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Anti-parasitic effect on *Toxoplasma gondii* induced by BnSP-7, a Lys49-phospholipase A_2 homologue from *Bothrops pauloensis* venom

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ABSTRACT

Toxoplasmosis affects a third of the global population and presents high incidence in tropical areas. Its great relevance in public health has led to a search for new therapeutic approaches. Herein, we report the antiparasitic effects of BnSP-7 toxin, a Lys49 phospholipase A_2 (PLA_2) homologue from *Bothrops pauloensis* snake venom, on *Toxoplasma gondii*. In an MTT assay, BnSP-7 presented significant cytotoxicity against host HeLa cells at higher doses (200 μg/mL to 50 μg/mL), whereas lower doses (25 μg/mL to 1.56 μg/mL) produced low cytotoxicity. Furthermore, the toxin showed no effect on *T. gondii* tachyzoite viability when evaluated by trypan blue exclusion, but decreased both adhesion and parasite proliferation when tachyzoites were treated before infection. We also measured cytokines in supernatants collected from HeLa cells infected with *T. gondii* tachyzoites previously treated with RPMI or BnSP-7, which revealed enhancement of only MIF and IL-6 cytokines levels in supernatants of HeLa cells after BnSP-7 treatment. Our results showed that the BnSP-7 PLA_2 exerts an anti-*Toxoplasma* effect at a lower dose than that required to induce cytotoxicity in HeLa cells, and also modulates the immune response of host cells. In this sense, the anti-parasitic effect of BnSP-7 PLA_2 demonstrated in the present study opens perspectives for use of this toxin as a tool for future studies on toxoplasmosis.

1. Introduction

Toxoplasma gondii, an obligate intracellular protozoan parasite of the phylum Apicomplexa, is widely distributed in nature. Toxoplasmosis, the disease caused by this parasite, is present throughout the world, affects one third of the global population, and increases in incidence with decreasing latitude (Montoya and Liesenfeld, 2004; Petersen, 2007). In Brazil, due to the presence of several atypical types of strains, the incidence of congenital toxoplasmosis is the highest in the world, affecting children and promoting severe symptoms (Gilbert et al., 2008; Dubey et al., 2012). Throughout its evolution, *T. gondii* has developed mechanisms that enable a long-lasting parasite-host interaction to assure its survival without inducing life-threatening disease in the intermediate host, making it extremely adapted for infection in humans (Lang et al., 2007).

In addition, the standard treatment against toxoplasmosis is a combination of two drugs: sulfadiazine and pyrimethamine. However, it is well known that this classical treatment induces serious side effects and toxicity in the host (Montoya and Liesenfeld, 2004; Carruthers, 2006; Martins-Duarte et al., 2006; Petersen, 2007); thus, alternative therapeutic compounds are needed. Many studies proposed alternative strategies for the treatment of toxoplasmosis, including plant extracts (Oliveira et al., 2009), azithromycin (Costa et al., 2009), toltrazuril (Kul et al., 2013) and enrofloxacin (Barbosa et al., 2012).

Our previous study demonstrated the potential anti-parasitic effect of BpLec, a C-type lectin isolated from *Bothrops pauloensis*

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snake venom, since this lectin reduced the *T. gondii* infection in human epithelial uterine cells (HeLa lineage) (Castanheira et al., 2015). In this sense, animal venoms, especially snake venoms, have been the subject of several studies that demonstrate their therapeutic potential (Costa et al., 2008; Passero et al., 2008; Nunes et al., 2013; Castanheira et al., 2015; Rodrigues et al., 2015). Most prominent among the different proteins present in snake venoms that have shown anti-parasitic effects are phospholipases A_2 (PLA_2) (Guillaume et al., 2004; Stábeli et al., 2006; Torres et al., 2010; Adade et al., 2010; Costa Torres et al., 2010; Castillo et al., 2012; Nunes et al., 2013; de Moura et al., 2014).

PLA_2s, which contain approximately 125 amino acid residues, are able to hydrolyze the 2-acyl ester bond of phospholipids by releasing fatty acids and lysophosphatides (Arni and Ward, 1996; Kini, 2003). The replacement of the amino acid aspartate by a lysine in position 49 provokes loss of enzymatic activity of these PLA_2, and therefore these variants have been referred to as Lys49 PLA_2 homologues (Lomonte et al., 2003). However, these Lys49-PLA_2s are capable of disrupting the integrity of membranes and provoking many pharmacological effects (Díaz et al., 1991; Rufini et al., 1992; Lomonte et al., 2003, 2012).

BnSP-7, a Lys49 PLA_2 homologue, is the most well studied toxin from *B. pauloensis* venom (Rodrigues et al., 1998; Soares et al., 2000; Magro et al., 2003; Oliveira et al., 2009; Nunes et al., 2013). Recently, Nunes et al. (2013) reported the growth inhibition of *Leishmania (Leishmania) amazonensis* promastigotes by BnSP-7, as well as a delay in amastigote-promastigote differentiation, ultrastructural changes in promastigote morphology and reduction in the infectivity index. Considering these promising anti-parasitic effects induced by BnSP-7, we describe herein the effects of BnSP-7 on the adhesion and proliferation of *T. gondii* tachyzoites. In addition, we demonstrated the cytokine profile induced by BnSP-7 and its relationship with parasitism control.

2. Material and methods

2.1. BnSP-7 PLA_2 purification

BnSP-7 PLA_2 was isolated from *B. pauloensis* snake venom according to the description of Soares et al. (2000) with minor modifications. Approximately 200 mg of *B. pauloensis* was resuspended in 2 mL of 50 mM ammonium bicarbonate NH_4HCO_3 buffer (pH 7.8) and centrifuged at 3000 g for 10 min at 4 °C. The supernatant was recovered and injected on a CM-Sepharose Fast Flow resin previously equilibrated and initially eluted with the same buffer at room temperature. Fractions were collected at 1 mL/tube 6.5 mL/h (flow rate by a Redifrac fraction collector). Fractions were monitored by Ultrospec 1000 spectrophotometer by absorbance at 280 nm. The fraction containing BnSP-7 PLA_2, according to the method of Soares et al. (2000), was lyophilized and stored at −20° C and then rechromatographed by reverse-phase chromatography (RP-HPLC) in a C18 column (GE Healthcare). The column was equilibrated with solvent A (0.1% trifluoroacetic acid, 4% acetonitrile) and eluted with a concentration gradient of solvent B (0.1% trifluoroacetic acid, 80% acetonitrile) from 0% to 100%, and a flow rate of 0.5 mL/min for 33 min. The single peak Abs 280 nm was lyophilized and analyzed on 12.5% SDS PAGE (v/v) according to Laemmli (1970) and its amino-terminal sequence was determined by the Edman degradation method (Edman and Begg, 1967) for confirmation of protein identity. This protein was assessed further in relation to *T. gondii* parasitism as described below.

2.2. Cell culture

Human epithelial uterine cells (HeLa cell line) were obtained from the American Type Culture Collection (ATCC® CCL-2™, Manassas, VA, USA) and cultured in 75 cm^3 flasks with RPMI-1640 medium supplemented with 25 mM HEPES, 23 mM sodium carbonate, 100 μg/mL streptomycin, 100 U/ml penicillin and 10% heat-inactivated fetal bovine serum (FBS) (complete medium), in a humidified incubator at 37 °C and 5% CO_2 (Barbosa et al., 2008).

2.3. Parasites

The *T. gondii* RH strain was maintained primarily in the peritoneal cavity of Swiss mice from the Animal Experimentation Vivarium of the Federal University of Uberlândia. Intraperitoneal inoculation was performed in mice by serial passages at intervals of 48–72 h in inoculum, with approximately 10^6 tachyzoites obtained from peritoneal exudates of previously infected mice (Mineo et al., 1980), obtained by washing the abdominal cavity with phosphate buffered saline 0.01 M phosphate (PBS) pH: 7.2. Then the parasitic suspensions were centrifuged at 720 × *g* for 5 min. These parasites were then transferred to bottles containing HeLa cells cultured in RPMI 1640 medium supplemented with penicillin, streptomycin and 2% FBS at 37 °C and 5% CO_2 (Barbosa et al., 2008).

T. gondii tachyzoites (2F1 clone) – which are derived from the RH strain, are highly virulent and express constitutively cytoplasmic β-galactosidase – were generously donated by Dr. Vern Carruthers from the School of Medicine at the University of Michigan (USA). These parasites were maintained in HeLa cells cultured in RPMI 1640 medium supplemented with penicillin, streptomycin and 2% FBS at 37 °C and 5% CO_2.

2.4. Cellular viability in HeLa cells

The cellular viability in the presence of BnSP-7 toxin was evaluated on HeLa by a colorimetric assay, based on mitochondrial oxidation of the MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue tetrazole) (Sigma), as previously described by Mosmann (1983) with some modifications.

Briefly, HeLa cells were cultured in 96-well plates (3×10^4 cells/200 μL/well) in complete medium for 24 h at 37 °C and 5% CO_2. Next, cells were treated with different concentrations of BnSP-7 (200 μg/mL to 1.5 μg/mL) by serial two-fold dilutions in RPMI medium. After 24 h, the supernatants were discarded and 10 μL of MTT and 90 μL of 10% FBS medium were added to each well for 3 h, under the same culturing conditions. Afterward, formazan crystals were solubilized in 10% sodium dodecyl sulfate (SDS) (Sigma) and 50% N, N- dimethyl formamide. The optical density was determined at 570 nm in a plate reader. The results were expressed as percentages of viable cells in relation to controls (100% of the viability). This assay was performed three times in triplicate.

2.5. Tachyzoite viability

The tachyzoite viability assay was performed according to Castanheira et al. (2015). Briefly, tachyzoites (4×10^6/mL) were treated with BnSP-7 (50 μg/mL, 25 μg/mL and 12.5 μg/mL) for 30 min and dyed by trypan blue. The control of the assay was comprised of tachyzoites treated with RPMI-1640 medium. Viable parasites that presented a clear cytoplasm were counted in an optical microscope (Strober, 2001). This assay was performed three times in triplicate.

2.6. Adhesion assay

To perform this assay, we chose two treatment approaches. In the first step of experiments, HeLa cells were cultured and treated with BnSP-7 (3.0 μg/mL and 1.5 μg/mL) for 1 or 24 h, after which the