Proliferation of *Toxoplasma gondii* (RH strain) is inhibited by the combination of pravastatin and simvastatin with low concentrations of conventional drugs used in toxoplasmosis


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**ABSTRACT**

*Toxoplasma gondii*, an etiologic agent of toxoplasmosis, is an obligate intracellular parasite, which exhibits an apicoplast organelle which assists in the metabolism of isoprenoids and other pivotal mediators for the parasite survival. Statins are drugs that inhibit cholesterol synthesis, blocking the conversion of the substrate HMG-CoA to mevalonate, thus preventing the initial processes of the biosynthesis of these precursors, both in humans and parasite. In the light of this information, we determined the effect of pravastatin and simvastatin associated with the current drugs (pyrimethamine and sulfadiazine) as a possible alternative treatment for this infection. Cytotoxicity was evaluated in HeLa cells by MTT assay, which was observed the drug combinations did not affect cell viability. HeLa cells (10⁵) were infected with *T. gondii* tachyzoites of RH strain (5 × 10⁵/C2) and treated with pravastatin and/or simvastatin combined with pyrimethamine and/or sulfadiazine for 24 h. Our data showed a significant reduction in cell adhesion, infection and mainly parasite proliferation in all treatments. Based on these results, the combination of statins with drugs used in current therapy showed to be a promising therapeutic alternative for toxoplasmosis treatment.

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**Introduction**

*Toxoplasma gondii* is a protozoan parasite from phylum Apicomplexa, which causes the toxoplasmosis infection, a disease that in most cases is asymptomatic, but can become severe and fatal in immunocompromised patients or congenital infection (Cenci-Goga et al., 2011; Dubey et al., 2012; McAuley, 2014).

The recommended treatment for toxoplasmosis is the combination of pyrimethamine and sulfadiazine, which act synergistically in blocking folate synthesis pathway, essential for the survival and replication of parasite (Anderson, 2005). However, this therapy has various side effects, including bone marrow suppression, which causes megaloblastic anemia, leukopenia, and granulocytopenia, which justifies the search for more effective therapeutic alternatives (Petersen, 2007).

*T. gondii* presents an organelle called apicoplast responsible for the biosynthesis of relevant mediators such as fatty acids and isoprenoids (Carruthers and Tomley, 2008; Seeber and Soldati-Favre, 2010). This pathway has been the subject of studies in the alternative therapy of toxoplasmosis by compounds that act on it.

Some studies have shown that statins, drugs used in hypercholesterolemia treatment due to the inhibitory role of cholesterol synthesis in the human body, present promising results in experimental infection with *T. gondii* (Cortez et al., 2009; García-Sabina et al., 2012).

Despite the fact that protozoan *T. gondii* does not promote cholesterol synthesis, there is evidence that isoprenoid synthesis...
occurs by via 1-deoxy-\textit{n}-xylulose-5-phosphate (DOXP) pathway in the apicoplast organelle, which is essential for several further functions in the parasite (growth control, transport of electrons, mitochondrial and rRNA synthesis). Thus, studies have shown that statins, besides acting on host cholesterol metabolism, can also act in apicoplast, interfering on fatty acids and cholesterol of the parasite, being able to act directly or indirectly in the inhibition of parasite replication (Coppens, 2014; Lim et al., 2010; Nishikawa et al., 2011; Qidwai and Khan, 2012).

A previous study has demonstrated that pravastatin showed an inhibitory activity in the \textit{Leishmania amazonensis} proliferation assay (Kuckelhaus et al., 2011). In addition, another study using simvastatin and pravastatin individually or in an association presented an inhibitory activity in parasite \textit{T. gondii} (RH strain) in a HeLa cells experimental infection model (Sanfelice et al., 2017).

Simvastatin (Cortez et al., 2009) and atorvastatin (Li et al., 2013) have been evaluated in a culture assay of macrophages infected with \textit{T. gondii}, decreasing the multiplications of parasites in \textit{in vitro} infection. Furthermore, Kessler et al. (2013) demonstrated that a treatment with lovastatin induces morphological changes in \textit{Trypanosoma cruzi} with consequent death of the protozoan.

The aim of this study was to verify the effect of statins – pravastatin and simvastatin associated with low concentrations of pyrimethamine and sulfadiazine, in order to improve the mechanism of action in toxoplasmosis therapeutic besides of decrease the toxicity present in the current drugs of toxoplasmosis treatment.

Materials and methods

HeLa cells culture

HeLa cells derived from a human uterine cervix tumour were acquired from the American Type Culture Collection (Manassas, VA, USA) and were maintained in culture in Medical Mycology of the RH strain of \textit{T. gondii}. After the treatments, the medium was replaced with fresh medium – 10 \textmu l of MTT (5 mg/ml) and the cells were incubated for another 3 h under the usual conditions. The medium was removed and the crystals solubilized in 10\% sodium dodecyl sulfate (SDS) (Sigma Chemical Co., Brazil) in 50\% dimethyl formamide (DMF) (Sigma Chemical Co.). After 30 min, absorbance was read at 570 nm in a microplate reader (Thermo Plate – TP-Reader). The results were expressed as a percentage relative to MTT reduction in the control group calculated with following formula:

% viable cells = \( \frac{\text{Abs of treated cells}}{\text{Abs of non-treated cells}} \times 100 \).

**Experimental infection**

The effect of pravastatin and simvastatin on adhesion, invasion and intracellular proliferation of \textit{T. gondii} in HeLa cells was evaluated using an experimental infection with tachyzoites of \textit{T. gondii} in HeLa cells with post treatment for 24 h.

Briefly, HeLa cells \( \times 10^5 \) maintained in 24-well plates containing 13 mm round coverslips (Cienorc Scientific) were infected with \( 5 \times 10^5 \) tachyzoites of \textit{T. gondii} RH strain. After 3 h of infection, the cells were washed and received the treatments according to Table 1. In addition, we used the association of pyrimethamine 8 \textmu g/ml and sulfadiazine 16 \textmu g/ml (Fig. 1).

Cells were fixed in 10\% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and washed in PBS to remove non-fixed material. Cells were stained with 1\% toluidine blue (Sigma Chemical Co.) for 5 min and mounted on glass slides for examination under a light microscope (e100, Nikon – led, magnification 1000 \times). We determined the \textit{T. gondii} infection

Pravastatin and simvastatin

Pravastatin, obtained from Laboratório Catarinense SA (Joinville, Brazil), was diluted in distilled water to 12.8 mg/ml. Simvastatin, obtained from the same company, was diluted in 0.25 M NaOH to 5 mg/ml and kept at 37 °C for 1 h with pH adjusted to 7.4 to cleave the pre-drug and release the active drug (Nyilai et al., 2010).

**HeLa cell viability by MTT assay**

The viability of HeLa cells after treatment with pravastatin and simvastatin was evaluated according to mitochondrial oxidation, by colorimetric tetrazolium salt assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) (MTT) (Sigma Chemical Co., Brazil) (Mosmann, 1983). HeLa cells were grown in 96-well plates \( 3 \times 10^4 \) cells/well/200 μl, for 24 h in CMH medium at 37 °C and 5% CO2. Afterward, the cells were treated according to Table 1. The treated cells were maintained in a CO2 incubator at for 24 h.

Cells with only culture medium served as the negative control. After the treatments, the medium was replaced with fresh medium with 10 \textmu l of MTT (5 mg/ml) and the cells were incubated for another 3 h under the usual conditions. The medium was removed and the crystals solubilized in 10\% sodium dodecyl sulfate (SDS) (Sigma Chemical Co.) in 50\% dimethyl formamide (DMF) (Sigma Chemical Co.). After 30 min, absorbance was read at 570 nm in a microplate reader (Thermo Plate – TP-Reader). The results were expressed as a percentage relative to MTT reduction in the control group calculated with following formula:

% viable cells = \( \frac{\text{Abs of treated cells}}{\text{Abs of non-treated cells}} \times 100 \).

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>Sulfadiazine (50 μg/ml) combined with pyrimethamine (25 μg/ml)</td>
</tr>
<tr>
<td>Negative control</td>
<td>HeLa cells with no treatment</td>
</tr>
<tr>
<td>Statin combination</td>
<td>Pravastatin (12 μg/ml) combined with simvastatin (3.125 μg/ml)</td>
</tr>
<tr>
<td>Pravastatin associations</td>
<td>Pravastatin (12 μg/ml) combined with pyrimethamine (8 μg/ml)</td>
</tr>
<tr>
<td>Pravastatin associations</td>
<td>Pravastatin (12 μg/ml) combined with sulfadiazine (16 μg/ml)</td>
</tr>
<tr>
<td>Simvastatin associations</td>
<td>Pravastatin (12 μg/ml) associated with pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml)</td>
</tr>
<tr>
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</table>

**Treatment approach to different groups to evaluate the effect of combinations of statin pravastatin and simvastatin associated with pyrimethamine and sulfadiazine in HeLa cells infected with \textit{T. gondii} RH strain.**
index (number of infected cells per 200 cells examined), the number of tachyzoites of *T. gondii* adhered per cell and intracellular proliferation of the parasite (parasite intracellular/cell) (Barbosa et al., 2012).

**Statistical analysis**

All data are given as the mean and standard deviation of three independent experiments carried out in triplicate. The differences between treatments and controls were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests, using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was considered when *P* < 0.05.

**Results**

**Combination of pravastatin and simvastatin does not alter HeLa cell viability**

In the first line, we used the pyrimethamine and sulfadiazine concentrations established by Barbosa et al. (2012) in vitro study. We observed that HeLa cells treated with the combination of pravastatin (12 μg/ml), pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml) for 24 h did not affect cell viability compared with positive control (Fig. 2A). The treatment with simvastatin (3.125 μg/ml), pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml) also did not affect the viability of HeLa cells (Fig. 2B). When we assessed the pravastatin and simvastatin combination, we did not see any difference in cell viability compared with negative control (Fig. 2C).

**Pravastatin and simvastatin inhibit adhesion, invasion and intracellular proliferation of *T. gondii* in HeLa cells**

We determined the effects of combinations in processes of adhesion, invasion, and proliferation using *T. gondii* tachyzoites infection model.

HeLa cells experimentally infected with *T. gondii* tachyzoites and treated with pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml) did not present a statistical difference from the negative control in adhesion, invasion, and proliferation of parasite (Fig. 1).

Regarding the associations it was observed that all tested concentrations present significantly reduced adhesion compared to the negative control (treated with only RPMI) (*P* < 0.0001) except when the cells were treated with the combination of pravastatin (12 μg/ml) and sulfadiazine (16 μg/ml) (Fig. 3A).

A significant reduction was also observed in the number of cells infected and treated with the following combinations of pravastatin and pyrimethamine, pravastatin, and sulfadiazine, as well as pravastatin, pyrimethamine, and sulfadiazine, compared to negative control (*P* < 0.0001) (Fig. 3B). When compared to positive control (pyrimethamine (25 μg/ml) associated with sulfadiazine (50 μg/ml)), a reduction in the number of infected cells was observed for the combinations of pravastatin (12 μg/ml) combined with pyrimethamine (8 μg/ml) as well as pravastatin (12 μg/ml), pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml) (*P* < 0.0001) (Fig. 3B).

There was also a significant reduction in the number of tachyzoites per cell when with all combinations of drugs compared to the negative control (*P* < 0.0001) (Fig. 3C). However, when compared to the positive control, the combinations of pravastatin (12 μg/ml) with pyrimethamine (8 μg/ml) (*P* < 0.0001) and...
pravastatin (12 μg/ml) associated with pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml) $(P < 0.0001)$ showed decreased levels of tachyzoites (Fig. 3C).

However, treatment with pravastatin (12 μg/ml) alone showed no significant reduction in the number of infected cells and the number of tachyzoites per cell when compared either to the negative or to the positive control (Fig. 3B and C).

We found that simvastatin (3.1 μg/ml) alone $(P < 0.0001)$ and also pyrimethamine (25 μg/ml) and sulfadiazine (50 μg/ml) $(P < 0.05)$, significantly decreased the number of tachyzoites adhered to cells when compared to the negative control (Fig. 4A). However, the combinations of simvastatin (3.1 μg/ml) and pyrimethamine (8 μg/ml), simvastatin (3.1 μg/ml) and sulfadiazine (16 μg/ml), as well as simvastatin (3.1 μg/ml) associated with pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml), were not effective in eliminating the adhered tachyzoites when compared to the positive control $(P < 0.0001)$ (Fig. 4A).

In turn, all treatments caused a significant decrease in the number of infected cells (Fig. 4B) and in the number of tachyzoites per cells (Fig. 4C), compared to the negative control $(P < 0.0001)$.

In addition, the number of parasites was significantly lower compared to the positive control, when the cells were treated with the combination of simvastatin (3.1 μg/ml) associated with pyrimethamine (8 μg/ml) and sulfadiazine (16 μg/ml) $(P < 0.0001)$ (Fig. 4C).

The Fig. 5 shows that the combination of pravastatin and simvastatin significantly decreased adhesion (Fig. 5A), the number of infected cells (Fig. 5B) and the number of tachyzoites per cell $(P < 0.0001)$ (Fig. 5C), when compared to the negative control. This was not observed when compared to the positive control.

**Discussion**

Statins are a group of drugs indicated for the treatment of hypercholesterolemias that act principally via inhibition of endogenous cholesterol synthesis by acting on the mevalonate pathway (García-Sabina et al., 2012). Regardless of their origin, all statins have in common the heptanoic acid as a pharmacophoric group, which mimics mevalonate (the natural substrate of hydroxymethylglutaryl-CoA-HMG-CoA reductase enzyme) acting as competitive inhibitors of this enzyme, inhibiting the initial processes of isoprenoids and consequently cholesterol biosynthesis (Mason, 2007).

It has been identified that *T. gondii* it produces isoprenoids through DOXP pathway in apicoplast (Coppens, 2013). In addition to using lipid components synthesized in the apicoplast, this protozoan also uses lipid compounds of host cell. In this way, statins present a direct and indirect action in replication of the parasite (Nishikawa et al., 2011; Li et al., 2013).

Therefore, in order to minimize the side effects caused by current therapy, our study evaluated the effect of combination of pravastatin/simvastatin with pyrimethamine and sulfadiazine in lower concentrations compared with positive control, when a higher concentration of pyrimethamine and sulfadiazine was used.

We observed that the effect of pyrimethamine and sulfadiazine on adhesion, on the number of infected cells and on the number of tachyzoites per cell was dependent on the concentration used (Fig. 1).

All combinations using the treatment with simvastatin demonstrated a decreased number of infected cells, mainly using lower concentrations of sulfadiazine and pyrimethamine.
combined. This result corroborates with data obtained in previous HeLa cells study, in which we demonstrated that simvastatin alone also reduced the number of infected cells and intracellular parasites (Sanfelice et al., 2017).

The combination of pravastatin and conventional drugs (pyrimethamine and sulfadiazine) caused a reduction up to 91% in the number of tachyzoites per cell when compared with positive control using higher concentrations of pyrimethamine and sulfadiazine treatment. This information is important since it is expected that lower doses of pyrimethamine and sulfadiazine would be less toxic to the host.

Our results showed a significant reduction in the number of infected cells, and especially in the intracellular parasite proliferation rate in cells treated with pravastatin and/or simvastatin combined with sulfadiazine and pyrimethamine in lower concentrations. We believe that decrease in intracellular proliferation of T. gondii using different combinations was due to a synergic effect of the drugs (statins associated with current therapy) with different mechanisms of action, such as, inhibition of lipid synthesis and blocking folate synthesis, respectively (Anderson, 2005).

Conclusion

Our results demonstrated that the associations with pravastatin and simvastatin presented an inhibitory activity in T. gondii parasite, which is similar to the positive control effect. In addition, some combinations of pravastatin/simvastatin and pyrimethamine and/or sulfadiazine in lower concentrations showed a reduction in the number of infected cells, as well as in the number of tachyzoites per cell. Thus, pravastatin and simvastatin showed up to be a promising alternative for toxoplasmosis treatment. This study is a promising pioneer work, due to the reduction in invasion as well decreased proliferation of parasite, as a consequence of the mechanism of action of two classes of different drugs and reduction of cytotoxic activity. Thus, these drugs together act on important targets for replication and survival of the parasite, either directly by inhibiting DNA synthesis of the parasite or indirectly by suppressing lipid supply.

Conflict of interests

The authors have no conflict of interest to disclose.

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