Veterinary Parasitology 177 (2011) 157–161

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Evaluation of three novel azasterols against *Toxoplasma gondii*

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ARTICLE INFO

Article history:
Received 24 May 2010
Received in revised form
19 November 2010
Accepted 23 November 2010

Keywords:
Azasterols
Toxoplasmosis
Chemotherapy
Ultrastructure

ABSTRACT

Previous studies from our group have demonstrated the high susceptibility of *Toxoplasma gondii* tachyzoites to the sterol analogues 22,26-azasterol and 24,25-(R,S)-epiminolanosterol. In this work we present data on testing in vitro three novel azasterols as potential agents for the treatment of toxoplasmosis. The three compounds inhibited parasite growth at micromolar concentrations, in a dose-dependent manner. Electron microscopy analysis of intracellular tachyzoites after treatment with the most effective compound showed drastic mitochondrion swelling associated with the appearance of an electron-lucent matrix and disrupted cristae. Parasite lysis also took place. The appearance of electron dense cytoplasmic structures similar to amylopectin granules distributed throughout the parasite suggests that azasterols might be inducing differentiation of those tachyzoites which were not lysed to the bradyzoite stage.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite found throughout the world. It infects members of the Felidae family and a wide range of animals, including humans (Tenter et al., 2000). The infection in humans is usually asymptomatic but can cause great morbidity and mortality in imunocompromissed or congenitally infected individuals. The disease in sheep is responsible for abortion and neonatal deaths, causing economic losses (Dubey and Jones, 2008). The most effective current therapy for the treatment of toxoplasmosis is the synergistic combination of pyrimethamine and sulfadiazine, which blocks both folic acid biosynthesis and folic acid metabolism. Folinic acid is added to the regimen to reduce the risk of bone marrow suppression (Remington et al., 2006). Although very effective, this treatment is commonly associated with many adverse effects and has no efficacy against tissue cysts, leading to the possibility of recurrence after treatment (Montoya and Liesenfeld, 2004). Thus the search for new chemotherapeutic drugs for the treatment of toxoplasmosis is very important.

Azasterols, initially developed as inhibitors of the sterol biosynthesis enzyme $\Delta^{24(25)}$-sterol methyl transferase (SMT), have been shown to have activity against many protozoan parasites, including *Trypanosoma cruzi*, *Leishmania* sp. (Rodrigues et al., 2002; Magaraci et al., 2003; Gros et al., 2006a) and *Giardia lamblia* (Maia et al., 2007). Although *T. gondii* lacks the sterol biosynthetic pathway (Coppens et al., 2000) two azasterols, inhibitors of the enzyme SMT, were able to inhibit proliferation of *T. gondii* and induce several ultrastructural changes (Dantas-Leite et al., 2004). The mode of action of the azasterols against *T. gondii* is still unknown. In this report, we present data for three novel

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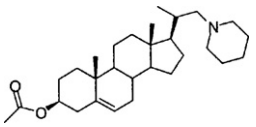
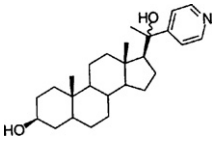
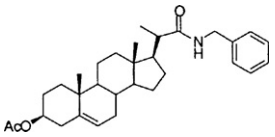
doi:10.1016/j.vetpar.2010.11.034

Table 1
Growth inhibition of *T. gondii* by azasterol analogues after 24 and 48 h of treatment.[a]

Compound	Structure	IC_{50} (μM) 24 h	48 h
1		>5	4.7 ± 1.7
2		3.8 ± 1.2	3.1 ± 0.7
3		2.6 ± 0.2	0.80 ± 0.5

[a] Results are expressed as mean ± standard error of three different experiments.

azasterols which showed selective activity against *T. gondii* in infected LLC-MK_2 cells.

2. Methodology

Tachyzoites from the virulent RH strain of *T. gondii*, isolated from human brain (Sabin, 1941) were used in the in vitro experiments and were maintained by intraperitoneal (i.p.) passages in Swiss mice. The cystogenic Me49 strain of *T. gondii*, was isolated from sheep (Lunde and Jacobs, 1983), and was used as a control for the carbohydrate detection experiments.

Preparation of compounds **1**–**3** has previously been described (Magaraci et al., 2003; Lorente et al., 2005). Compound **1** is compound **9b** in the original reference (Lorente et al., 2005); compound **3** is compound **3c** (Lorente et al., 2005); and compound **2** is compound **10** (Magaraci et al., 2003). The compounds were dissolved in dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany) and added directly to the growth medium; the final concentration of DMSO in the medium never exceeded 0.1% (v/v) and had no effect either on the proliferation of intracellular parasites or on the host cells (data not shown).

LLC-MK_2 cell cultures (kidney, Rhesus monkey, *Macaca mulata* – ATCC CCL7, Rockville, MD/EUA) were maintained in RPMI medium with 5% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2. For the in vitro anti-proliferative assays approximately 5×10^5 cells were cells placed in a 24-well tissue culture plate one day before. The cells were infected with freshly obtained parasites, re-suspended in RPMI without fetal bovine serum (FBS) at a ratio of 3:1 parasite/host cell. Tachyzoites were allowed to interact for 1 h and then the cell monolayers were washed twice with phosphate-buffered saline (PBS) to remove non-adherent extracellular parasites. Different concentrations of azasterols were added to the infected cells 6 h after infection and incubated for 24 or 48 h at 37 °C (assays were performed in triplicate). The parasite proliferation was evaluated using selective [5,6-^{3}H] uracil incorporation by the parasites (Pfefferkorn and Pfefferkorn, 1977). Thus, after treatment, 0.2 μCi of [5,6-^{3}H]uracil/well (specific activity 42 Ci/mmol; Amersham Biosciences UK Limited) was added to the infected cultures and incubated for an additional 4 h. Uracil incorporation by parasites was evaluated by liquid scintillation and was carried out as previously described (Martins-Duarte et al., 2008). For IC_{50} (concentration for 50% parasite growth inhibition) calculations, the percentage of growth inhibition was plotted as a function of the drug concentration by fitting the values to non-linear curve analysis. The regression analyses were performed using Sigma Plot 8.0 software (Systat Software Inc., Chicago, IL, USA).

Morphological changes induced by the compounds on the ultrastructure of *T. gondii* tachyzoites were examined by transmission electron microscopy. For these experiments LLC-MK_2 cultures were infected with tachyzoites at a ratio of 5:1 parasites/host cell. Infected cells (controls or treated with the azasterols) were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Cells were post-fixed for 1 h in the dark with a solution containing 1% osmium tetroxide, 1.25% potassium ferrocyanide and 5 mM $CaCl_2$, in 0.1 M sodium cacodylate buffer (pH 7.4). Samples were dehydrated with increasing concentrations of acetone, and then embedded in PolyBed (Polyscience Inc., Warrington, PA, USA). Ultrathin sections were stained with uranyl acetate and lead citrate and then observed using a Zeiss 900 Electron Microscope (Carl Zeiss, Inc.).

For detection of polysaccharide inclusions, ultrathin sections of samples prepared for transmission electron microscopy, as described above, were processed for cytochemical detection of carbohydrates (Thiéry, 1967). Tissue cysts were used as a positive control for amylopectin granules. Cysts were obtained from mice previously infected with *T. gondii* strain Me49 for at least 4 weeks, based on the protocol established by Freyre (1995). Ultrathin sections collected on 200-mesh gold grids were incubated in 1% periodic acid for 30 min, washed in distilled water and incubated with 1% thiosemicarbazide in 10% acetic acid for 72 h. Next, the sections were washed in 10%, 5% and 2% acetic acid and 3 times in distilled water for 10 min. Afterwards, the sections were incubated for 30 min with 1% silver proteinate in the dark and washed abundantly in distilled water. For control assays, periodic acid was omitted. The sections were observed in a Jeol 1200 EX transmission electron microscope operating at 80 kV.

For imunofluorescence assays, LLC-MK2 cells infected with tachyzoites at a ratio of 3:1 parasite/host cell were treated with compounds **1**, **2** or **3** for 48 h. At the end of treatment, infected cells were fixed in 3.7% freshly prepared formaldehyde, permeabilized with 0.5% Triton X-100 for 15 min and blocked with 3% bovine serum albumine in PBS pH 7.4 for 1 h at room temperature. Cells were then incubated for 1 h in the presence of *Dolichos biflorus* lectin conjugated with FITC (DBA-FITC) 10 μg/ml (Sigma–Aldrich Co., St. Louis, MO, USA). After lectin labeling, the coverslips

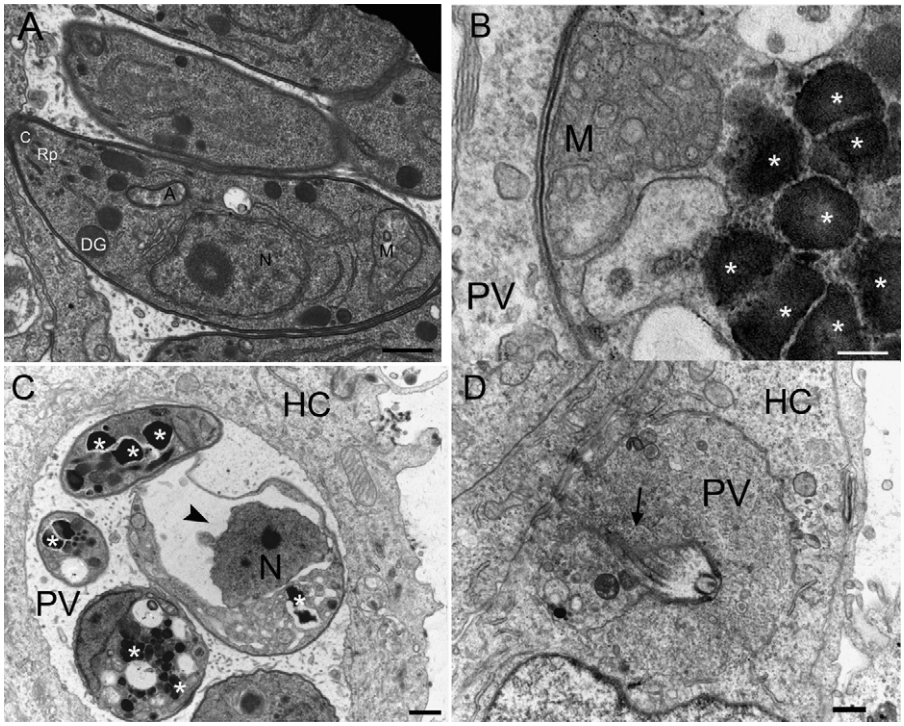


Fig. 1. Effect of azasterol treatment in the ultrastructure of *T. gondii*-infected LLC-MK_2 (A) *T. gondii* tachyzoites in the absence of treatment, showing the characteristic features of parasite morphology. C: conoid, Rp: rhoptry, DG: dense granule, A: apicoplast, M: mitochondrion, N: nucleus. (B) Swelling of *T. gondii* mitochondrion (M) and the appearance of electron-dense structures in the cytoplasm (*) can be observed after treatment with 3 μM compound 1 for 48 h. (C) A large vacuole (arrowhead) can be seen in one of the tachyzoites after treatment with 3 μM compound 3 for 48 h. The appearance of amylopectin-like structures (*) can also be seen. (D) Lyses of a tachyzoite after treatment with 3 μM compound 3 for 48 h. The conoid can be identified (arrow). PV: parasitophorous vacuole, HC: host cell. Bars = 500 nm.

were mounted and observed in a Zeiss Axioplan microscope using the fluorescein filters.

3. Results and discussion

The azasterols inhibited *T. gondii* proliferation with IC_{50} values in the micromolar range. Table 1 shows the in vitro anti-proliferative activity of the azasterols. Compound **3** was the most active, showing an IC_{50} at nanomolar range after 48 h. The anti-proliferative activity range of the new compounds (0.8–4.7 μM) was of the same order as that previously obtained by our group for 22,26-azasterol and 24,25-(R,S)-epiminolanosterol (Dantas-Leite et al., 2004). These results confirm that azasterols can cause growth inhibition of *T. gondii*, across a variety of different structural types. Interestingly, compounds do not necessarily need to have a basic nitrogen as can be seen from compounds **2** and **3**, which has implications for the mode of action.

In order to investigate the selective effect of the azasterols against *T. gondii* over host cell, LLC-MK_2 cell monolayers were treated for 48 h with the compounds at concentrations five times higher than the IC_{50} obtained

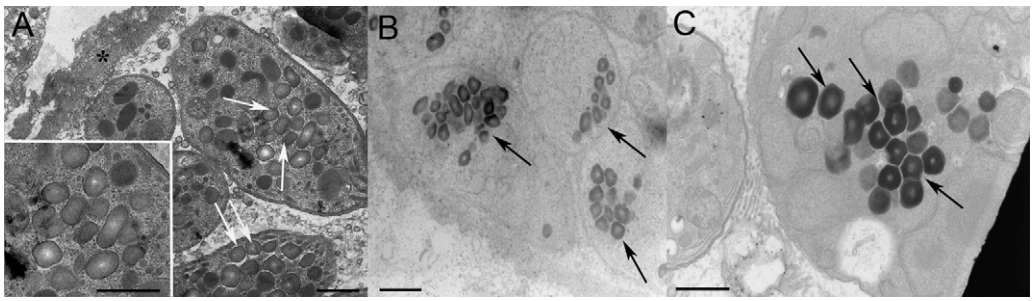


Fig. 2. Electron micrographs showing polysaccharide granules after treatment of infected cells with 3 μM compound 3 for 48 h. (A) Control preparation of cyst containing bradyzoites showing several amylopectin granules (white arrows) in the parasite cytoplasm. Cyst wall (asterisk). Inset: higher magnification of the granules. (B) Bradyzoite processed for the detection of polysaccharide granules (black arrows) following the Thiery technique. (C) Tachyzoite treated with 3 μM compound 3 and processed for Thiéry technique. Polysaccharide granules (black arrows). Bars = 500 nm.

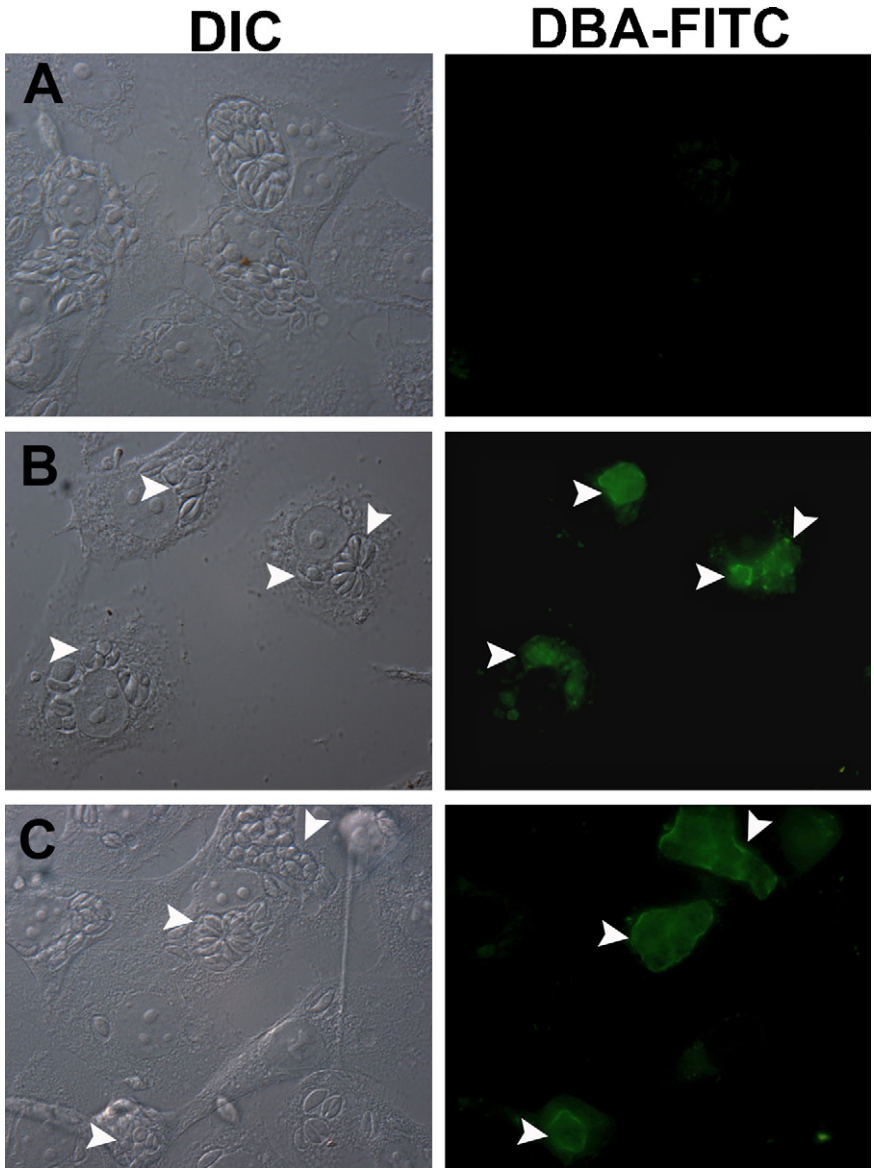


Fig. 3. Fluorescence microscopy of tachyzoites of *T. gondii* stained with DBA-FITC after treatement with azasterols: control (A), 3 μM compound **2** (B), 1 μM compound **3** (C). Treatment with azasterol led to the staining of parasitophorous vacuole with DBA-FITC, demonstrating that the compounds are possibly inducing the formation of cysts. Arrow head: vacuoles stained for DAB-FITC.

after 48 h. The analysis by dye exclusion test with 0.4% Trypan blue in PBS, demonstrated that only less than 5% of cells treated with azasterols lost their viability. This result was similar to that obtained for control cells, demonstrating the selective effect of azasterols on *T. gondii* over host cells. Previous toxicity investigation using KB cells also demonstrated that compounds **1**–**3** are well-tolerated by animal cells and selective against protozoan parasites (Magaraci et al., 2003; Lorente et al., 2005).

The morphological changes observed by electron microscopy demonstrated that compounds **1** and **3** had similar effects on the parasite, when compared with control preparations (Fig. 1A), causing: mitochondrial swelling (Fig. 1B); appearance of large granules (Fig. 1B and C, asterisks), morphologicaly similar to the amylopectin granules found in the bradyzoite stage (Fig. 2A); and parasite lysis (Fig. 1C). The carbohydrate nature of these granular inclusions was confirmed using the technique established by Thiéry (1967). In this experiment, the tissue cyst bradyzoites were used as a positive control, as they are known to contain carbohydrate granules (Fig. 2B). The granules in the azasterol-treated tachyzoites (Fig. 2C) stained positively in this assay, confirming that they were of carbohydrate nature. In a further experiment, azasterol-treated cultures were stained with DBA-FITC (a lectin from *Dolichos biflorans*) (Fig. 3A–C), which specifically binds cyst wall N-acetyl-galactosamine residues which are found in bradyzoites (Zhang et al., 2001), but not in the tachyzoites

parasitophorous vacuole. The treatment for 48 h with compounds **2** and **3** in concentrations near to the IC_{50} led to the positive staining of many vacuoles (Fig. 3B and C), indicating the presence of cysts forming in response to azasterol treatment.

Taken together, these results suggest that the azasterols could possibly induce the conversion of tachyzoites to bradyzoites. This differentiation to bradyzoite stage could be an adaptive response of the parasite to the compounds, as the bradyzoite stage is less susceptible to drugs (Araujo et al., 1991). Thus, tachyzoites that did not succumb to treatment with azasterols might be differentiating to bradyzoites as a mechanism to escape lysis. This is consistent with previous work that has demonstrated differentiation to the bradyzoite stage can be stimulated by drug pressure (Tomavo and Boothroyd, 1995; Gross and Pohl, 1996).

The molecular target for these compounds in *T. gondii* is not known; a possible mode of action may be the similarity of the sterol core in both azasterols and cholesterol molecules, leading to indiscriminate uptake of these compounds by the parasite. The excess of intracellular sterol molecules can cause many deleterious effects, principally to membranes (Urbina et al., 1995). Many morphological changes observed in *T. gondii* by ultrastructural analysis are associated with damage to cellular membranes, such as mitochondrial swelling and rupture of the parasite plasma membrane. Another possibility, which deserves further investigation, is that azasterols may have an effect on methylation during phospholipid biosynthesis (Palmié-Peixoto et al., 2006). Similarly, the effect of azasterols against the bloodstream form of *Trypanosoma brucei rhodesiense*, which utilises host sterols (Coppens and Courtoy, 2000), also demonstrated that, these compounds can inhibit the growth of these protozoa by a mechanism of action other than inhibition of ergosterol biosynthesis (Gros et al., 2006b). Our results demonstrate that azasterols are very active and selective against *T. gondii* in vitro and suggest further investigation of this class of molecules as potential agents against toxoplasmosis.

Acknowledgements

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Programa de Núcleos de Excelência-Pronex-Faperj-CNPq.

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