In contrast, in DB745-treated samples the overall number of counted parasites was reduced to 664 tachyzoites and only 66 (11·2%) were found to be intracellular (Fig. 4B). No such effect was seen when *T. gondii-DB750* tachyzoites were exposed to DB745 treatment, and when non-adapted *T. gondii Me49* tachyzoites were exposed to DB750 (data not shown).

In vitro *adaptation of* T. gondii Me49 *tachyzoites to DB745*

In order to explore the potential of *T. gondii Me49* tachyzoites to adapt to DB745 treatment, the concentration of the drug during the culture of infected HFF monolayers, starting at 0·02 μM, was increased stepwise by 0·02 μM every 3–4 days (see Table 2). As for DB750, *T. gondii Me49* tachyzoites exhibited an astonishing capacity to also adapt to DB745, albeit at a much slower speed, but finally still undergoing proliferation up to a concentration of 0·46 μM. Rapid adaptation to increasing concentrations of DB745 as previously observed for DB750 was not possible. At higher concentrations, exposure to DB745 during periods of 10 days or longer lead to deterioration of the HFF monolayers. Thus, besides exhibiting a higher anti-parasitic activity compared to DB750, DB745 also showed an increased host cell toxicity. The IC_{50} of DB745 for this DB745-adapted strain was not determined.

Table 2. Schedule for the establishment of a DB745-adapted *Toxoplasma gondii* Me49 line

(At indicated time-points the medium was replaced with new medium containing either slightly elevated concentrations (+0·02 μM) of DB745, or (at the later time-points) medium was changed with drug concentration unchanged as indicated. Every 6–10 days cultures were trypsinized and seeded onto fresh HFF monolayers. At day 111 and a DB745 concentration of 0·46 μM, host cell monolayers exhibited clear signs of disintegration and the adaptation process was stopped.)

Time-points of treatment (days)	DB745 (μM)
0	0·02
3	0·02
7	0·04
10	0·06
14	0·08
17	0·10
21	0·12
24	0·14
27	0·14
31	0·16
35	0·18
38	0·20
42	0·22
45	0·24
48	0·26
52	0·28
56	0·30
59	0·32
63	0·34
66	0·36
69	0·38
73	0·40
76	0·40
80	0·40
83	0·42
86	0·42
90	0·42
93	0·42
97	0·42
101	0·42
104	0·42
108	0·44
111	0·46

Effects of DB745 on the ultrastructure of T. gondii *tachyzoites*

Differences in efficacy of DB745 against non-adapted and DB750- adapted *T. gondii_DB750* tachyzoites were also detectable at the ultrastructural level. HFF monolayers infected with *T. gondii_DB750* or non-adapted *T. gondii* were treated with 1 μM DB750 and DB745, respectively, for 24 h, and were processed for TEM (Figs 5 and 6). In non-adapted *T. gondii Me49* both, DB750 (Fig. 5B) and DB745 (Fig. 5C, D) induced severe morphological alterations in parasite ultrastructure, as can be seen by, for example, the formation of increased cytoplasmic vacuoles with membranous and often granular content, the presence of membrane stacks, and electron-dense inclusions. In DB750-adapted *T. gondii_DB750* tachyzoites (Fig. 6) there were no obvious differences observed in the ultrastructure of untreated (Fig. 6A) and DB750-treated *T. gondii_DB750* tachyzoites (Fig. 6B), confirming the complete adaptation of these parasites to the drug. In contrast, populations with different degrees of structural alterations were detected in cultures infected with *T. gondii_DB750* tachyzoites and treated with DB745. In some instances seemingly viable parasites with normal or only slightly aberrant ultrastructural organization (Fig. 6C and D) could be seen, often in the direct vicinity of tachyzoites that exhibited extensive cytoplasmic distortions such as increased vacuolization and membranous inclusions (Fig. 6C, E). These aberrant structural changes point towards severe metabolic impairment of affected tachyzoites. In addition, numerous non-viable tachyzoite-remnants were detected in DB745-treated cultures (Fig. 6F).

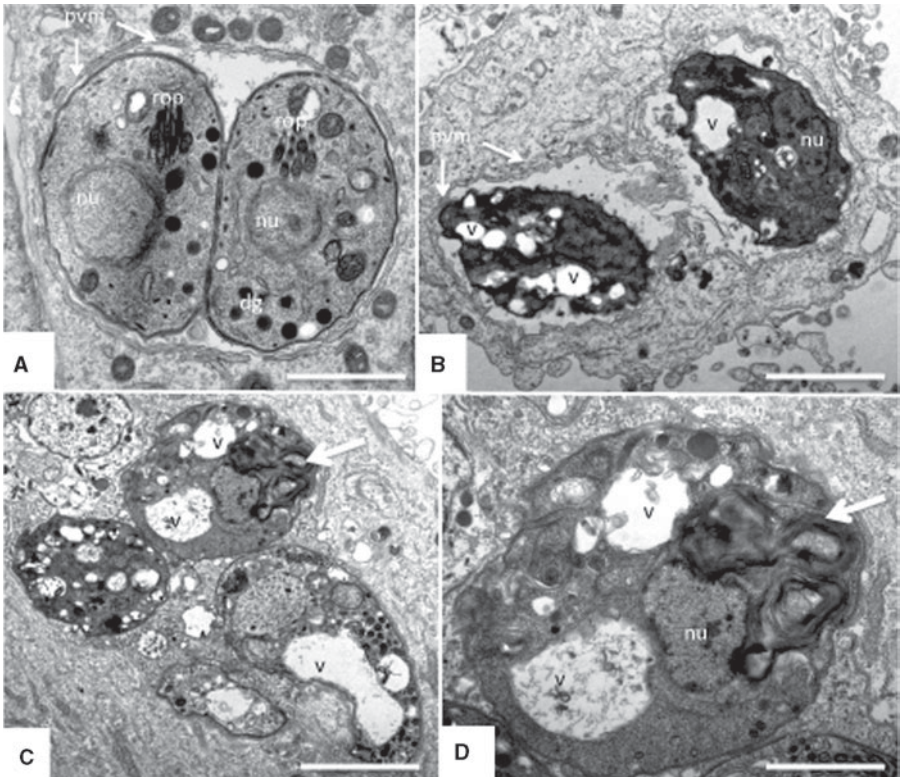


Fig. 5. TEM of the effects of DB750 and DB745 (1 μM, 24 h) against *Toxoplasma gondii Me49* tachyzoites cultured in HFF. (A) Control without drug treatment, with *T. gondii Me49* tachyzoites surrounded by a parasitophorous vacuole membrane (pvm) and exhibiting cytoplasmic organelles such as nucleus (nu), rhoptries (rop) and dense granules (dg). Scale bar = 0·7 μm. (B) *T. gondii Me49* tachyzoites incubated in the presence of DB750, exhibiting extensive vacuolization, and substantial degradation of the cytoplasmic architecture (v). Scale bar = 0·9 μm. (C) Shows a group of *T. gondii Me49* tachyzoites incubated in the presence of DB745 (Scale bar = 0·9 μm), and (D) displays a higher magnification view of the parasite indicated by the large arrow (Scale bar = 0·4 μm). In (D), note the presence of cytoplasmic vacuoles, either seemingly empty or filled with a meshwork of filamentous and granular material of unknown origin (v) and the large stacks of membrane adjacent to the nucleus (nu). In all drug-treated samples, the pvm appeared still largely intact and clearly discernible.

DISCUSSION

Although pentamidine was synthesized already in the 1930s, it has remained the only aromatic diamidine that is widely used in the clinic, especially against human stage 1 African trypanosomiasis caused by *Trypanosoma brucei gambiense*, but also against Pneumocystis pneumonia caused by *Pneumocystis jiroveci*, and against leishmaniasis, and *Candida albicans* infections (Werbovetz, 2006). A number of arylimidamides, including DB702, DB750, DB766, DB786, DB811 and DB889 exhibited good *in vitro* activity against *Trypanosoma cruzi, N. caninum, B. besnoiti or L. donovani* (Stephens *et al*. 2003; Silva *et al*. 2007*a*,*b*; Leepin *et al*. 2008; Cortes *et al*. 2011), and DB766 effectively reduced the parasite burden in the blood and heart of *T. cruzi*-infected mice at a dose of 100 mg/kg/day given orally and intraperitoneally (Batista *et al*. 2010*a*). Thus, the goal of this investigation was to study the interactions of *T. gondii* tachyzoites with DB750, which had shown interesting properties in a previous study (Leepin *et al*. 2008), and possibly identify other compounds with good *in vitro* activity. This included effects of these compounds on intracellular proliferation, host cell toxicity, parasiticidal activity, the involvement of the host cells in drug activity, and the potential of these parasites to adapt to the adverse conditions during *in vitro* drug treatment.

The di-cationic molecules used here have been designed to have improved pharmacokinetic properties by including aryl groups on one of the amidine nitrogen atoms (Wang *et al*. 2010) and DB750 was an obvious drug candidate due to its low IC_{50} against *T. gondii Rh* (0·23 μM), and low toxicity in HFF monolayers (Leepin *et al*. 2008). Previous studies had also shown that the HFF host cells actually participated in the activity against closely related *N. caninum* tachyzoites, most likely by taking up the drug and providing the host cell with a 'memory effect', thus exerting the anti-parasitic activity against intracellular tachyzoites even in the absence of added drug (Leepin *et al*. 2008). However, DB750 did not affect the viability and invasive capacities of extracellular *N. caninum* and *T. gondii Rh* tachyzoites (Leepin *et al*. 2008). Moreover, recent *in vivo* studies in mice

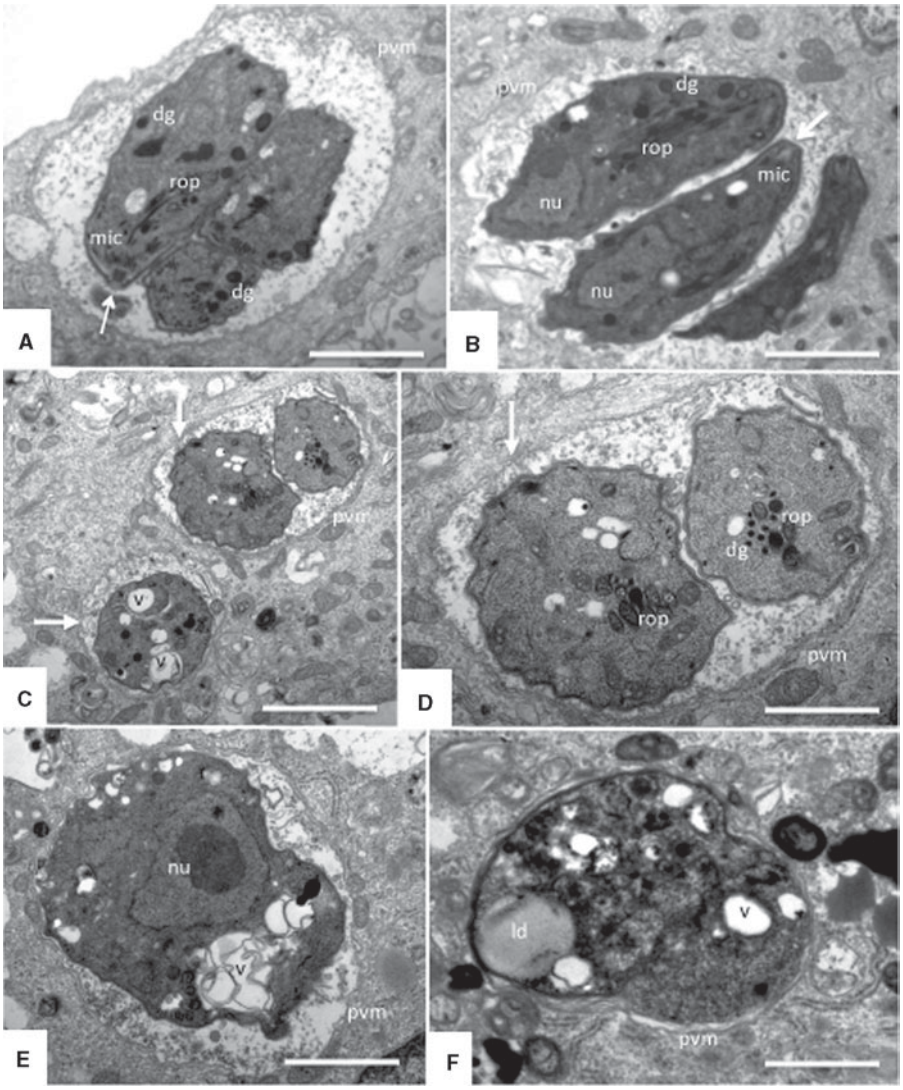


Fig. 6. TEM of the effects of DB750 and DB745 (1 μM, 24 h) against DB750-adapted *Toxoplasma gondii-DB750* tachyzoites cultured in HFF. (A) Control, displaying *T. gondii_DB750* tachyzoites without drug treatment. Typical components such as the parasitophorous vacuole membrane (pvm), rhoptries (rop), dense granules (dg) and micronemes (mic) at the apical pole of the tachyzoites are clearly discernible. The arrow points towards the conoid. Scale bar = 1 μm. (B) *T. gondii_DB750* tachyzoites incubated in the presence of DB750. Note that these parasites seem largely unaffected by the drug treatment. (C–F) *T. gondii_DB750* cultured in the presence of DB745; in (C) tachyzoites exhibiting only moderate alterations and still exhibiting a largely intact cytoplasmic organization (vertical arrow), and another tachyzoite with increased vacuole formation (horizontal arrow) are shown. (D) Higher magnification view of (C). Scale bars in (C) = 0·9 μm, in (D) = 0·4 μm. (E) and (F) Show tachyzoites that exhibit more severe alterations due to drug treatment such as intracytoplasmic vacuoles (v), lipid droplet formation (ld) and a general desintegration of the cellular architecture. Scale bars in (E) and (F) = 0·5 μm. In all drug-treated samples, the pvm appeared still intact and clearly discernible.

experimentally infected with the *N. caninum* tachyzoites showed that DB750-treatment, applied intraperitoneally at 2 mg/kg/day for a period of 14 days, had a benefitial impact by reducing clinical signs of neosporosis and diminishing the cerebral parasite load (Debache *et al*. 2011). Thus, we attempted to characterize the effects mediated by DB750 in 2 different strains of *T. gondii,* Rh and Me49, which represent strains of high and low virulence in mice, respectively (Dardé, 2008).

By employing 72 h-growth assays, DB750 IC_{50} values for the two *Toxoplasma* strains, *T. gondii β-gal* (Rh background) and *T. gondii Me49,* were shown to be in the similar low range (0·11–0·16 μM) of the previously reported IC_{50} for *T. gondii Rh* (Leepin *et al*. 2008). Thus, the assessments of parasite proliferation by a colourimetric assay based on the quantification of β-galactosidase activity (McFadden *et al*. 1997) and the quantification of parasite numbers by quantitative real time PCR produced similar results.

Similar to what was previously reported for *N. caninum,* DB750 retained its anti-parasitic activity against *T. gondii* also upon pre-treatment of host cells

for prior to infection and subsequent culture for 72 h in the absence of the drug. In addition, no anti-parasitic activity could be seen in the corresponding HFF-medium supernatant. The lack of DB750-activity in medium supernatant after 12 h of incubation with HFF could be potentially explained (i) by metabolic degradation of DB750 or (ii) by highly efficient drug-uptake by the host cells. The fact that the anti-parasitic effect was retained in these pre-treated HFF cells but was no longer evident in the corresponding medium supernatant supports the notion of DB750 being actively taken up by HFF monolayers and subsequently retained in the host cell cytoplasm. How this is achieved is not known; passive and active transport could be involved. Ming *et al*. (2008) identified a human organic cation transporter for which pentamidine and furamidine act as substrates, and possibly such transporters could mediate DB750 uptake into HFF. In addition, intracellular accumulation of DB750 within the HFF monolayers could be responsible for the toxicity that was detected upon culture of infected fibroblasts for more than 10 days in the presence of DB750 at concentrations higher than 1·2 μM.

Although the promising IC_{50} values achieved in 72 h assays indicated that *T. gondii* tachyzoites were highly susceptible to DB750 treatments, it became clear that these parasites exhibited a surprising ability to rapidly adapt to approximately 10 times higher drug concentrations within 5–6 days following initiation of treatment. The resulting DB750-adapted *T. gondii* strain, *T. gondii_DB750*, had an IC_{50} of 0·48 μM and could still undergo proliferation in HFF at a drug concentration of 1·2 μM. In comparison to *T. gondii*, the closely related *N. caninum* (Nc-1 and Nc-Liv isolates) did not exhibit the ability to rapidly adapt to such high concentrations of DB750. DB750 adaptation of *Neospora* populations could only be achieved after several weeks of careful and stepwise increase of drug concentrations up to 0·2–0·3 μM. Thus, although phylogenetically closely related, the two species seem to exhibit a different adaptation profile. Most likely, *T. gondii* has a more sophisticated metabolic machinery to deal with adverse life conditions, and it would be interesting to investigate the molecular mechanisms involved. In any case, the lower potential of *Neospora* to react to environmental changes could be a determining factor that limits the range of potential intermediate hosts of this parasite in comparison to *T. gondii* (Hemphill *et al*. 2006; Dubey *et al*. 2007). Thus, a higher degree of flexibility on the transcriptional and translational level (reviewed by Bougdour *et al*. 2010) may have enabled *T. gondii* tachyzoites to adapt to the arylimidamide compound DB750 within this short time span.

By monitoring the growth of *T. gondii Me49* and *T.gondii_DB750* in the presence of DB750 over time, the capacity of *T. gondii_DB750* to proliferate at a slightly higher rate compared to non-adapted parasites was demonstrated. However, the fact that *T. gondii Me49* tachyzoites were able to adapt to 1·2 μM DB750 and resume proliferation in such a short time frame was surprising. Adaptation could be accompanied by an increased expression of genes coding for components of the *Toxoplasma* detoxification machinery, such as e.g. ATP-binding cassette (ABC) transporters, which represent an important family of membrane proteins involved in drug resistance and other biological activities (Sauvage *et al*. 2009). A prominent ABC transporter, P-glycoprotein (TgABC.B1 and TgABC.B2) potentially mediating transport of the drug out of the cell, are associated with the membrane in tachyzoites, and is constitutively expressed in all 3 virulent types. On the other hand, another adaptation mechanism could involve the reduced uptake of drug, due to the down-regulation of certain adenosine transporter activities, which are involved in diamidine uptake (Matovu *et al*. 2003; Witola *et al*. 2004). For instance, homologues to the P2 aminopurine transporter in *T. brucei* are found in the *Toxoplasma* genome (www.toxodb.org). This protein normally transports essential adenosine and adenine, but is also responsible for the efficient transfer of pentamidine and the diamidine DB75 into the trypanosome interior (Lanteri *et al*. 2006). Down regulation of the expression of such adenosine transporters would then, besides limiting diamidine uptake, also limit the uptake of other essential molecules such as aminopurines, which are important for proliferation. This could explain the initial reduced proliferation of *T. gondii_DB750* compared to *T. gondii Me49* in the absence of DB750.

Evidently, these findings on DB750 did not allow high expectations in terms of a potential *in vivo* efficacy of DB750 against *T. gondii* infection. As a consequence, we aimed to search for additional active di-cationic compounds with increased anti-parasitic activity, leading to the identification of DB745.

DB750 and DB745 exhibit a highly similar molecular structure. However, the change of the hydroxyl groups on the benzene ring to ether-coupled ethyl groups could have provided DB745 with an increased metabolic resistance and membrane permeability, since the compound is slightly more lipophilic (log P at pH 7·4 = 3·82; Wang *et al*. 2010), which could in turn increase the chances of the drug passing several layers of membrane, including the host cell plasma membrane, parasitophorous vacuole membrane, parasite membrane, and the membrane of a potential target organelle. Which target is actually affected by DB745 is not clarified to date. Current evidence suggests that aromatic diamidines bind to AT-rich sites in the DNA minor groove, and thus inhibit transcription or the interaction with DNA-binding enzymes such as topoisomerases or nucleases (Wilson *et al*. 2008). This indicates that these compounds could influence gene expression, and thus many diverse cellular functions could be affected.

In kinetoplastids, fluorescence microscopy had demonstrated that these drugs associate with the kinetoplast DNA, which is in agreement with the observed mitochondrial swelling seen in *T. cruzi* upon incubation with diamidines (Batista *et al*. 2010*b*). However, neither DB750 nor DB745 exhibit fluorescent properties, thus it was not possible to localize these drugs, neither within the host cells nor within the parasites.

DB745 did not only affect the proliferation of intracellular *T. gondii* tachyzoites, but also exhibited pronounced effects upon treatment of extracellular parasites. This showed that, in order to be active DB745 is not required to be metabolized by the host cell. In contrast, DB750 did not affect the viability of extracellular *N. caninum* and *T. gondii Rh* tachyzoites (Leepin *et al*. 2008) nor did it have an impact on the invasive properties of *T. gondii Me49* tachyzoites. Thus, the decrease in the IC_{50} (from 0·13 μM (DB750) to 0·03 μM (DB745)) against *T. gondii Me49* could be due to an added effect such as the impairment of extracellular parasites. As a consequence, *T. gondii Me49* adaptation to the effects of DB745 treatment took place much less efficiently and only after extended periods of time. Rapid adaptation to DB745, as seen for DB750, was not possible.

The impact of pre-treatments on the viability of *T. gondii Me49* tachyzoites with DB745 at 37 °C was time dependent, and these effects were less pronounced upon pre-treatment at 4 °C and, at lower temperature, time independent. By employing an adhesion/invasion assay that allowed distinction between adhering tachyzoites and intracellular tachyzoites, the negative impact of DB745-treatments of extracellular tachyzoites was confirmed microscopically. The fact that the IC_{50} of DB745 for *T. gondii_DB750* was about 2 times higher compared to the IC_{50} for *T. gondii Me49* (0·07 μM versus 0·03 μM) can be explained by the similarities in structure of DB750 and DB745, thus adaptation to DB750 must have had some cross-protective effect. HFF monolayers infected with *T. gondii Me 49* and *T. gondii_DB750* were exposed to either DB750 or DB745, both at 1 μM for 24 h, and the effects were monitored by TEM. As expected, DB750 did not affect *T. gondii_DB750*, while DB745 clearly had a much higher impact. However, seemingly structurally unharmed parasites were occasionally visible. This indicates and confirms that a certain degree of cross-adaptation had taken place in some tachyzoites, but not in all. This also clearly shows that DB745, although highly effective in 3 day-growth assays, does not readily kill all parasites, even at high concentrations, allowing adaptation to take place in some tachyzoites.

In conclusion, we have demonstrated an astonishing ability of the apicomplexan parasite *T. gondii* to adapt to the effects of the arylimidamide DB750 within a time span of 5–6 days in culture. DB750 had previously provided encouraging data on selective anti-*Toxoplasma* efficacy *in vitro*. Thus our findings question the value of short-term (e.g. 72 h) *in vitro* assays for the evaluation of anti-*Toxoplasma* properties, and corresponding investigations that include longer-term *in vitro* drug exposure could provide more reliable results. *T. gondii* could also adapt to (less) elevated levels of DB745, although only after many weeks in cell culture. Drug adaptation is also likely to happen *in vivo*, since chemotherapeutical treatment failures when employing pyrimethamine, sulfadiazine and clindamycine have been reported frequently (Dedicoat and Livesley, 2006; Ribera Pasquet *et al*. 1998). Clearly, *Toxoplasma* represents a true survival artist, and this illustrates the inherent difficulties in obtaining reliable and efficacious drugs for the treatment of toxoplasmosis. Arylimidamides, however, represent a class of compounds that could serve this cause. DB745 should be followed up in an appropriate *in vivo* model, and the molecular mechanisms of the outstanding adaptive potential of *T. gondii* must be elucidated in future studies.

ACKNOWLEDGEMENTS

Many thanks are addressed to Norbert Müller for critical comments on the manuscript. We are grateful to Furio Spano (Istituto Superiore di Sanità, Rome, Italy) for providing us with *T. gondii Me49,* and to David Sibley (Washington University, St Louis, MO, USA) for his gift of *T. gondii β-gal*. This study was financed through the Swiss National Science Foundation (grant no. 31003A_127374/1).

REFERENCES

Barratt, J. L., Harkness, J., Marriott, D., Ellis, J. T. and Stark, D. (2010). Importance of nonenteric protozoan infections in immunocompromised people. *Clinical Microbiology Reviews* **23**, 795–836.

Batista, D. G., Batista, M. M., de Oliveira, G. M., do Amaral, P. B., Lannes-Vieira, J., Britto, C. C., Junqueira, A., Lima, M. M., Romanha, A. J., Sales Junior, P. A., Stephens, C. E., Boykin, D. W. and Soeiro, M. N. (2010*a*). Arylimidamide DB766, a potential chemotherapeutic candidate for Chagas' disease treatment. *Antimicrobial Agents and Chemotherapy* **54**, 2940–2952.

Batista, D. G., Pacheco, M. G., Kumar, A., Branowska, D., Ismail, M. A., Hu, L., Boykin, D. W., and Soeiro, M. N. (2010*b*). Biological, ultrastructural effect and subcellular localization of aromatic diamidines in *Trypanosoma cruzi*. *Parasitology* **137**, 251–259.

Bougdour, A., Braun, L., Cannella, D. and Hakimi, M. A. (2010). Chromatin modifications: implications in the regulation of gene expression in *Toxoplasma gondii*. *Cellular Microbiology* **12**, 413–423.

Buckner, F. S. and Navabi, N. (2010). Advances in Chagas disease drug development: 2009–2010. *Current Opinion in Infectious Diseases* **23**, 609–616.

Carruthers, V. B., and Suzuki, Y. (2007). Effects of *Toxoplasma gondii* infection on the brain. *Schizophreny Bulletin* **33**, 745–751.

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

Costa, J. M., Pautas, C., Ernault, P., Foulet, F., Cordonnier, C. and Bretagne, S. (2000). Real-time PCR for diagnosis and follow-up of *Toxoplasma* reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. *Journal of Clinical Microbiology* **38**, 2929–2932.

Dabritz, H. A. and Conrad, P. A. (2010). Cats and *Toxoplasma*: implications for public health. *Zoonoses and Public Health* **57**, 34–52.

Dardé, M. L. (2008). *Toxoplasma gondii,* "new" genotypes and virulence. Parasite 15, 366–371.

Debache, K., Guinaud, C., Kropf, C., Boykin, D., Stephens, C. E. and Hemphill, A. (2011). Experimental treatment of *Neospora caninum*-infected mice with the arylimidamide DB750 and the thiazolide nitazoxanide. *Experimental Parasitology* **129**, 95–100.

Dedicoat, M. and Livesley, N. (2006). Management of toxoplasmic encephalitis in HIV-infected adults (with an emphasis on resource-poor settings). *Cochrane Database Systemic Reviews* **19;3**, CD005420.

Dubey, J. P. (2009). History of the discovery of the life cycle of *Toxoplasma gondii*. *International Journal for Parasitology* **39**, 877–882.

Dubey, J. P., Schares, G. and Ortega-Mora, L. M. (2007). Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Reviews* **20**, 323–367.

Feldman, D. M., Timms, D. and Borgida, D. F. (2010). Toxoplasmosis, parvovirus, and cytomegalovirus in pregnancy. *Clinical Laboratory Medicine* **30**, 709–720.

Hemphill, A. (1996). Subcellular localization and functional characterization of Nc-p43, a major *Neospora caninum* tachyzoite surface protein. *Infection and Immunity* **64**, 4279–4287.

Hemphill, A., Gottstein, B. and Kaufmann, H. (1996). Adhesion and invasion of bovine endothelial cells by *Neospora caninum*. *Parasitology* **112**, 183–197.

Hemphill, A., Vonlaufen, N., Naguleswaran, A., Keller, N., Riesen, M., Guetg, N., Srinivasan, S. and Alaeddine, F. (2004). Tissue culture and explant approaches to studying and visualizing *Neospora caninum* and its interactions with the host cell. *Microscopy and Microanalysis* **10**, 602–620.

Hemphill, A., Vonlaufen, N. and Naguleswaran, A. (2006). Cellular and immunological basis of the host-parasite relationship during infection with *Neospora caninum*. *Parasitology* **133**, 261–278.

Henriquez, F. L., Woods, S., Cong, H., McLeod, R. and Roberts, C. W. (2010). Immunogenetics of *Toxoplasma gondii* informs vaccine design. *Trends in Parasitology* **26**, 550–555.

Laliberté, J. and Carruthers, V. B. (2008). Host cell manipulation by the human pathogen *Toxoplasma gondii*. *Cellular and Molecular Life Science* **65l**, 1900–1915.

Lanteri, C. A., Stewart, M. L., Brock, J. M., Alibu, V. P., Meshnick, S. R., Tidwell, R. R. and Barrett, M. P. (2006). Roles for the *Trypanosoma brucei* P2 transporter in DB75 uptake and resistance. *Molecular Pharmacology* **70**, 1585–1592.

Leepin, A., Stüdli, A., Brun, R., Stephens, C. E., Boykin, D. W. and Hemphill, A. (2008). Host cells participate in the *in vitro* effects of novel diamidine analogues against tachyzoites of the intracellular apicomplexan parasites *Neospora caninum* and *Toxoplasma gondii*. *Antimicrobial Agents and Chemotherapy* **52**, 1999–2008.

Lindsay, D. S., Blagburn, B. L., Hall, J. E. and Tidwell, R. R. (1991). Activity of pentamidine and pentamidine analogs against *Toxoplasma gondii* in cell cultures. *Antimicrobial Agents and Chemotherapy* **35**, 1914–1916.

Matovu, E., Stewart, M. L., Geiser, F., Brun, R., Mäser, P., Wallace, L. J., Burchmore, R. J., Enyaru, J. C., Barrett, M. P., Kaminsky, R., Seebeck, T. and de Koning, H. P. (2003). Mechanisms of arsenical and diamidine uptake and resistance in *Trypanosoma brucei*. *Eukaryotic Cell* **2**, 1003–1008.

McFadden, D. C., Seeber, F. and Boothroyd, J. C. (1997). Use of *Toxoplasma gondii* expressing beta-galactosidase for colorimetric assessment of drug activity *in vitro*. *Antimicrobial Agents and Chemotherapy* **41**, 1849–1853.

Ming, X., Ju, W., Wu, H., Tidwell, R. R., Hall, J. E. and Thakker, D. R. (2009). Transport of dicationic drugs pentamidine and furamidine by human organic cationic transporters. *Drug Metabolism and Disposition* **37**, 424–430.

Montoya, J. G. and Liesenfeld, O. (2004). Toxoplasmosis. *Lancet*. **363**, 1965–1976.

Müller, J., Limban, C., Stadelmann, B., Missir, A. V., Chirita, I. C., Chifiriuc, M. C., Nitulescu, M. G. and Hemphill, A. (2009). Thioureides of 2-(phenoxymethyl)benzoic acid 4-R substituted: a novel class of anti-parasitic compounds. *Parasitology International* **58**. 128–135.

Naguleswaran, A., Müller, N. and Hemphill, A. (2003). *Neospora caninum* and *Toxoplasma gondii:* a novel adhesion/invasion assay reveals distinct differences in tachyzoite-host cell interactions. *Experimental Parasitology* **104**, 149–158.

Pereira-Chioccola, V. L., Vidal, J. E. and Su, C. (2009). *Toxoplasma gondii* infection and cerebral toxoplasmosis in HIV-infected patients. *Future Microbiology* **4**, 1363–1379.

Ribera Pascuet, E., López Aldeguer, J., Pérez Elías, M. J. and Podzamczer Palter, D. (1998). Cerebral toxoplasmosis. *Enfermeria*. *Infecciology*. *Microbiology Clinica* **16** Suppl 1, 45–51.

Sauvage, V., Aubert, D., Escotte-Binet, S. and Villena, I. (2009). The role of ATP-binding cassette (ABC) proteins in protozoan parasites. *Molecular and Biochemical Parasitology* **167**, 81–94.

Scheidegger, A., Vonlaufen, N., Naguleswaran, A., Gianinazzi, C., Müller, N., Leib, S. L. and Hemphill, A. (2005). Differential effects of interferon-gamma and tumor necrosis factor-alpha on *Toxoplasma gondii* proliferation in organotypic rat brain slice cultures. *Journal of Parasitology* **91**, 307–315.

Silva, C. F., Batista, M. M., Mota, R. A., de Souza, E. M., Stephens, C. E., Som, P., Boykin, D. W. and Soeiro, M. N. (2007*a*). Activity of "reversed" diamidines against *Trypanosoma cruzi* "*in vitro*". *Biochemical Pharmacology* **15**, 1939–1946.

Silva, C. F., Meuser, M. B., De Souza, E. M., Meirelles, M. N., Stephens, C. E., Som, P., Boykin, D. W. and Soeiro, M. N. (2007*b*). Cellular effects of reversed amidines on *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **51**, 3803–3809.

Soeiro, M. N., De Souza, E. M., Stephens, C. E. and Boykin, D. W. (2005). Aromatic diamidines as antiparasitic agents. *Expert Opinion in Investigational Drugs* **14**, 957–972.

Stephens, C. E., Brun, R., Salem, M. N., Werbovetz, K. A., Tanious, F., Wilson, W. D. and Boykin, D. W. (2003). The activity of diguanidino and 'reversed' diamidino 2,5-diarylfurans versus *Trypanosoma cruzi* and *Leishmania donovani*. *Bioorganic Medicinal Chemistry Letters* **16**, 2065–2069.

Tenter, A. M., Heckeroth, A. R. and Weiss, L. M. (2000). *Toxoplasma gondii*: from animals to humans. *International Journal for Parasitology* **30**, 1217–1258.

Wang, M. Z., Zhu, X., Srivastava, A., Liu, Q., Sweat, J. M., Pandharkar, T., Stephens, C. E., Riccio, E., Parman, T., Munde, M., Mandal, S., Madhubala, R., Tidwell, R. R., Wilson, W. D., Boykin, D. W., Hall, J. E., Kyle, D. E. and Werbovetz, K. A. (2010). Novel arylimidamides for treatment of visceral leishmaniasis. *Antimicrobial Agents and Chemotherapy* **54**, 2507–2516.

Werbovetz, K. (2006). Diamidines as antitrypanosomal, antileishmanial and antimalarial agents. *Current Opinion in Investigational Drugs* **6**, 147–157.

Wilson, W. D., Tanious, F. A., Mathis, A., Tevis, D., Hall, J. E. and Boykin, D. W. (2008). Antiparasitic compounds that target DNA. *Biochimie* **90**, 999–1014.

Witola, W. H., Inoue, N., Ohashi, K. and Onuma, M. (2004). RNA-interference silencing of the adenosine transporter-1 gene in *Trypanosoma evansi* confers resistance to diminazene aceturate. *Experimental Parasitology* **107**, 47–57.