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Bothrops pirajai snake venom L-amino acid oxidase: *in vitro* effects on infection of *Toxoplasma gondii* in human foreskin fibroblasts

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Abstract: The effect of an L-amino acid oxidase isolated from *Bothrops pirajai* snake venom (BpirLAAO-I) was investigated on infection of *Toxoplasma gondii* in human foreskin fibroblasts (HFF). The cytotoxic activity of BpirLAAO-I on HFF cells showed a dose-dependent toxicity with median cytotoxic dose (TD50) of 11.8 µg/mL. BpirLAAO-I induced considerable dose-dependent decrease in the *T. gondii* infection index under two different conditions, treatment of tachyzoites before infection or treatment of HFF cells after infection. A maximal inhibition of infection (56%) was found for treatment before infection, with a median inhibitory dose (ID50) at 1.83 µg/mL and selectivity index (SI) at 6.45. For treatment after infection, it was observed a maximal inhibition of infection at 65%, ID50 of 1.20 µg/mL and SI of 9.83. The treatment before infection was also effective to reduce intracellular parasitism up to 62%, although presenting higher values of ID50 (3.14 µg/mL) and lower values of SI (3.76). However, treatment after infection was not effective, suggesting that the enzyme seems to have no effect on the parasite intracellular replication for this condition. In conclusion, BpirLAAO-I was more effective to inhibit the infection of neighboring cells and consequently parasite dissemination than primary infection and parasite replication. Thus, the effect of BpirLAAO-I described herein could be taken into account for the development of new synthetic anti-parasite therapeutic agents.

Article

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Introduction

Bothrops pirajai snake is an endemic species from the South region of Bahia state, Brazil, and it belongs nowadays to a national list of Brazilian fauna species threatened of extinction (Martins & Molina, 2008). Its venom is rich in proteins such as phospholipases A_2, desintegrins, metalloproteases, serinoproteases, L-amino acid oxidases and others (Rodrigues et al., 2009). L-amino acid oxidases (LAAO, EC 1.4.3.2) are enantioselective flavoenzymes catalyzing the oxidative deamination of a wide range of L-amino acids (Stábeli et al., 2007). During the reductive half-reaction, the amino acid substrate is oxidized to the imino acid with concomitant reduction of the flavin adenine dinucleotide (FAD) cofactor. The imino acid product of oxidation undergoes a non-enzymatic hydrolysis to give the respective α-keto acid and ammonia. An oxidative half-reaction completes the catalytic cycle re-oxidizing the FAD with molecular oxygen and producing hydrogen peroxide (Moustafa et al., 2006).

BpirLAAO-I is an L-amino acid oxidase isolated from *Bothrops pirajai* snake venom by molecular exclusion, affinity and hydrophobic chromatography (Izidoro et al., 2006). It is a homodimeric acid glycoprotein with molecular mass of 130 kDa and isoelectric point (pl) 4.9. Also, it was shown that BpirLAAO-I demonstrates bactericidal activity against *Escherichia coli* and *Pseudomonas aeruginosa*, in addition to cytotoxic activity against tumor cells, mouse paw edema, and typical fago (M13mp18) DNA fragmentation (Izidoro et al., 2006).

Anti-protozoan activities have been

investigated in many snake venom components. For example, L-amino acid oxidases from *Bothrops* spp were responsible for *in vitro* killing of *Plasmodium falciparum* (Zieler et al., 2001), *Leishmania* spp (Izidoro et al, 2006), and *Trypanosoma cruzi* (França et al., 2007). Neuwiedase, a metalloproteinase from *Bothrops neuwiedi*, showed a considerable effect against *Toxoplasma gondii* infection *in vitro* (Bastos et al., 2008).

T. gondii is a ubiquitous obligate intracellular parasite of the Phylum Apicomplexa and it is unusual within this group due to its capacity to infect a diverse array of cell types and virtually any warm-blooded animal. It is also remarkable the diversity of strategies that the parasite achieves for its essential intracellular survival (Miller et al., 2009). There are three infectious stages in the life cycle of *T. gondii i.e.* tachyzoites, bradyzoites contained in tissue cysts, and sporozoites contained in sporulated oocysts (Tenter et al., 2000). Tachyzoites actively and rapidly enter cells, and colonize within a highly modified parasitophorous vacuole that resists endosomal acidification. After six to eight parasite divisions the host cell lyses, and released tachyzoites infect surrounding cells and tissues, characterizing the acute phase of infection. Chronic infection is associated with differentiation into bradyzoites that form quiescent cysts within tissues of the central nervous system and skeletal muscle (Denkers, 2003).

T. gondii has both asexual and sexual phases in its life cycle, but the sexual stages of the parasite only occur in the intestine of felids, mainly the cat, as its definitive host, resulting in the excretion of oocysts in the feces. Thus, one way that humans and other warm-blooded animals can be infected is by swallowing oocysts that contaminate food, water or the environment. A second way of transmission of *T. gondii* to humans is by the consumption of undercooked meat containing tissue cysts from a variety of animals that are susceptible to infection, including sheep, pigs, and poultry. Finally, *T. gondii* tachyzoites can also be transmitted from mother to developing fetus, leading to congenital disease (Black & Boothroyd, 2000). Toxoplasmosis in humans is commonly asymptomatic, but the symptoms can vary depending on the immune status of the patient and the clinical setting, that is, ocular or congenital toxoplasmosis (Montoya & Liesenfeld, 2004; Sharif et al., 2007).

Current therapies for toxoplasmosis have been limited due to host toxicity and side effects, particularly for sulfa drugs (Kim et al., 2007). Considering that the treatment shows limited efficacy due to their substantial side effects, particularly in immunocompromised individuals and pregnant women, alternative therapies have been investigated by using chemical substances derived from plants, animals and microorganisms, as well as natural toxins and their derivatives (Tempone et al., 2007; Bastos et al., 2008). The aim of this study was to evaluate the effects of BpirLAAO-I on infection of *Toxoplasma gondii* in human foreskin fibroblasts *in vitro*.

Materials and Methods

Bothrops pirajai *snake venon and BpirLAAO-I toxin*

Crude *Bothrops pirajai* snake venom was obtained from Serpentário Proteínas Bioativas Ltda., Batatais, Brazil. BpirLAAO-I toxin was isolated and purified using a combination of molecular exclusion, affinity, and hydrophobic chromatography steps, as previously described (Izidoro et al., 2006). BpirLAAO-I is a homodimeric acid glycoprotein with approximate molecular weight of 130 kDa and pI of 4.9, displays high specificity toward hydrophobic/aromatic amino acids, and deglycosylation does not alter its enzymatic activity (Izidoro et al., 2006). In the present study, the protein concentration of the purified enzyme was determined by Bradford (1976).

Toxoplasma gondii *tachyzoites*

Tachyzoites of the virulent RH strain of *T. gondii* were maintained by intraperitoneal serial passages in Swiss mice at regular 48 h intervals (Mineo et al., 1980). Peritoneal exudates were harvested and washed twice (720 x *g*, 10 min, 4 °C) in RPMI 1640 medium (Gibco, Paisley, UK). The pellet was suspended in 5 mL of medium and viable parasites were counted in hemocytometric chamber by Trypan blue dye exclusion.

Human foreskin fibroblast culture

Human foreskin fibroblasts (HFF) were obtained from American Type Culture Collection (ATCC, Manassas, USA) and cultured in 25 cm^2 flasks until confluence in RPMI medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma Chemical Co., St. Louis, USA) and 10% heat-inactivated fetal calf serum (Cultilab, Campinas, Brazil) (complete RPMI) in a humidified incubator at 37 °C and 5% CO_2.

Cytotoxicity assay

Cytotoxicity of BpirLAAO-I was assessed by determining HFF cell viability using MTT assays (Mosmann, 1983). HFF cells were cultured in 96-well plates (1×10^5 cells/well) in triplicate in complete RPMI

medium in the presence of double serial dilutions of BpirLAAO-I (from 20 to 0.3 µg/mL) in complete medium, for 24 h at 37 °C and 5% CO_2. As controls, cells were incubated with complete medium alone. Cells were washed and pulsed with 10 µL of thiazolyl blue (MTT, Sigma Chemical Co.) at 5 mg/mL in 90 µL of complete RPMI medium 4 h prior to the end of the culture. Formazan particles were solubilized in 10% sodium dodecyl sulfate and 50% N,N-dimethyl formamide. The optical density was determined after 30 min at 570 nm in a plate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, USA). Results were expressed as percentage of cell viability in relation to controls. The median cytotoxic dose of BpirLAAO-I for HFF cells (50% cytotoxic dose - TD50) was calculated by extrapolation of a sigmoidal dose-response curve on a nonlinear regression plot with 95% confidence intervals (CI), corresponding to the cell viability 50% point, by using the GraphPad Prism software (GraphPad Software Inc., San Diego, USA) as previously described (Jones-Brando et al., 2006).

BpirLAAO-I treatment of T. gondii *tachyzoites before infection of HFF cells*

HFF cells were cultured on 13-mm round glass coverslips into 24-well plates ($1x10^5$ cells/well/200 µL) for 24 h at 37 °C and 5% CO_2. *T. gondii* tachyzoites were pretreated for 1 h at 37 °C and 5% CO2 with double serial dilutions of BpirLAAO-I (from 5 to 0.3 µg/mL) or with medium alone. Next, parasites were centrifuged (720 x *g*, 10 min), washed once with medium, and then incubated with HFF cell monolayers on glass coverslips at a 5:1 (parasite:host-cell) ratio of infection. After 24 h of incubation at 37 °C and 5% CO_2, cells were washed with phosphate-buffered saline (PBS, pH 7.2), fixed in 10% buffered formalin for 2 h and stained with 1% toluidine blue. Coverslips were mounted on glass slides and cells were examined under light microscope with regards to the infection index (percentage of infected cells per 100 examined cells) and parasite intracellular replication (mean number of parasites per cell in 100 infected cells) (Bastos et al., 2008). Results were also expressed as percentages of inhibition of infection or inhibition of intracellular replication of each treatment in relation to controls (100% of infection). Three slides of each treatment condition were assessed by two independent observers. The median inhibitory dose (50% inhibitory dose - ID50) was calculated as described for TD50. For each treatment, a selectivity index (SI) was also calculated as described elsewhere (Nan et al., 2004), representing the ratio of the median cytotoxic dose (TD50) by the median inhibitory dose (ID50) for *in vitro* experiments.

*BpirLAAO-I treatment of HFF cells after infection wit*h T. gondii *tachyzoites*

HFF cell monolayers were washed with RPMI medium and infected with *T. gondii* RH strain tachyzoites at a 5:1 (parasite:host-cell) ratio of infection. After 3 h of incubation, cells were again washed to remove non-adherent parasites and then treated in triplicate with double serial dilutions of BpirLAAO-I (from 5 to 0.3 µg/mL). As controls, infected cells were incubated with medium alone. After 24 h of incubation at 37 °C and 5% of CO_2, cells were examined and results were expressed as described above. Three slides of each treatment condition were assessed by two independent observers.

Statistical analysis

Statistical analysis and graphs were performed using the GraphPad Prism software version 4.0 (GraphPad Software Inc.). Comparison between groups was done using ANOVA and Bonferroni´s multiple comparison test. Statistical significance was established when $p<0.05$.

Results

Cytotoxic activity was determined by screening different concentrations (0.3-20 µg/mL) of BpirLAAO-I on HFF cells (Figure 1). The dosing interval (0.3 to 5 µg/mL) of BpirLAAO-I resulted in cell viability above 80%, which was adopted as criteria for further experiments. The TD50 of BpirLAAO-I was established in 11.8 µg/mL (Figure 1; Table 1).

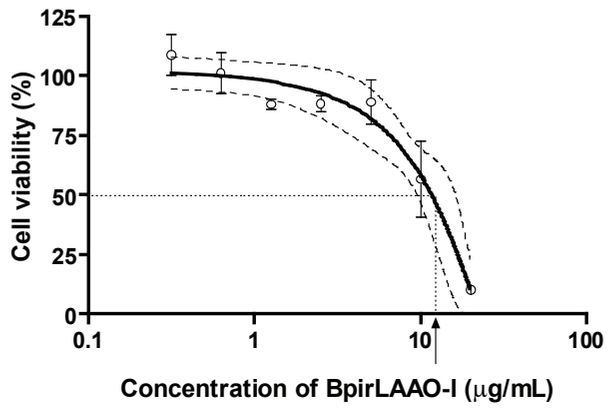

Figure 1. *In vitro* cytotoxic activity of BpirLAAO-I determined by MTT assays. Human fibroblasts were cultivated in the absence or presence of different concentrations (20 to 0.3 µg/mL) of BpirLAAO-I for 24 h. Results are expressed as mean and standard deviations of percentages of viable cells in relation to the controls and are plotted in a nonlinear regression sigmoidal dose-response curve with 95% confidence intervals. Dotted lines show BpirLAAO-I concentration that corresponds to 50% of cell viability and

Table 1. *In vitro* inhibition of *Toxoplasma gondii* infection in human fibroblasts under different treatment conditions with BpirLAAO-I isolated from *Bothrops pirajai* venom snake.

Treatment	TD_{50}[c] (µg/mL)	ID_{50}[d] (µg/mL)		SI[e]	
		Infection	Replication	Infection	Replication
Before infection[a]	11.80	1.83	3.14	6.45	3.76
After infection[b]	11.80	1.20	ND[f]	9.83	ND[f]

[a]Treatment of *T. gondii* RH strain tachyzoites with different concentrations of BpirLAAO-I (serial double dilutions from 5 to 0.3 µg/mL) before infection of human fibroblasts; [b]Treatment of human fibroblasts with different concentrations of BpirLAAO-I (serial double dilutions from 5 to 0.3 µg/mL) after infection with *T. gondii* tachyzoites; [c]TD50: Toxic dose of 50% in human fibroblasts; [d]ID50: Inhibitory dose of 50% in relation to the parameters of infection and parasite intracellular replication; [e]SI: Selectivity index = TD50/ID50; [f]Not determined as no 50% inhibition was obtained.

the arrow indicates the median cytotoxic dose.

The treatment of *T. gondii* tachyzoites with different BpirLAAO-I concentrations before infection of host cells showed a dose-dependent decrease in the infection index (Figure 2A). A significant effect of BpirLAAO-I was observed for concentrations from 1.25 to 5 µg/mL in relation to untreated controls (Figure 2A). A similar profile with significant dose-dependent decrease in the infection index, starting from 1.25 to 5 µg/mL of BpirLAAO-I was found for treatment of host cells after parasite infection (Figure 2C).

The mean number of intracellular parasites in host cells infected with tachyzoites previously treated with different concentrations of BpirLAAO-I decreased when enzyme concentrations increased, although significant differences were found only at 5 µg/mL of enzyme as compared to untreated controls (Figure 2B). In contrast, when *T. gondii*-infected HFF cells were treated with BpirLAAO-I, no enzyme concentration was effective in reducing the mean number of intracellular parasites as compared to untreated controls (Figure 2D). As illustrated in Figure 3, the amount of parasites within the parasitophorous vacuole decreased when parasites were treated before infection (Figure 3A), as compared to untreated infected cells (Figure 3B). Also, the treatment with the enzyme did not alter the host cell

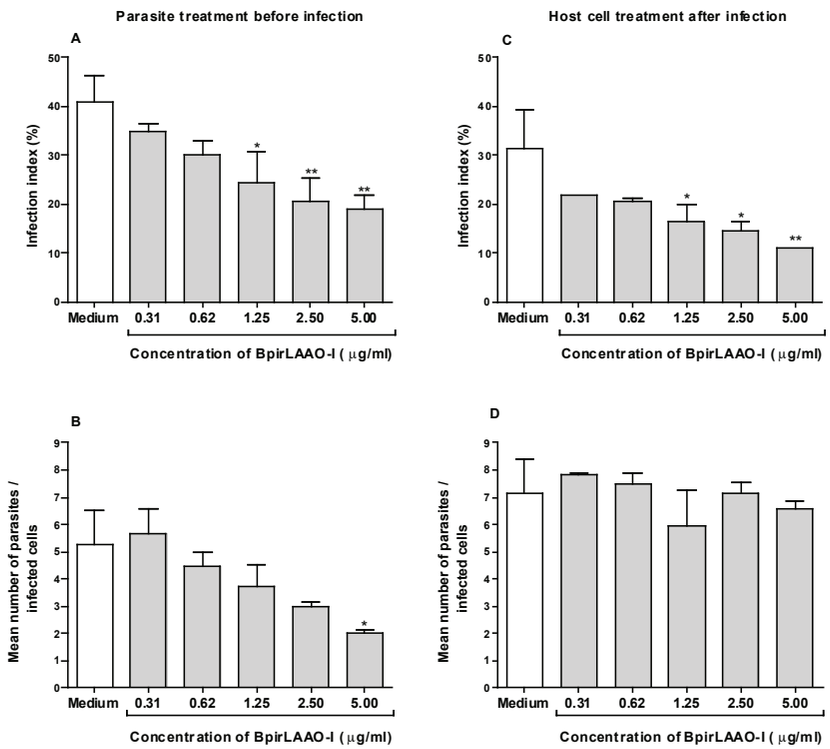

Figure 2. *In vitro* effects of BpirLAAO-I treatment on *T. gondii* infection and intracellular replication. The index of infection (percentage of infected cells in 100 examined cells) and the parasite replication (mean number of parasites per 100 infected cells) on human fibroblasts were evaluated under two different experimental conditions. (A, B) BpirLAAO-I treatment of *T. gondii* tachyzoites before infection of fibroblasts. (C, D) BpirLAAO-I treatment of fibroblasts after *T. gondii* infection. Bars represent mean and standard deviation of three analyses for each condition. Statistically significant differences in relation to medium (*p<0.05; **p<0.01).

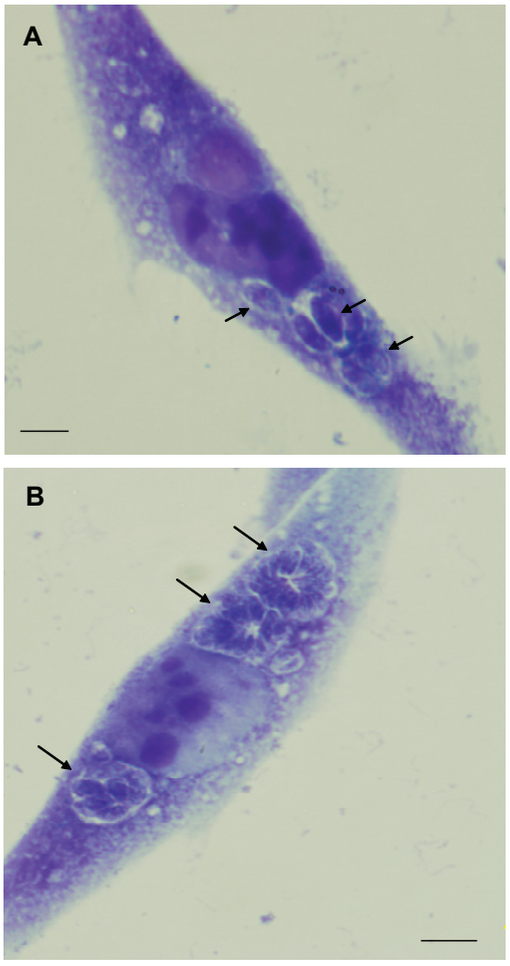

Figure 3. Photomicrographs of human fibroblasts infected with *T. gondii* tachyzoites previously treated with BpirLAAO-I at 5 µg/mL (A) and untreated infected cells (B) after 24 h of infection. The presence of parasitophorous vacuoles (arrows) inside a single host cell was observed in both conditions. Note the considerable decrease in the number of parasites within the parasitophorous vacuoles after BpirLAAO-I treatment (A) compared to multiple generations of tachyzoites inside the parasitophorous vacuoles in untreated cells (B). Bar scale: 10 µm.

morphology or its replication.

The curves of inhibition of *T. gondii* infection on HFF cells when parasites were pretreated with different concentrations of BpirLAAO-I (Figure 4A) or when previously infected cells were treated with BpirLAAO-I (Figure 4C) showed a dose-dependent inhibition in relation to untreated controls. The ID50 of BpirLAAO-I was calculated and established in 1.83 µg/mL for treatment before infection and 1.20 µg/mL for treatment after infection (Table 1).

Concerning the inhibition of parasite intracellular replication, the treatment of *T. gondii* tachyzoites before infection of host cells with BpirLAAO-I showed a curve of dose-dependent inhibition in relation to untreated controls, with ID50 of enzyme determined at 3.14 µg/mL (Figure 4B; Table 1). In contrast, the treatment of *T. gondii*-infected cells with the tested concentrations of BpirLAAO-I showed no inhibition of intracellular parasite replication in relation to untreated controls, and the ID50 could not be determined (Figure 4D; Table 1).

The selectivity indexes (SI) calculated for the infection index and intracellular replication were 6.45 and 3.76, respectively, for the BpirLAAO-I treatment of parasites before infection. In contrast, a higher SI (9.83) was obtained for the infection index when BpirLAAO-I treatment was performed after infection, whereas SI could not be calculated for the parasite intracellular replication, since no 50% inhibition was obtained (Table 1).

Discussion

Snake venoms belong to the most concentrated secretory fluids of vertebrates, consisting mainly of proteins and peptides generally termed toxins. These are primarily responsible for well-known biological effects and employed in several diagnostic and therapeutic approaches (Oliveira et al., 2009; Costa et al., 2009). The toxicity of the snake venom has been attributed to a class of toxins, the L-amino acid oxidases (svLAAO), although the precise mechanism of action of these enzymes and their role in snake toxicity are not well understood. Previous studies have reported that svLAAO may be related to defense mechanisms against natural agents, as parasites and bacteria (Iijima et al., 2003; Nuutinen & Timonen, 2008; Kitani et al., 2008).

The anti-parasite effect of snake venoms can be also attributed to LAAO as previously described in the killing of *Leishmania* spp. (Tempone et al., 2001; Izidoro et al., 2006), *Plasmodium falciparum* (Zieler et al., 2001), and *Trypanosoma cruzi* (França et al., 2007). In the present study, we demonstrated for the first time the *in vitro* effects of a svLAAO isolated from *Bothrops pirajai* (BpirLAAO-I) on infection of *T. gondii* in human fibroblasts. Initially, we evaluated the cytotoxic activity of BpirLAAO-I on host cells, showing a dose-dependent cytotoxicity with TD50 established at 11.8 µg/mL. The probable mechanisms of cytotoxicity induced by LAAO involve necrosis, apoptosis, or depletion of essential amino acids in the culture medium by enzymatic oxidation (Butzke et al., 2005). The process of necrosis could be related to the direct action of hydrogen peroxide on the cell plasma membrane, since the mechanism of apoptosis in the development of morphological and biochemical changes leads to cell death (Ande et al., 2006). Therefore, most of the biological effects of LAAO may be due to the secondary effect of hydrogen peroxide generated

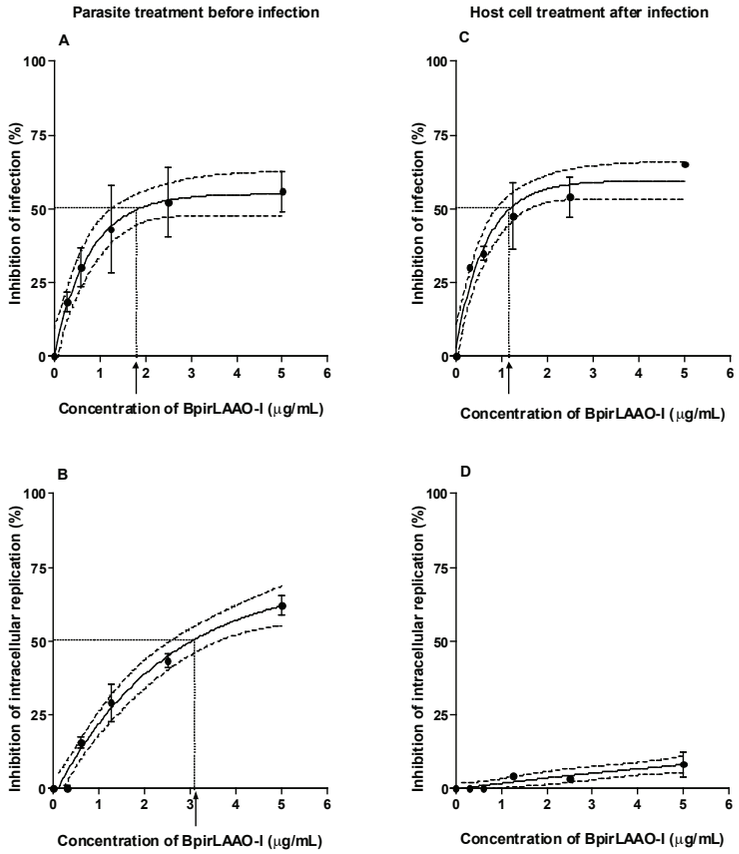

Figure 4. Effect of BpirLAAO-I treatment on inhibition of *T. gondii* infection and intracellular replication. Results of BpirLAAO-I treatment before (A, B) or after (C, D) infection are expressed as mean and standard deviations of percentages of infection inhibition in relation to the untreated controls and plotted in a nonlinear regression sigmoidal dose-response curve with 95% confidence intervals. Dotted lines show the BpirLAAO-I concentration that corresponds to 50% of infection or intracellular replication inhibition. The arrows indicate the median inhibitory doses (ID50).

during the specific catalytic activity (Ponnudurai et al., 1994). This hypothesis can be supported when catalase is added to the medium in experimental conditions, when the toxic activity is totally abolished (Toyama et al., 2006; Izidoro et al., 2006; Rodrigues et al., 2009; Ciscotto et al., 2009).

In the present study, the effects of BpirLAAO-I were also evaluated on the infection index of *T. gondii* in human fibroblasts. It was demonstrated that BpirLAAO-I induced considerable dose-dependent decrease in this infection parameter in the two experimental conditions, treatment of tachyzoites before infection and treatment of host cells after parasite infection. However, the values of ID50 and SI indicate lower inhibitory dose and higher selectivity index when LAAO was given to previously infected cell cultures, indicating that BpirLAAO-I seems to have higher efficacy in inhibiting the infection index in this experimental condition. These findings could be due to a probable effect of BpirLAAO-I on tachyzoites freshly released from cellular lysis, thus preventing the infection of new surrounding fibroblasts, rather than a direct effect or interaction with the host cell. Although all infection indexes were lower when the host cell treatment was done after infection, including the untreated controls, the dose-dependent decrease in this infection parameter was similar when the parasite treatment was done before infection. It is worthy to note that the two groups of experiments were performed independently in triplicate samples, with their respective untreated controls. Therefore, the differences observed in the infection index between both groups of experiments could be attributed to intra-assay variations of each experimental condition that were minimized by using the untreated controls, as well as by analyzing the inhibition percentages in relation to controls for each experimental group.

Accordingly, *T. gondii* tachyzoite replication inside the host cells happens at each 6 and 8 h, which may result in host cell lysis and infection of new

neighboring cells (Black & Boothroyd, 2000). It is worthy to note that in our experimental design we used a parasite:host-cell ratio relatively high (5:1) that could lead to invasion of plus than one tachyzoite to a single host cell. Consequently, multiple generations of daughter-parasites may be replicating by endodyogeny inside a single host cell, which may result in its lysis even before 24 h of infection. However, when the parasite intracellular replication was analyzed, the condition of treatment before infection was also successful in reducing parasitism, although with higher values of ID50 and correspondently lower values of SI. These findings suggest that this reduction in parasite numbers could also be the result of the initial infection inhibition rather than a replication specific inhibitory effect. On the other hand, the treatment after infection was not effective, since no ID50 or SI values could be determined, suggesting that the enzyme seems to cause no effect on the parasite intracellular replication for this condition. It is noteworthy that BpirLAAO-I is a glycoprotein with molecular mass of 130 kDa and possibly does not easily enter cells and reach parasitophorous vacuoles lodging *T. gondii* tachyzoites. Therefore, these findings reinforce that the effect of BpirLAAO-I is more likely directed to tachyzoites freshly released from infected lysed cells, with consequent decrease of the infection index of new host cells.

Recently, we observed anti-parasitic activity against tachyzoites of *T. gondii* with another class of snake toxin, a metalloproteinase named neuwiedase purified from *Bothrops neuwiedi* (Bastos et al., 2008). This enzyme acts by degradating proteins expressed in fibroblast extracellular matrix responsible to maintain adhesion of the cellular monolayer, especially laminin, which is involved in the process of parasite invasion (Rodrigues et al., 2009; Bastos et al., 2008). In this context, BpirLAAO-I can also act in the extracellular matrix by inducing oxidative changes and preventing its use by the parasite as a mediator to access the receptors of laminin found in the membrane of the host cells, thus reducing the infection rates in fibroblasts.

When the rates of infection inhibition were compared between both studies, maximal infection inhibition rates around 71 and 61% were found for the neuwiedase treatments after and before infection, respectively, which were slightly higher as compared to the BpirLAAO-1 treatments (65 and 56%, respectively). However, when the selectivity indexes for infection inhibition were compared, higher SI values (6.45 and 9.83) were found for BpirLAAO-I treatments before and after infection, respectively, as compared to lower SI values (1.62 and 2.58) for the neuwiedase toxin (Bastos et al., 2008), indicating that BpirLAAO-I seems to be more effective than neuwiedase for this infection parameter.

After invasion of the host cell, *T. gondii* tachyzoites are located inside parasitophorous vacuoles constituted mostly by membranous material coming from the parasite (Dubey et al., 1998; Black & Boothroyd, 2000). During the endodyogeny process, the original plasmalemma of the invading tachyzoites is used to involve the generated daughter cells. In the present study, when the tachyzoite was pretreated with BpirLAAO-I before infection, it is possible that some parasite components might have been submitted to the oxidative action of this enzyme. As a result, these components can lose the integrity of some proteins, which might harm the functional activity of the vacuole as much as the formation of the plasmalemma of the daughter cells, thus reducing parasite intracellular replication. It is also possible that the enzyme signals the parasite to induce premature egress. Further studies should be conducted to clarify the actual mechanisms of action of BpirLAAO-I on *T. gondii* infection and replication, by analyzing the early time points after infection. Bastos et al (2008) found similar results with dose-dependent inhibition of *T. gondii* intracellular replication after previous treatment of tachyzoites with the snake metalloproteinase neuwiedase.

The research for new active principles and the development of more effective drugs has been largely investigated in the treatment for toxoplasmosis, due to the considerable side effects of the conventional therapy (Montoya & Liesenfeld, 2004; Carruthers, 2006; Martins-Duarte et al., 2006; Petersen, 2007). Thus, the effects of the BpirLAAO-I described herein could be taken into account for the development of new generation of synthetic or recombinant therapeutic anti-parasitic agents. In addition, these findings support the importance to study ophidian enzymes not only for a better understanding of their role in the envenomation mechanism, but also because of their potential applications as biotechnological tools.

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