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Research Paper

Potent anti-*Toxoplasma gondii* activity of 4-chlorophenylthioacetone-derived thiosemicarbazones: Involvement of CCR2 and CCR5 receptors and 5-lipoxygenase in the mode of action

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ABSTRACT

Toxoplasmosis is a disease requiring therapeutic innovation, and thiosemicarbazones with antimicrobial activity are candidates to control *Toxoplasma gondii* infection. Here, the anti-*T. gondii* activities of (*E*)-2-(1-(4-chlorophenylthio)propan-2-ylidene)-hydrazinecarbothioamides (**Ca** and **Cb**) were investigated. *T. gondii*-infected macrophages (MOs) or glial cells treated with **Ca** or **Cb** showed a decrease in the number of intracellular parasites. A deficiency in the chemokine receptor CCR2, but not CCR5, partially reduced anti-*T. gondii* activity induced by **Ca** or **Cb**. In contrast, a deficiency in 5-lipoxygenase (5-LO) activity potentiated anti-*T. gondii* activities induced by these compounds. *In vivo* treatment with **Ca** increased the survival of *T. gondii*-infected wild-type mice, and this was associated with increased IFN-γ and IL-12 production. A deficiency in CCR5 or CCR2 abolished the protective effect of **Ca** treatment *in vivo*, while a deficiency in 5-LO increased **Cb** anti-*T. gondii* effects. Collectively, our data suggest that **Ca** and **Cb** are potential therapeutic candidates for the treatment of toxoplasmosis.

1. Introduction

T. gondii is the causative agent of toxoplasmosis and is an obligate intracellular parasite with global distribution [1,2]. Even though toxoplasmosis is considered asymptomatic in immunocompetent individuals, studies have demonstrated that, due to the neurotropic nature of this parasite, immunocompetent individuals may develop mental and behavioral disorders, with ocular toxoplasmosis causing focal necrotizing retinochoroiditis and myocarditis or polymyositis also occurring in some cases [3]. In immunocompromised patients (e.g., patients with acquired immunodeficiency syndrome, transplant patients, or cancer patients) and newborns infected by the transplacental route, toxoplasmosis can provoke encephalitis, changes in mental state, focal motor deficits, cranial nerve disorders, sensory abnormalities, and/or neuropsychiatric findings and may progress to death [4–8].

The host's innate immune response attenuates parasite replication and spread, promoting the development of an adaptive immune response, which is involved in prolonged resistance during *T. gondii* infection [9]. The antiparasitic response is provided by a T helper 1 (Th1)-type profile with the production of several inflammatory mediators, such as chemokines, cytokines including interleukin (IL)-12, tumor necrosis factor (TNF), and interferon-gamma (IFN-γ), nitric

Abbreviations: 5-LO, 5-Lipoxygenase; CCR2, chemokine receptor 2; CCR5, chemokine receptor 5; CNS, central nervous system; DPI, day post infection; IFN-γ, interferon-gamma; I.P, intraperitoneally; KO, knockout; LDH, lactate dehydrogenase; LXAs, lipoxins; MOs, macrophages; NO, nitric oxide; ROS, reactive oxigem species; Tg, *Toxoplasma gondii*; TNF, tumor necrosis factors; WT, wild-type.

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oxide (NO), and reactive oxygen species (ROS). These mediators are important activators of phagocytic cells that are responsible for parasite processing and antigen presentation [10,11].

Chemokines belong to a broad family of chemotactic proteins that signal through G protein-coupled receptors (GPCRs) to mediate cell migration, differentiation, and recruitment [12,13]. These proteins are involved in the immune response in inflammatory diseases and play a crucial role in homeostatic processes [12,13]. CCR2 is a chemokine receptor that is important for resistance to *T. gondii* infection and is expressed on monocytes and T cells. CCL2 is a ligand of CCR2 and is produced by various cell subtypes, including macrophages (MOs), dendritic cells, and fibroblasts, following inflammatory stimuli such as from IL to 1β, TNF, and IL-4 [14]. Another chemokine receptor important for resistance to *T. gondii* infection is CCR5. This receptor has an affinity for CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) ligands that are produced during various inflammatory processes, including infections with parasites such as *T. gondii* [15]. Notably, *T. gondii* cyclophilin-18 activates dendritic cells through CCR5, thereby inducing IL-12 production [16].

Besides cytokines and chemokines, other molecules that participate in the regulation of host immunity during infection are the eicosanoids [17,18]. Eicosanoids are known to participate in a range of biological responses, such as platelet aggregation, edema, smooth muscle contraction, inflammation, cancer, and immune responses [19]. Moreover, these molecules have been reported to participate in the pathogenesis of human and experimental toxoplasmosis [17,18]. Examples of eicosanoids include thromboxanes, prostaglandins, leukotrienes, and lipoxins (LXAs) [20].

LXAs have anti-inflammatory activity in various immune system disorders [21] and the 5-lipoxygenase (5-LO) enzyme has been described as the key enzyme for their synthesis. 5-LO is a member of the iron-containing enzyme family that catalyzes the oxidation of polyenic fatty acids to lipid hydroperoxides [22].

Despite the worldwide prevalence of toxoplasmosis, its treatment is limited owing to the small number of medications available and the high occurrence of side effects [23,24]. Therefore, it is necessary to develop alternative and efficient treatments for this infection.

Among new drug candidates for the treatment of this disease, compounds derived from thiosemicarbazones have a prominent place. These compounds have a high affinity for transition metal ions and this underlies some properties that have been extensively studied, including antitumor, antiviral, antibacterial, antimalarial, antituberculosis, fungicidal, antiparasitic, and anticonvulsant activities [25,26]. Thiosemicarbazones are considered potential tools in the medicinal chemistry of drug discovery to treat several diseases including infections. Our group has previously shown that 4-chlorophenylthioace tone-derived thiosemicarbazones ((*E*)-2-(1-(4-chlorophenylthio)pro pan-2-ylidene)-hydrazinecarbothioamides) exhibit potent anti-trypanosomal activity [25]. Here, the anti-*T. gondii* activity of these compounds and their interactions with CCR2, CCR5, and 5-LO were investigated *in vitro* and *in vivo*. Our results reveal potent anti-*T. gondii* activities for new compounds in this class (**Ca** and **Cb)**, suggesting the involvement of CCR2, CCR5, and 5-LO in modulating these responses, paving the way for new therapeutic compounds for this important clinical problem.

2. Materials and methods

2.1. Ethics statement and animals

This study was conducted in strict accordance with the Brazilian Guidelines on Animal Work and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The Animal Ethics Committee (CEUA) of the Universidade Federal de Minas Gerais (UFMG), Minas Gerais, Brazil, approved all experiments and procedures, including euthanasia and fluid and organ removal (Permit Number 414/2018). For *in vitro* and *in vivo* experiments, C57BL/6 wild-type (WT) female mice, aged 9–11 weeks, were obtained from the Animal Care Facilities of UFMG. CCR2 knockout (CCR2 KO) and CCR5 KO female mice were bred on a C57BL/6 genetic background, and 5-LO KO mice were bred on an SV.129 (WT) genetic background under pathogen-free conditions at the Instituto de Ciências Biológicas (ICB), UFMG.

2.2. Synthesis of [(E)-2-(1-(4-chlorophenylthio)propan-2-ylidene)-N-methylhydrazinecarbothioamide] (Ca) and [(E)-2-(1-(4-chlorophenylthio) propan-2-ylidene)hydrazinecarbothioamide] (Cb)

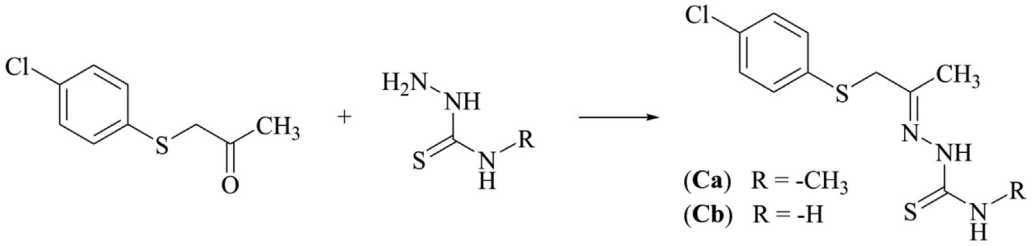
Thiosemicarbazones **Ca** and **Cb**, previously identified as C1 and C3, were prepared as described in the literature. Briefly, 207 mg (1 mmol) of (4-chlorophenylthio)acetone was solubilized in a 50 mL flask containing 10 mL of methanol. Three drops of acetic acid were added, and after 10 min of stirring, 1 mmol of the appropriate thiosemicarbazide was added. The reaction mixture was stirred magnetically and refluxed for 4 h. After cooling to room temperature, the mixture was kept in a freezer for 24 h, filtered, washed with a minimum of methanol and diethyl ether, and subsequently dried in a desiccator under reduced pressure (see Scheme 1). The structures of **Ca** and **Cb** were confirmed by ^{1}H NMR spectroscopy and high-resolution mass spectrometry [ESI(+), IT-TOF] by comparison with published data [25]. The compounds were additionally characterized using elemental analysis. The carbon atom numbering scheme and the spectra of **Ca** and **Cb** are shown in the Supplementary Material.

2.3. MO cultures

Briefly, C57BL/6, SV.129, CCR2 KO, CCR5 KO, and 5-LO KO female mice were injected intraperitoneally (i.p.) with 2 mL of sodium thioglycolate solution (3 %) (Difco™ fluid). After 72 h, MOs were harvested from the peritoneal cavity by washing with sterile cold phosphate-buffered saline 1× (PBS), centrifuged at 290 × g for 10 min, and counted in a Neubauer chamber. Cells (1×10^6 cells/well) were plated onto 24-well culture plates (Nunc, Rochester, New York, USA) with or without circular coverslips. Cells were incubated at 37 °C, 5 % CO_2 in the presence of Roswell Park Memorial Institute 1640 culture medium (RPMI, Gibco, BLR Life Technologies), supplemented with 5 % fetal bovine serum (Cultilab, Campinas, SP/BR), 1 % (200 mM) L-glutamine (Gibco) and 1 % penicillin/streptomycin (Gibco) (complete medium – RPMIc) for 3 h. After incubation, the plates were washed with RPMI medium and incubated overnight for subsequent infection and/or treatment.

2.4. Glial cells culture

For extraction and cultivation of glial cells, C57BL/6 neonatal mice (up to 3 days after birth) were euthanized by decapitation. Brain pieces were added to a trypsin/EDTA (Sigma) (0.5 %; 0.2 %) solution, followed by incubation at 37 °C in 5 % CO_2 for 10 min. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum was added to neutralize trypsin activity. The suspension was then centrifuged at 290*g* for 10 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in 10 mL 10 % DMEM. The cell suspension was transferred to a culture flask previously coated with 1 % porcine skin gelatin (G2500, Sigma), followed by incubation at 37 °C in 5 % CO_2 for 24 h. After this period, the medium was replaced every three days up to 14 days (the time at which the cells displayed 90–100 % confluency), then the cells were extracted with trypsin, washed, and plated (5×10^6 cells/well, 24-well plates).



Scheme 1. Syntheses of (4-chlorophenylthio)acetone-derived thiosemicarbazones (**Ca** and **Cb**).

2.5. T. gondii culture and infection in vitro

Tachyzoites of *T. gondii*, RH strain, were provided by the Laboratory of Toxoplasmosis, Department of Parasitology, ICB-UFMG, and were cultured in the kidney epithelial cell line LLCMK2 (*Macaca mulatta* – "Rhesus") in the presence of RPMIc. Parasites were centrifuged twice (50*g* for 5 min, then 2438*g* for 10 min), counted, and used to infect MOs (1:1 *T. gondii*/cell ratio) for 2 h, followed by a wash (RPMIc), treatment with **Ca** or **Cb** freshly prepared in dimethyl sulfoxide (DMSO) at 0.5 % v/v for 4 or 48 h, and treatment with pyrimethamine (**P**) plus sulfadiazine (**S**) (**P** at 1 μM, and **S** at 30 μM) for 48 h. Intracellular tachyzoites were quantified in MOs fixed and stained (*Panótico Rápido*; LB Laborclin, Pinhais, PR/BR) after 4 or 48 h of infection (supernatants were stored at −20 °C for further analysis). After being stained, the coverslips, still in the wells, were washed twice with distilled water and removed from the wells for drying at room temperature. The coverslips were then fixed in triplicate on microscopy slides using Entellan® Fixer (MERCK). For each coverslip, a total of 300 cells were counted, distinguishing between uninfected and infected cells and counting the number of tachyzoites in cells using an optical microscope (40× objective). For parasite pretreatment experiments, tachyzoites were incubated in the presence of **Ca**, **Cb**, (**P** + **S**) or RPMIc alone for 1 h, washed with RPMIc, and used for MO infection (as described above). For the MO pretreatment experiment, cells were incubated in the presence of **Ca**, **Cb,** or RPMc alone for 1 h, washed with RPMIc and infected with the parasite as described above.

2.6. T. gondii-infection and treatment with Ca or Cb in vivo

C57BL/6, SV129, CCR2 KO, CCR5 KO, and 5-LO KO female mice were infected with 20 cysts of *T. gondii* (Me49 strain) intraperitoneally (i.p.). Treatment with **Ca** or **Cb** freshly prepared in DMSO at 0.5 % v/v (gavage; at a dose of 1 mg/kg) started 8 h after infection and lasted until the 7th day post-infection (dpi), at a 12/12 h interval. Animals were monitored throughout the experiment (survival), and at the 5th and 7th dpi, blood samples were collected for cytokine analysis in the serum.

2.7. Cytokine determinations

Cytokines IFN-γ and IL-12 were assayed in animal serum at the 5th and 7th dpi, by a sandwich enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, Minnesota). Optical densities were measured at 450 nm using a spectrophotometer (Elx800, BioTek).

2.8. Nitric oxide (NO) and lactate dehydrogenase (LDH) cytotoxicity assay

NO measurement was performed in supernatants by the Griess colorimetric method as previously described [25]. Optical densities were measured at 540 nm using a spectrophotometer (BioTek - Elx 800). The LDH assay was performed in the culture supernatants according to the manufactures' instructions (Bioclin® Kit, Belo Horizonte, MG/BR).

2.9. Cytotoxicity assay in blood cells

Blood from C57BL/6 female mice was collected (800 μl per mouse) into an anticoagulant (EDTA) and the red blood cells was lysed or not with ACK (Ammonium-Chloride-Potassium), resuspended in RPMIc (800 μl), plated onto 96-well plates (200 μl/well), and treated with **Ca**, **Cb**, **P** + **S** (**Ca, Cb,** and **S** at 30 μM and **P** at 1 μM) or medium alone, and incubated at 37 °C, 5 % CO_2. After 24 and 48 h, the number of leukocytes, lymphocytes, granulocytes and red blood cells was determined in blood samples (30 μl) using the a Celltac MEK-6500 K hemocytometer (Nihon Kohden). Additionally, after incubation, 20 μl of sample was collected for LDH assay (as described above).

2.10. Statistical analysis

Statistical analyses were performed by comparing controls to treated samples using Student's *t*-test or Two-way ANOVA with the Bonferroni test. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Ca and Cb induce potent anti-Toxoplasma activity in MOs.

MOs are among the first cells of the immune system to combat *T. gondii* infection. Therefore, the microbicidal capacity of **Ca** and **Cb** in this cell type was investigated *in vitro*. At the concentrations tested (10, 20, 30, 40, and 50 μM), both compounds reduced intracellular replication of tachyzoites in MOs after 48 h of challenge (having IC50 values of 8,001 μM R^2 0,961 and 6,317 μM R^2 0,988 respectively), compared to the infected/untreated group (Fig. 1**A**). To ensure that the microbicidal activity of these compounds was not associated with toxicity in MOs, an assay was performed to measure the release of the LDH enzyme. At all tested concentrations, **Ca** and **Cb** did not induce toxicity in uninfected MOs (data not shown), consistent with previous results [25], and at 30 μM was not toxic in uninfected blood cells (including lymphocytes, granulocytes and red blood cells.; Supplementary Fig. 1A-E). Fig. 1**B** demonstrates that *T. gondii*-infection increases the release of LDH enzyme by MOs compared to control cells. The presence of **Ca** or **Cb** at 30 μM significantly reduced LDH levels compared to infected untreated cells. Of great relevance, **Ca** or **Cb** at 30 μM reduced the number of parasites similarly to combined pyrimethamine (**P**) plus sulfadiazine (**S**) treatment (**P** + **S**; used as positive control – Supplementary Fig. 2A). Thus, a 30 μM concentration of **Ca** and **Cb** was selected for subsequent experiments. As shown in Fig. 1**C**, **Ca** or **Cb** treatment also reduced the number of parasites in MOs 4 h after infection (uptake/invasion assay), suggesting that the compounds were able to control not only the replication of the parasite but also its invasion into host cells.

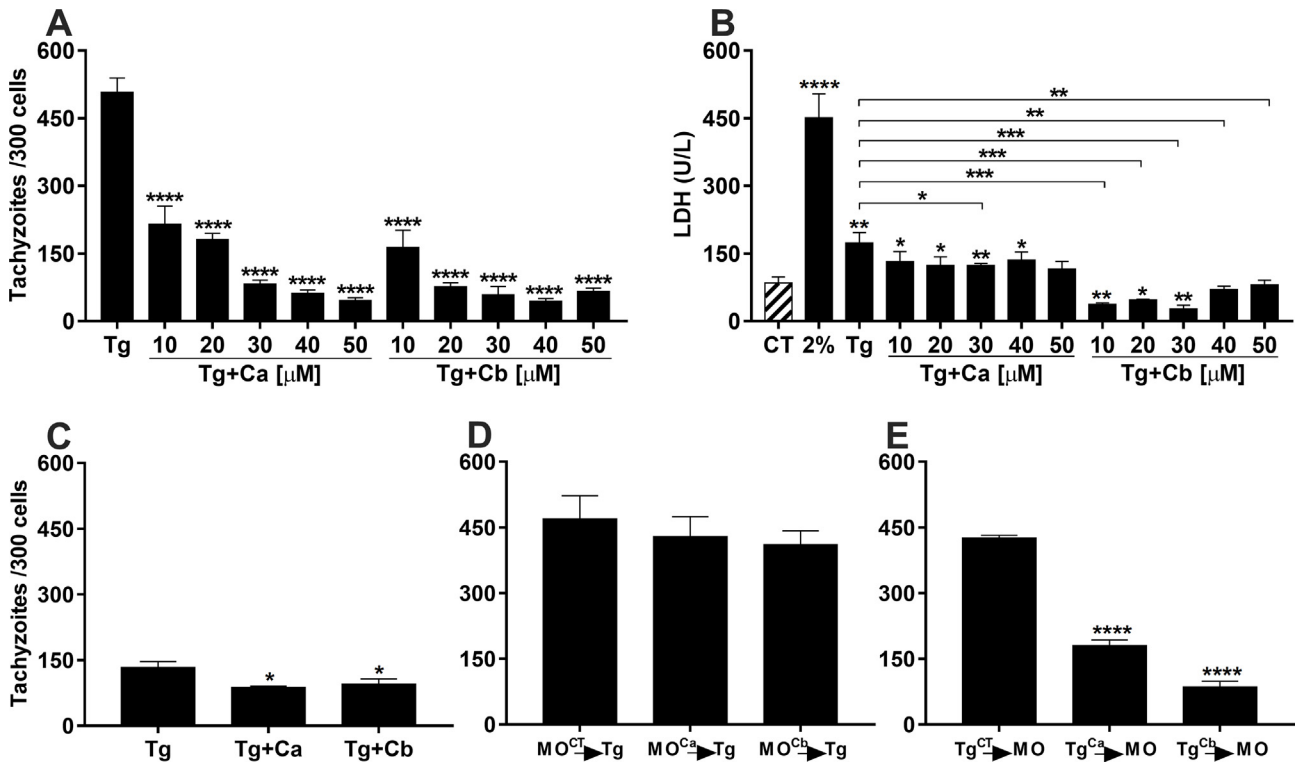


Fig. 1. Ca and Cb stimulate potent anti-*Toxoplasma* activity in MOs without inducing host cell toxicity, and have a direct effect on the parasite. Peritoneal MOs were obtained from C57BL/6 female mice and infected with tachyzoites of *T. gondii* (RH strain; 1:1 parasite:cell) and treated with **Ca** or **Cb**. After 48 h, intracellular parasite counts were performed on fixed and stained cells (Rapid Panotic) (**A**). A viability test (LDH) was performed on cell supernatant after 48 h of *T. gondii* infection and/or treatment with **Ca** or **Cb** (**B**). For the uptake assay, intracellular parasites were counted 4 h after infection and treatment with **Ca** or **Cb** (30 μM) in fixed and stained cells (**C**). MOs were preincubated with **Ca** or **Cb** (30 μM) for 1 h, followed by washing and infection with tachyzoites of *T. gondii* (RH strain) (1:1 parasite: cell). After 48 h the intracellular parasites count was performed in fixed and stained cells (**D**). The tachyzoites were preincubated with **Ca** or **Cb** (30 μM) for 1 h, then washed and used to infect macrophages. After 48 h the intracellular parasite count was performed in fixed and stained cells (**E**). "CT" = control (non-treated cells); "2 %" = cell treated with Triton (positive control); "Tg" = *T. gondii*-infected MOs; "$MO^{CT} \rightarrow Tg$" = untreated MOs infected with *Tg*; "$MO^{Ca} \rightarrow Tg$" = **Ca**-pretreated MOs infected with *Tg*; "$MO^{Cb} \rightarrow Tg$" = **Cb**-pretreated MOs infected with *Tg*; "$Tg^{CT} \rightarrow MO$" = Medium-pretreated *Tg* used to infect MOs; "$Tg^{Ca} \rightarrow MO$" = **Ca**-pretreated *Tg* used to infect MOs; "$Tg^{Cb} \rightarrow MO$" = **Cb**-pretreated *Tg* used to infect MOs. Data from one experiment representative of two independent experiments are shown as mean ± SEM (standard error of the mean) ($*p < 0,05$, $**p < 0,01$, $***p < 0,001$ e $****p < 0,0001$). The *t*-test and Two-way ANOVA were used for statistical analysis.

3.2. Ca and Cb act directly on parasite

The direct action of **Ca** and **Cb** on *T. gondii* and the ability of these molecules to activate MOs before infection were investigated. Tachyzoites or MOs were pretreated with **Ca** or **Cb** for 1 h. Fig. 1**D** demonstrated that when MOs were pretreated with **Ca** or **Cb** followed by *T. gondii* infection, the host cells did not gain the ability to control parasite replication. However, MOs infected by parasites that were pretreated with **Ca** or **Cb** presented a reduced number of intracellular tachyzoites (similar to **P** + **S** treated parasite, used as positive control - Supplementary Fig. 2B) as compared with cells that were infected with the untreated parasite (Fig. 1**E**). The data suggest that the two compounds have a direct effect on the parasite.

3.3. Ca and Cb treatment reduce T. gondii replication in glial cells.

CNS cells actively participate in infection control and pathogenesis during *T. gondii* infection [27]. Therefore, the effects of **Ca** and **Cb** on infected glial cells were investigated. Fig. 2**A** shows that **Ca** or **Cb** treatment (15 and 30 μM) reduced the number of parasites in glial cells. Notably, the anti-*T. gondii* effect observed was not due to glial cell toxicity, since levels of LDH detected in supernatants from **Ca**- or **Cb**-treated and infected cells were similar to levels found in untreated cells (Fig. 2**B**).

3.4. CCR2 and 5-LO modulate the Ca and Cb anti-Toxoplasma activities in macrophages

Next, molecular targets and receptors that could be involved in the protective effect of **Ca** and **Cb** during *T. gondii* infection were investigated, including CCR2 and CCR5 chemokine receptors that are related to resistance to this infection [17,28]. MOs were isolated from WT, CCR2 KO, or CCR5 KO mice and infected and/or treated with **Ca** or **Cb**. Deficiency in CCR2 (Fig. 3**A**), but not CCR5 (Fig. 3**B**), in MOs resulted in a reduced ability to control *T. gondii* replication after 48 h of **Ca** or **Cb** treatment, compared with treated WT cells. The results suggest that the effects of **Ca** and **Cb** were CCR5-independent but partially dependent on the CCR2 receptor. CCR2 KO MOs (Fig. 3**A**), but not CCR5 KO MOs, (Fig. 3**B**) showed reduced parasite internalization as compared to WT, and the presence of **Ca** or **Cb** did not change this profile in KO cells. Of note, **Ca** and **Cb** reduced parasite invasion in WT cells (C57BL6 background) **(**Fig. 3**A and 3B)**.

Based on the relatively small differences in **Ca-** and **Cb-**induced anti-Toxoplasma activity in CCR2 KO and CCR5 KO MOs, other pathways may be involved in regulating the action of these compounds. Thus, the involvement of the 5-LO enzyme was investigated because it is known to trigger leukotriene and lipoxin production, which are important mediators for infection control and the resolution of inflammatory responses. Our results demonstrated that 5-LO-deficient MOs show a reduction in parasite replication compared with WT control,

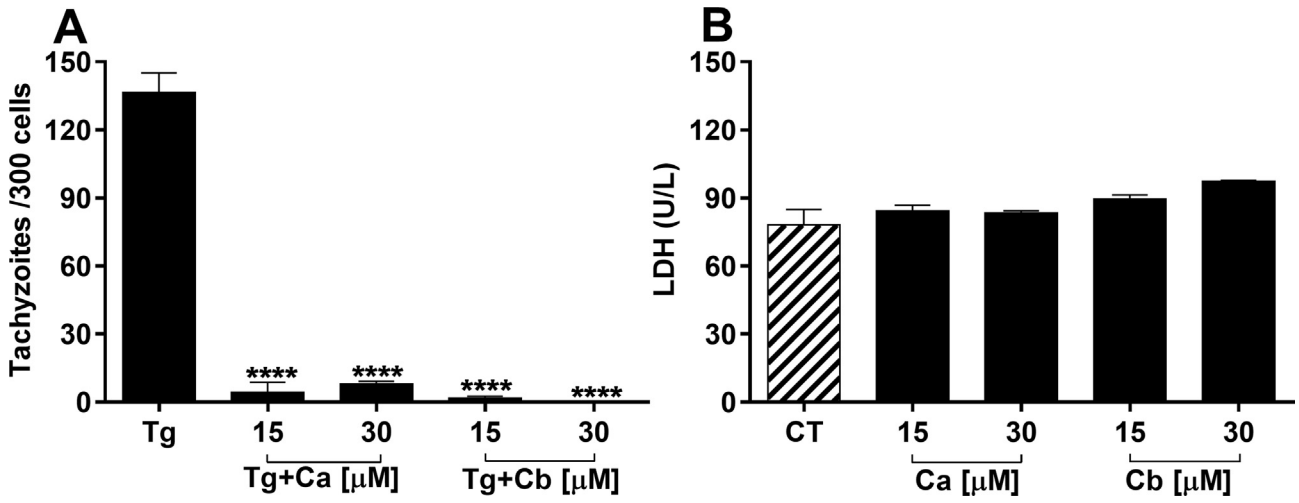


Fig. 2. Ca and Cb reduce *T. gondii* replication in glial cells. Total glial cells were obtained from C57BL/6 neonate mice, plated (2×10^5/well), infected with tachyzoite of *T. gondii* (RH strain; 1:1 parasite: cell), and treated with **Ca** or **Cb** (15 and 30 μM). After 24 h the intracellular parasite counts were performed on fixed and stained cells (**A**), and the cell viability assayed in the supernatants (**B**). 'Tg" = *T. gondii*-infected MOs; "CT" = control (non-treated cells). Data from one experiment representative of two independent experiments are shown as mean ± SEM (****$p < 0{,}0001$). The *t*-test was used for statistical analysis.

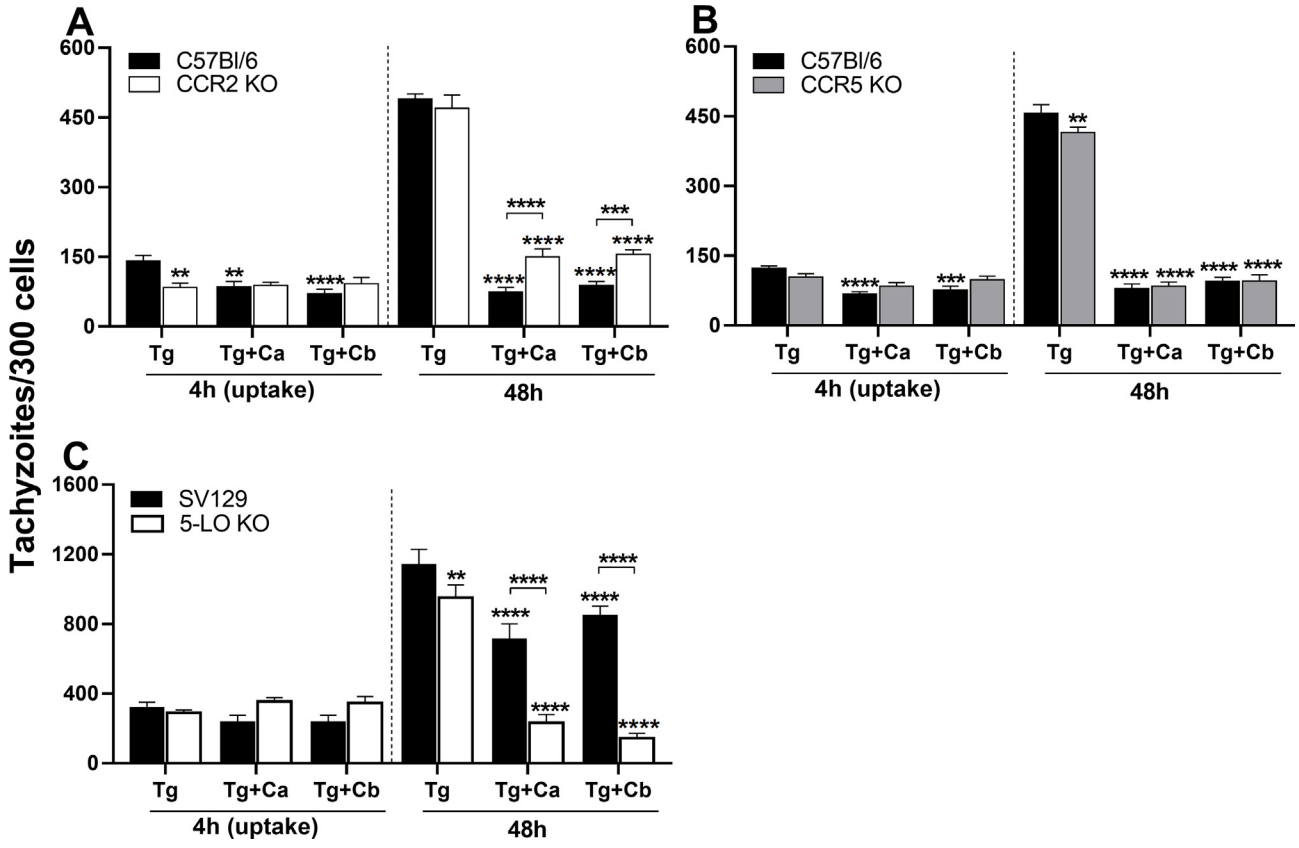


Fig. 3. CCR2 and 5-LO modulate the anti-Toxoplasma activities of Ca and Cb in MOs. MOs were obtained from C57BL/6 (**A, B**) and CCR2 KO (**A**), CCR5 KO (**B**), and SV.129 and 5-LO KO (**C**) mice and infected with tachyzoite of *T. gondii* (RH strain; 1:1 parasite: cell), and treated with **Ca** or **Cb** (30 μM), and after 4 h (uptake) and 48 h intracellular parasite counts were obtained for in fixed and stained cells. Data from one experiment representative of two independent experiments are shown as mean ± SEM (*$p < 0{,}05$, **$p < 0{,}01$, ***$p < 0{,}001$ e ****$p < 0{,}0001$). The *t*-test and two-way ANOVA were used for statistical analysis.

despite presenting similar parasite uptake/invasion levels (Fig. 3**C**). Moreover, the absence of 5-LO potentiated **Ca** (Fig. 3**C**) and **Cb** (Fig. 3**C**) microbicidal capacity as compared with the WT counterpart. Of note, the parasite uptake/invasion assay demonstrated that **Ca** and **Cb** did not affect tachyzoite internalization by MOs WT (SV.129 background) or 5-LO deficiency (Fig. 3**C**).

3.5. Ca increases IL-12 and IFN-γ production and the survival rate of T. gondii-infected mice

Anti-*T. gondii* activities of **Ca** and **Cb** were evaluated *in vivo*. WT mice (C57BL/6) were infected with *T. gondii*, and after 8 h, they were treated with **Ca** or **Cb** at a dose of 1 mg/kg every 12 h, up to the 7th dpi.

Fig. 4**A** demonstrates that infected animals treated with **Ca** presented increased survival rates during infection (finalized at the 45th dpi). Notably, treatment with **Cb** increased the mortality of *T. gondii*-infected mice compared to that of untreated-infected mice (Fig. 4**A**). These data demonstrate that, *in vivo*, **Ca**, but not **Cb**, can enhance the survival of *T. gondii*-infected mice. Thus, **Cb** was not tested in the subsequent experiments with the C57BL/6 mouse background because of the increased mortality induced.

To analyze the possible molecular targets involved in the increased protection induced by **Ca** during *T. gondii* infection *in vivo*, C57BL/6, CCR2 KO, and CCR5 KO mice were infected and treated with **Ca** as described above. CCR2 KO and CCR5 KO mice were highly susceptible to infection as compared to WT mice (Fig. 4**B**). Moreover, CCR2 or CCR5 deficiency abolished the protection conferred by **Ca** treatment during infection (Fig. 4**B**). These results suggest that *in vivo*, the protection conferred by **Ca** treatment was partially dependent on CCR2 and CCR5 receptors.

Cytokines IL-12 and IFN-γ are critical for resistance to *T. gondii* infection. Therefore, the participation of these cytokines in the anti-*T. gondii* activity exerted by **Ca** *in vivo* was investigated. At the 5th dpi, but not the 7th dpi, **Ca**-treated C57BL/6 mice showed increased serum levels of IL-12 and IFN-γ compared to the untreated group (Fig. 4**C-F**). Upon **Cb** treatment, increased levels of IL-12 induced by

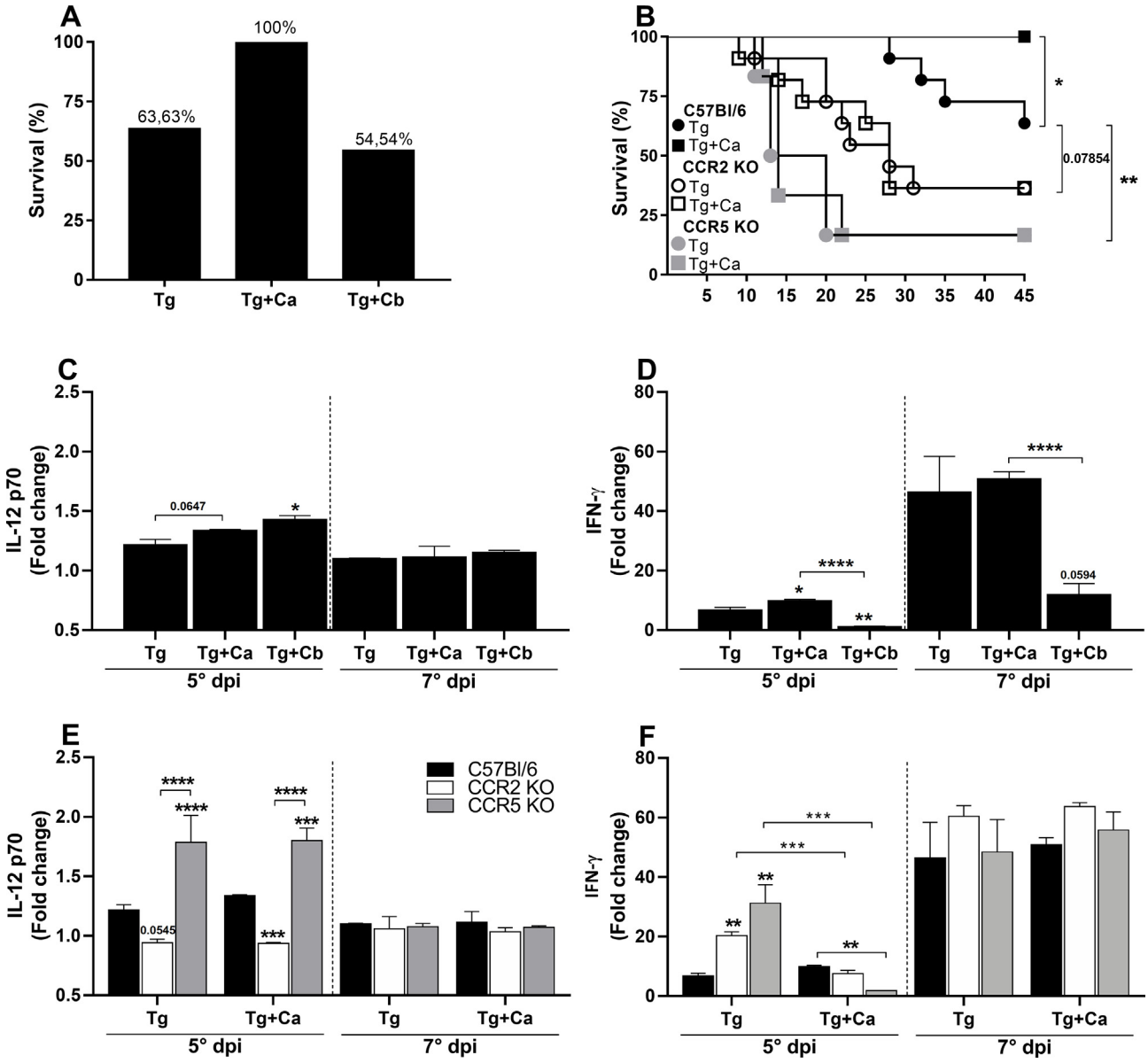


Fig. 4. Ca increases the production of IL-12 and IFN-γ and increases the survival of *T. gondii*-infected mice. C57BL/6 mice (n = 11/group) were infected with 20 cysts of *T. gondii* (ME49 strain) and treated with **Ca** or **Cb** (1 mg/Kg) at 8 h after infection until the 7th- dpi of each 12/12 h. The survival was monitored until the 45th dpi (**A**). C57BL/6, CCR2 KO, and CCR5 KO mice were infected and treated with **Ca** as described above. The survival was monitored from the 5th to the 45th dpi (**B**). The cytokines IL-12p70 (**C, E**) and IFN-γ (**D, F**) were quantified in the serum of animals at the 5th and the 7th dpi. Data from one experiment representative of two independent experiments are shown as mean ± SEM (*p < 0,05, **p < 0,01, ***p < 0,001 e ****p < 0,0001). The *t*-test and two-way ANOVA were used for statistical analysis.

infection were observed at the 5th but not the 7th dpi in WT mice (**Figure C**). In contrast, **Cb** treatment reduced the levels of IFN-γ at the 5th and 7th dpi as compared with the WT untreated-infected group (Fig. 4**D**).

CCR5 KO-infected mice, at the 5th dpi, presented higher levels of IL12 (Fig. 4**E**) and IFN-γ (Fig. 4**F**) than their WT counterparts. CCR2 KO-infected mice presented in serum, at the 5th dpi, lower levels of IL-12 (Fig. 4**E**) and higher levels of IFN-γ (Fig. 4**F**) when compared to the WT counterparts. **Ca** treatment reduced the levels of IFN-γ, but not IL-12, at the 5th dpi, in CCR2 KO and CCR5 KO mice when compared to untreated-infected CCR2 KO and CCR5 KO groups, respectively (Fig. 4**E and 4F**). At the 7th dpi, the levels of IL-12 and IFN-γ were similar in the CCR2 KO, CCR5 KO, and WT groups (Fig. 4**E and 4F**).

3.6. The absence of the 5-LO enzyme increases the protection induced by Ca and Cb during experimental T. gondii infection in vivo

To evaluate the involvement of the 5-LO enzyme in the protection induced by **Ca** and **Cb** during *T. gondii* infection *in vivo*, 5-LO knockout mice with an SV.129 background were used. Notably, using a different mouse background is extremely important to verify that **Ca** and **Cb** treatment *in vivo* reproduces the same effect found in the C57BL/6 background. Fig. 5**A** demonstrates that SV.129 (WT) mice were more susceptible to *T. gondii* infection than C57BL/6 (WT) mice, displaying a decreased survival rate (Fig. 4**A** versus Fig. 5**A**; C57BL/6 63.63 % × SV129 9.09 %). Moreover, in contrast to the results obtained in C57BL/6 mice, treatment with **Cb**, but not **Ca**, increased protection against *T. gondii* infection (Fig. 5**A**). Furthermore, a deficiency in 5-LO resulted in greater resistance to *T. gondii* infection than in SV.129 (WT) mice (Fig. 5**B**). Treatment with **Ca** or **Cb** increased the survival rate of infected 5-LO KO mice. Notably, treatment with **Cb** protected 100 % of the 5-LO KO mice from death (Fig. 5**B**).

Treatment of SV.129 wt mice, but not of 5-LO KO mice, with **Ca** or **Cb** increased serum levels of IL-12 when compared to the WT-infected untreated group, at the 7th and 5th dpi, respectively**.** At the 7th dpi, **Cb**-treated 5-LO KOs mice (Fig. 5**C**) produced higher levels of IL-12 than their WT counterparts did. Regarding IFN-γ, at the 5th dpi, but not at the 7th dpi, treatment with **Cb** reduced the levels of this cytokine in the serum of the WT-infected group (Fig. 5**D**). In the absence of 5-LO, at the 5th dpi, but not at the 7th dpi, treatment with **Cb**, but not with **Ca**, reduced the levels of IFN-γ in serum when compared with untreated-infected 5-LO KO mice (Fig. 5**D**).

4. Discussion

Currently, treatment options for toxoplasmosis are scarce and have serious toxic effects on the host. Medicinal chemistry involves areas of medical and exact sciences as an alternative source for new drug development or adaptations of existing ones [28]. The thiosemicarbazones have several outstanding properties, such as; antitumor, antimicrobial, antiprotozoal, antioxidant, anticonvulsant, and herbicidal activities [23,24] arousing great interest in their development as new drugs.

Control of *T. gondii* infection involves several cell types including MOs, dendritic cells, and lymphocytes [29]. MOs are critical for controlling parasite proliferation and dissemination and for linking the innate immune response to the acquired immune response. Our results demonstrated that **Ca** and **Cb** were not toxic to MOs *in vitro* and *T. gondii*-infected MOs treated with these molecules showed a reduced number of intracellular parasites. Thus, **Ca** and **Cb** are potent therapeutic candidates for controlling *T. gondii* infections.

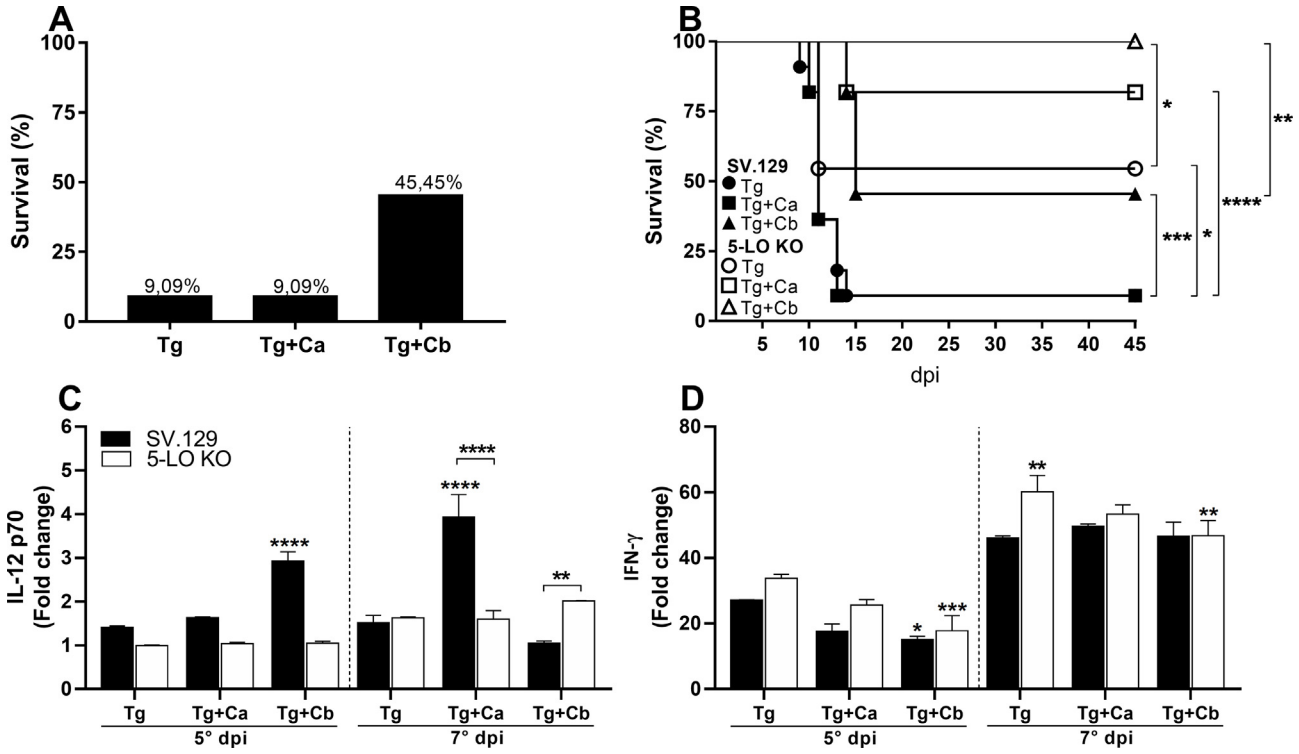


Fig. 5. Lack of the 5-LO enzyme increases the protection induced by Ca and Cb during experimental *T. gondii* infection *in vivo*. SV.129 mice (n = 11/group) were infected with 20 cysts of *T. gondii* (ME49 strain) and treated with **Ca** or **Cb** (1 mg/kg) at 8 h after infection until the 7th-dpi of each 12/12 h. The survival was monitored until the 45th dpi (**A**). SV.129 and 5-LO KO mice were infected and treated as described above. The survival was monitored from the 5th to the 45th dpi (**B**). The cytokines IL-12p70 (**C**) and IFN-γ (**D**) were quantified in the serum of animals at the 5th and the 7th dpi. Data from one experiment representative of two independent experiments are shown as mean ± SEM (*p < 0,05, **p < 0,01, ***p < 0,001 e ****p < 0,0001). The *t*-test and two-way ANOVA were used for statistical analysis.

Production of NO by several cell types in the immune system is an important mechanism for controlling pathogen replication/dissemination [30]. **Ca** and **Cb** are not good inducers of NO production by MOs, as recently demonstrated by our group [25] and confirmed here, where the increased toxoplasma activity induced by the compound was also independent of NO production (data not shown). Nevertheless, **Ca** and **Cb** decreased the ability of parasites to invade MOs. This may be linked both to greater MO activation and to direct action on the parasite. Our results demonstrated that parasites preincubated with **Ca** and **Cb**, before MO infection reduced their replication capacity in MOs. These results suggest that the compounds may act directly on tachyzoites of the parasite in addition to triggering MO activation.

Glial cells participate in CNS homeostasis and, in addition to supporting neuronal function, are also targeted in parasite infection and are involved in the pathogenesis of toxoplasmosis [31]. Here, we demonstrated that **Ca** or **Cb**-treated glial cells inhibited *T. gondii* replication in a NO-independent manner (data not shown). Studies have demonstrated that the main mechanism of action of the compounds, in general, is through direct or indirect interaction with DNA [32], causing the death of the targets (e.g., parasites, tumor cells, viruses, fungi, and bacteria) [32,16]. Our group recently demonstrated that **Ca** and **Cb** inhibit *Trypanosoma cruzi* replication, but this process cannot be reversed by inhibiting the DNA damage response [25].

Herein, we also explored other possible targets involved in the activity of **Ca** and **Cb**, focusing on immunological targets that are known to participate in the regulation of inflammatory responses and, so, in the outcome of the infection. The chemokine receptors are involved in the body's homeostasis, and also participate in the development of inflammatory responses to pathogens, being important in resistance to infections [16,33].

It has been reported that the chemokine receptor CCR5 not only recognizes endogenous ligands (CCL3, CCL4, and CCL5) but also *T. gondii* immunogenic molecules (e.g., cyclophilin-18). This recognition contributes to the efficient development of the response against the parasite[34,33].

Our results demonstrated that MOs deficient in CCR5 reduced the number of intracellular parasites compared to infected WT MOs. Notably, the absence of CCR5 did not alter the observed anti-*T. gondii* activity of the compounds, and did not change the parasite invasion process in MOs. These results demonstrate that the absence of the CCR5 chemokine receptor in MOs does not impair the anti-*T. gondii* activity *in vitro*. The CCR2 receptor is another chemokine receptor that is important during infection [35,36]. In contrast to CCR5, in the absence of CCR2, MOs partially lost anti-*T. gondii* activity triggered by **Ca** and **Cb**. Moreover, our data for parasite uptake/invasion indicated that CCR2, but not CCR5, is involved in *T. gondii*-MO invasion. Studies have demonstrated that, besides recognition functions, some receptors are also involved in the pathogen/host cell interaction, functioning as an "anchoring" mechanism favoring the pathogen to invade the host cell.

Besides cytokines and chemokines, the production of eicosanoids during *T. gondii* infection is critical in disease outcomes [17,18]. The 5-LO enzyme is crucial in both pro- and anti-inflammatory mechanisms, triggering the production of lipid mediators, such as leukotrienes and lipoxins, during *T. gondii* infection. Our results demonstrated that in the absence of 5-LO, the anti-Toxoplasma activities of **Ca** and **Cb** in MOs were potentiated.

The treatment with **Ca**, but not **Cb**, protected C57BL/6 (WT) mice during *T. gondii* infection *in vivo*. During *Trypanosoma cruzi* infection, these and other compound construction treatments have been shown to reduce parasitemia in mice [37].

At the dose tested, only **Ca** increased the survival of *T. gondii*-infected animals, but we cannot discard the possibility that other doses of **Cb** would be able to protect the infected mice. Thus, more experiments would be necessary to definitively show whether **Cb**, in the C57BL/l6 background, has any protective effect during *T. gondii* infection. Further, another dose level should be tested to determine whether the **Ca** protective effect observed can be improved upon.

Studies have demonstrated that IL-12 and IFN-γ productions are critical for controlling *T. gondii* infection *in vivo*. Mice deficient in IL-12 or IFN-γ are highly susceptible to this infection [38,39]. Thus, to understand the relationship between the increased survival rates and these cytokines, their serum levels were evaluated. C57BL/6 mice infected and treated with **Ca** showed increased production of IL-12 and IFN-γ when compared to the control group at the 5th dpi.

In the absence of CCR5 or CCR2 receptors, the mice demonstrated lower resistance to *T. gondii* infection, and treatment with **Ca** did not reverse this phenotype. We hypothesized that in these KO mice, a higher dose of **Ca** would be necessary to promote anti-Toxoplasma activity. Notable, our *in vitro* results demonstrated that, in MOs, **Ca** anti-*T. gondii* activity is partially dependent on CCR2 expression Our results, *in vivo*, with CCR5 and CCR2 KO mice are consistent with studies demonstrating that animals deficient in CCR5 or CCR2 are highly susceptible to *T. gondii* infection. CCR5 or CCR2 deficiency results in higher parasitic load and lower tissue expression of IL-12p40, IFN-γ, TNF, IL-6, iNOS, Foxp3, TGF-beta, GATA-3, and PPARα in mice [34,40,15].

Here, **Ca**-treated infected CCR5 KO mice at the 5th dpi, but not at the 7th dpi, had higher production of IL-12 and IFN-γ compared to WT, but these did not ensure mouse survival. In CCR2 KO animals, unlike in CCR5 KO mice, **Ca** treatment decreased IFN-γ at the 5th dpi. At the 7th dpi, all groups showed similar serum IFN-γ levels. However, it is not possible to discount a possible increase or decrease of these cytokines at the late stage of the infection, as well as a relationship with the death of mice.

In this study, we demonstrated that depending on the mouse background, Schiff base treatment resulted in a different outcome *in vivo*. In C57BL/6 mice, treatment with **Ca**, but not with **Cb**, efficiently ensured the survival of *T. gondii*-infected mice. In the SV.129 background, we found the opposite results: treatment with **Cb**, but not **Ca**, increased the survival rate of infected mice. Notably, the mice in the SV.129 background were more susceptible to *T. gondii* infection, presenting a higher rate of death when compared to the C57BL/6 background.

Deficiency in 5-LO increased the survival of *T. gondii*-infected mice (during the period of investigation), and **Ca** treatment potentiated this resistance. Of great relevance, **Cb** treatment guaranteed 100 % survival in infected 5-LO KO mice. Thus, these results demonstrate that the absence of 5-LO improved the **Ca** and **Cb** anti-Toxoplasma activity.

Interestingly, a previous study demonstrated that *T. gondii* is capable of transforming a downstream intermediate metabolite of arachidonic acid, produced by 5-LO enzyme activity, into lipoxin, which is a potent anti-inflammatory mediator that attenuates inflammatory responses that, in turn, contribute to parasite replication. Thus, we hypothesized that in cells and animals deficient in 5-LO, the ability of the parasite to bypass microbicidal activity by inducing lipoxin production is diminished; thus, the activities of **Ca** and **Cb** are potentiated. Moreover, our results also demonstrated that *in vivo*, the regulation of **Ca** and **Cb**-induced IL-12 and IFN-γ production during *T. gondii* infection is dependent on the 5-LO enzyme.

5. Conclusion

Taken together, our results suggest that the Schiff bases **Ca** and **Cb** are promising compounds for the treatment of toxoplasmosis, attenuating *T. gondii* replication without altering host cell viability. In the absence of 5-LO, the thiosemicarbazones studied here were able to increase the survival of infected mice, suggesting that the association of **Ca** or **Cb** with 5-LO inhibitors is a promising new therapeutic approach for the treatment of the pathogeneses associated with toxoplasmosis, an important clinical problem.

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Authors contribution

Rayane A.N.R., Diego R.R.A, Heloísa B. and Fabiana S.M. conceived experiments. Rayane A.N.R., Alexandre A.O., Mauro M.T., Ricardo W. A.V., Heloísa B. and Fabiana S.M. wrote the manuscript. Rayane A.N. R., Diego R.R.A, Alexandre A.O., César L.N.B., Rafaela D.P. and Wiliam C.B.R. performed experiments. Heloísa B., Ricardo W.A.V., Mauro M. T., and Fabiana S.M. supervised and provided expertise and funding.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.medidd.2023.100157.

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Erratum

Erratum to "Potent anti-*Toxoplasma gondii* activity of 4-chlorophenylthioacetone-derived thiosemicarbazones: Involvement of CCR2 and CCR5 receptors and 5-lipoxygenase in the mode of action" [Med. Drug Discovery 18 (2023) 100157]

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