Antiproliferative activities of two novel quinuclidine inhibitors against *Toxoplasma gondii* tachyzoites *in vitro*

Érica S. Martins-Duarte¹, Julio A. Urbina², Wanderley de Souza¹ and Rossiane C. Vommaro¹*

¹Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, CCS Universidade Federal do Rio de Janeiro, 21949-900-Rio de Janeiro–RJ, Brazil; ²Laboratório de Química Biológica, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020A, Venezuela

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**Objectives:** To study the antiproliferative effects of ER119884 and E5700, two quinuclidine-based inhibitors of squalene synthase (SQS), against *Toxoplasma gondii* tachyzoites in epithelial cells.

**Methods:** The antiproliferative effects of the quinuclidine derivatives, alone or in combination with epiminolanosterol or antifolates, were analysed, resulting in the construction of isobolograms. The ultrastructure of treated tachyzoites was analysed by transmission electron microscopy.

**Results:** The quinuclidine derivatives demonstrated selective anti-*T. gondii* activity, arresting parasite growth with IC₅₀ values of 0.66 and 0.23 mM for ER119884 and E5700, respectively, after 24 h of interaction and 0.44 and 0.19 mM after 48 h of interaction. Both compounds induced remarkable alterations in the parasite ultrastructure, such as mitochondrial swelling and the presence of autophagosome-like structures, after 24 h of treatment. Combination of these quinuclidine derivatives with the antifolates sulfadiazine and pyrimethamine produced a synergic effect. When epiminolanosterol was combined with E5700, the effect observed was synergic, whereas the combination with ER119884 produced no interaction.

**Conclusions:** E5700 and ER119884 demonstrated selective activity against *T. gondii* tachyzoites and are a possible alternative to be used in association with the current therapy. The ultrastructural alterations observed suggest a possible interference with lipid metabolism.

Keywords: Apicomplexa, chemotherapy, ultrastructure, antifolates, sterol biosynthesis inhibitors

**Introduction**

*Toxoplasma gondii* is the aetiologic agent of toxoplasmosis, a widespread disease worldwide. Toxoplasmosis affects principally immunocompromised individuals and newborns with congenital disease. The clinical manifestations of congenital toxoplasmosis include hydrocephalus, chorioretinitis and blindness.¹,² Toxoplasmosis in immunocompromised individuals can be the result of the reactivation of chronic (latent) infection.³ The presence of the parasite in the CNS causes necrotizing encephalitis and in AIDS patients it is the main cause of focal cerebral lesions.⁴,⁵ In immunocompetent individuals the disease is unnoticed in most of the patients, becoming latent with the formation of cysts principally in the CNS.

The mainstays of toxoplasmosis chemotherapy are antifolate drugs, such as the combination of pyrimethamine and sulfadiazine. Although this therapy is frequently successful, it is associated with many side effects including bone marrow suppression, minimized by concomitant administration of folic acid.⁶ However, sometimes the discontinuation of antifolate treatment is necessary and in cases of immunocompromised individuals the disease can be life-threatening requiring replacement therapy with another regimen. The sulfa component is also frequently not well tolerated, requiring its substitution by other drugs such as clindamycin.⁷ Therefore, the development of alternative single or combination therapies is necessary.

Sterol biosynthesis inhibitors (SBIs) azasterols have already been demonstrated to have a selective effect on *T. gondii* proliferation, when used alone and in combination with antifolates.⁸,⁹ A new class of SBIs, squalene synthase (SQS) inhibitors, has been tested, showing promising anti-trypanosomatid activity *in vitro* and *in vivo*.¹⁰–¹³ In this work we evaluated two quinuclidines,

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*Corresponding author. Tel/Fax: +55-21-2260-2364; E-mail: vommaro@biof.ufrj.br

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ER119884 and E5700, inhibitors of SQS, against proliferation of *T. gondii in vitro*.

**Materials and methods**

**Parasites and host cells**

Tachyzoites from the virulent RH strain of *T. gondii* were used in this study and were maintained by intraperitoneal passages in Swiss mice. After 48 h of infection the parasites were collected in phosphate-buffered saline (PBS) at pH 7.2. The ascitic fluid obtained from infected mice was centrifuged at 200 g for 10 min at room temperature to remove cells and debris. The supernatant, which contained the parasites, was collected and centrifuged at 1000 g for 10 min. The pellet obtained was washed with PBS at pH 7.2 and then in RPMI medium without fetal bovine serum (FBS). The parasites were used within 30–40 min of their removal from the peritoneal cavity. The experimental protocol was approved by the Instituto de Biofísica Carlos Chagas Filho (Universidade Federal do Rio de Janeiro) Ethics Committee for animal experimentation.

LLCMK2 cell cultures (kidney, Rhesus monkey, *Macaca mulata*) were maintained in RPMI medium with 5% FBS at 37°C in an atmosphere of 5% CO2. The viability of the parasite and host cell during the experiments was determined as described previously. For these experiments LLCMK2 cultures were infected with *T. gondii* and had no effect on the proliferation of intracellular parasites. The viability of the parasite and host cell during the experiments was determined as described previously. After 48 h of infection the parasites were collected in phosphate-buffered saline (PBS) at pH 7.2. The viability of the parasite and host cell was determined as described previously. For these experiments LLCMK2 cultures were infected with *T. gondii* and had no effect on the proliferation of intracellular parasites. The viability of the parasite and host cell during the experiments was determined as described previously. Antiproliferative assays

Approximately 2 x 10⁴ LLCMK2 cells/well were placed in a 24-well tissue culture plate 2 days before the assay. The cells were infected with freshly obtained parasites and resuspended in RPMI without FBS at a multiplicity of 3:1 parasite/host cell. Tachyzoites were allowed to interact for 1 h and then the cell monolayers were washed twice with PBS to remove non-adherent extracellular parasites. The drugs were added to the infected cells after 6 h of interaction. After treatment, samples were fixed with Bouin, stained with Giemsa and observed in a light microscope. The percentage of infected cells and the parasite proliferation index were determined by examination of at least 400 cells of two different coverslips. Data shown are representative of three experiments. The parasite proliferation index was determined by multiplying the percentage of infected cells by the total number of intracellular parasites per total number of cells.

**Effect of the drugs on the ultrastructure of *T. gondii*-infected cells**

For these experiments LLCMK2 cultures were infected with parasites at a multiplicity of 5. The control and drug-treated cells 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, 0.8% potassium ferrocyanide and 5 mM CaCl2, in 0.1 M sodium cacodylate buffer (pH 7.4). Dehydration was carried out with acetone; samples were embedded in Polybed (Polyscience Inc.). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Jeol 1200 electron microscope.

**Results and discussion**

**Antiproliferative effects of quinuclidine derivatives**

The drugs added after 6 h of infection in LLCMK2 cell cultures were capable of reducing *T. gondii* tachyzoite proliferation in a dose-dependent manner (Figures 1 and 2). The IC₅₀ values were 0.66 ± 0.1 and 0.44 ± 0.08 µM for ER119884 and 0.23 ± 0.06 and 0.19 ± 0.03 µM for E5700 after 24 and 48 h of interaction, respectively. We also tested the antiproliferative effects after different times (1–24 h) of infection: both drugs maintained their antiproliferative activity even 12 h post-infection. When the drugs were added after 24 h, reductions of 58% and 48% were observed with 3 µM E5700 and ER119884, respectively, demonstrating that the drugs could efficiently reduce parasite burdens after the infection is fully established (Figure 3). However, if the drugs at the same concentrations were added 6 h post-infection and removed from the growth medium after 42 h of incubation, it was found that the parasites began to grow normally, showing that at this concentration the drugs had a static rather than cidal effect or that there was a remaining subpopulation of parasites not affected by the treatment under these conditions.

The IC₅₀ values of the two quinuclidines derivatives observed in this work against *T. gondii* were somewhat higher than those previously reported against *Trypanosoma cruzi*, but in both studies the compounds were active at submicromolar levels and E5700 was the most active one. The results obtained, although preliminary, demonstrated that the novel quinuclidine derivatives have antiproliferative activity against *T. gondii* at lower concentrations than those of the
standard drugs, sulfadiazine and pyrimethamine (IC\textsubscript{50} 16.4/0.067 μM after 48 h), under identical experimental conditions. Despite the fact that quinuclidine derivatives inhibit mammalian SQS\textsuperscript{12,18–23} the treatment with these drugs did not provoke any alteration in the morphology or loss of viability (data not shown) in host cells submitted to concentrations five times higher than the maximum concentration used during parasite antiproliferative tests. As discussed elsewhere\textsuperscript{12} this fact is probably explained by the capacity of the host cells to compensate for the blockade of \textit{de novo} cholesterol synthesis by up-regulating the expression of low-density lipoprotein (LDL) receptors and taking this sterol from the growth medium. The drugs were demonstrated to be well tolerated by mice in \textit{in vivo} tests\textsuperscript{12}.

Although the quinuclidine derivatives had excellent effects against \textit{T. gondii} at submicromolar levels, the mechanism of action is still unclear. A search in the \textit{T. gondii} database

**Quinuclidine inhibitors against \textit{T. gondii}**

Figure 1. Effect of E5700 on proliferation of \textit{Toxoplasma gondii} tachyzoites. The drug was added to LLCMK\textsubscript{2} monolayers after 6 h of infection. Control, closed circles; E5700 0.1 μM, closed squares; E5700 1.0 μM, closed triangles; E5700 3 μM, crosses. Parasite proliferation index (see Materials and methods) was determined by light microscopy evaluation of duplicated cover slips. Results are expressed as means (±SD) of three experiments.

Figure 2. Proliferation of \textit{Toxoplasma gondii} tachyzoites in the presence of ER119884. The drug was added to the cultures after 6 h of infection. Control, closed circles; ER119884 0.1 μM, closed squares; ER119884 1.0 μM, closed triangles; ER119884 3 μM, crosses. Results are expressed as means (±SD) of three experiments.

Figure 3. E5700 and ER119884 inhibited \textit{T. gondii} proliferation when added after different times of infection. LLCMK\textsubscript{2} monolayers infected with \textit{T. gondii} were incubated, after 1, 6, 12 and 24 h of infection, with 3 μM E5700 (white bars) and 3 μM ER119884 (black bars) or without any drugs (grey bar). The effect was evaluated 48 h post-infection. Results are expressed as means of three different experiments.
Figure 4. Ultrathin sections of Toxoplasma gondii-infected LLCMK2 cells in the absence and presence of the quinuclidines. In (a) typical structures of tachyzoites, such as rhoptry (R), dense granules (DG) and mitochondria (M), can be observed. (b and c) show infected cells treated with ER119884 3 µM. In (b) several swelled mitochondria (M) profiles are spread for the whole cell. In (c) an autophagosome-like structure can be seen with different cellular structures inside (*). (d, e and f) show infected cells treated with 3 µM E5700 for 24 h. In (d) one of the tachyzoites presented disorganized cytoplasm, several swelled profiles of the mitochondria (M) and ruptured pellicle (arrow head). In (e) the tachyzoite presented single membrane (arrow) and deformed intumescent mitochondria (M). In (f) whorls of membrane (*) appeared inside a large vacuole originating from disarrangement of endoplasmic reticulum (arrow head). HC, host cell; N, nucleus; R, rhoptry; M, mitochondrion; DC, daughter cell. Bar, 0.5 µm.
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Ultrastructure alterations induced by quinuclidine derivatives

Electron microscopy analysis demonstrated many alterations in the ultrastructure of tachyzoites treated with quinuclidine derivatives. In contrast with untreated parasites (Figure 4a), tachyzoites treated with 3 μM ER119884 displayed an abnormal vacuolization of the parasite cytoplasm, mitochondrial swelling, disrupted cristae and the appearance of autophagosome-like structures after 24 h (Figure 4b and c). The presence of lamellar bodies in the parasitophorous vacuole was continuously observed (data not shown). However, when the infected cells were treated with 3 μM E5700 more remarkable effects were observed after 24 h of treatment: the parasites were seen with portions of their pellicle, a system composed of a plasma membrane unit and an inner membrane complex composed of two lipid bilayers, disarranged (Figure 4d). This effect could be caused by an alteration in the physicochemical properties of this membrane system, making it more susceptible to disruption. Mitochondrial swelling and the presence of whorls of membrane profiles in vacuoles were also visualized (Figure 4d–f). The enlargement of Golgi complex cisternae (data not shown) and complete cytoplasm disorganization could frequently be observed in *T. gondii* treated with both drugs.

Treatment of infected cells with quinuclidine derivatives did not seem to affect the parasitophorous vacuole membrane. The ultrastructural alterations did not pinpoint any specific primary site of action of the drugs in *T. gondii*, although effects on membranes of cellular structures appear even in shortest times of treatment. It is likely that components of parasite membranes are being affected. Therefore a biochemical investigation of the neutral and polar lipid profiles is currently underway by our group in order to determine the possible molecular target.
Effects of quinuclidine derivatives in combination with EIL

EIL is a known inhibitor of Δ^2(25) sterol methyl transferase (SMT). The antiproliferative effects of this compound against *T. gondii* tachyzoites and other protozoan parasites have been demonstrated in previous studies. In the present study we sought to verify the anti-*T. gondii* activity of quinuclidine derivatives in combination with EIL.

EIL alone inhibited parasite growth with an IC₅₀ of 0.36 ± 0.10 μM. When the SMT inhibitor was combined with 0.1 μM ER119884 the EIL IC₅₀ was reduced to 0.132 ± 0.028 μM, producing a slightly concave isobologram, with an FICI of 0.59 ± 0.08, indicating the absence of interaction between these drugs (Figure 5, left-hand panel). The association with 0.04 μM E5700 was able to reduce the IC₅₀ of EIL from 0.36 to 0.091 ± 0.023 μM; this drug combination produced a fully concave isobologram, with an FICI of 0.46 ± 0.06, demonstrating synergic effects (Figure 5, right-hand panel). The molecular mechanism of EIL, as for quinuclidines derivatives, is still unknown, but the results obtained here show that these drugs possibly affect different molecular targets, otherwise a synergic effect would not be observed.

Effect of the association of quinuclidine derivatives and antifolates

The effects of the combination of ER119884 and E5700 with antifolates were also studied. The IC₅₀ obtained for sulfadiazine/pyrimethamine was 16.4 ± 4.2 μM for sulfadiazine and 0.067 ± 0.016 μM for pyrimethamine after 48 h of treatment. The association of quinuclidine derivatives with sulfadiazine/pyrimethamine induced a remarkable enhancement of their antiproliferative activities; both compounds acted synergically with antifolates. The IC₅₀ of sulfadiazine/pyrimethamine decreased to 3.24 ± 0.36 μM/12.87 ± 1.43 nM with the addition of 0.04 μM E5700, with an FICI of 0.34 ± 0.09 (Figure 6, left-hand panel). The presence of 0.1 μM ER119884 reduced the sulfadiazine/pyrimethamine IC₅₀ to 3.3 ± 0.13 μM/13.19 ± 0.52 μM, with an FICI of 0.43 ± 0.01 (Figure 6, right-hand panel). These results are similar to those reported previously for the association of EIL with sulfadiazine/pyrimethamine and suggest the possibility of lowering the antifolate doses when used in combination with novel drugs, such as SBIs, thus reducing the side effects associated with this therapy without loss of efficacy.

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Transparency declarations

None to declare.

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