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Crotoxin and phospholipases A_2 from *Crotalus durissus terrificus* showed antiviral activity against dengue and yellow fever viruses

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A B S T R A C T

Dengue is the most important arbovirus in the world with an estimated of 50 million dengue infections occurring annually and approximately 2.5 billion people living in dengue endemic countries. Yellow fever is a viral hemorrhagic fever with high mortality that is transmitted by mosquitoes. Effective vaccines against yellow fever have been available for almost 70 years and are responsible for a significant reduction of occurrences of the disease worldwide; however, approximately 200,000 cases of yellow fever still occur annually, principally in Africa. Therefore, it is a public health priority to develop antiviral agents for treatment of these virus infections. *Crotalus durissus terrificus* snake, a South American rattlesnake, presents venom with several biologically actives molecules. In this study, we evaluated the antiviral activity of crude venom and isolated toxins from *Crotalus durissus terrificus* and found that phospholipases A_2 showed a high inhibition of Yellow fever and dengue viruses in VERO E6 cells.

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1. Introduction

Dengue virus (DENV) and yellow fever virus (YFV), members of the genus *Flavivirus*, family *Flaviviridae*, are two of the most important arboviruses in public health (Gould and Solomon, 2008). Dengue is the most rapidly spreading arbovirus disease in the world. In the last 50 years, incidence has increased 30-fold with increasing geographic expansion to new countries and, in the present decade, from urban to rural settings. An estimated 50 million dengue infections occur annually and approximately 2.5 billion people live in dengue endemic countries (WHO, 2010). Infection with any of the four DENV serotypes (DENV-1, -2, -3 and -4) can be asymptomatic or can lead to a wide spectrum of disease, in some cases with fatal outcome (Harris et al., 2000). YFV is the causative agent of severe acute hemorrhagic fever with high mortality. Effective vaccines against yellow fever have been available for almost 70 years and are responsible for a significant reduction of occurrences of the disease worldwide; however, approximately 200,000 cases of yellow fever still occur annually, principally in Africa (Robertson et al., 1996; WHO, 2010). There is no specific drug therapy for DENV and YFV infections; therefore, the development of antiviral agents to reduce the morbidity and mortality causes by these two viruses is a public health priority (Hombach et al., 2005).

Snake venoms are complex mixtures of toxins and enzymes that show different activities on biological

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systems, such as cytotoxicity, hemorrhage activity, bradykinin-releasing activity, thrombin-like activity, hemolysis, cardiovascular and hypotensive effects, tissue necrosis and neurotoxic effects (Castro et al., 2004; Debnath et al., 2010; Gutiérrez et al., 2005; Lee, 1977; Montecucco et al., 2008; Oyama et al., 2008; Vital-Brazil, 1982). The venom of *Crotalus durissus terrificus* snake, a South American rattlesnake, has shown several biological activities, including antiviral activity against measles virus (Bercovici et al., 1987; Petricevich and Mendonça, 2003). This venom is composed by neurotoxins, crotoxin (Slotta and Fraenkel-Conrat, 1938), crotamin (Gonçalves and Vieira, 1950), phospholipase A_2 "inter-cro" (PLA_2-IC) (Vieira, 2009), gyroxin (Alexander et al., 1988) and convulxin (Prado-Franceschi and vital Brazil, 1981). The fraction which pathophysiological aspects are better characterized is the crotoxin. It represents 40–60% of the dry weight of venom and it is the main toxic component with neurotoxic effects (Faure and Bon, 1988; Vital-Brazil, 1966). This component presents two different subunits no covalently linked: crotapotin, an acid component with a molecular weight of ∼9,000 Da and the phospholipase A_2, a basic component (PLA_2-CB) with a molecular weight of 16,400 Da (Hendon and Fraenkel-Conrat, 1971; Rubsamen et al., 1971). Faure et al. (1994) isolated and characterized several isoforms of each subunit of crotoxin in the venom collected from numerous snakes. Crotoxin is, in fact, a mixture of variants deriving from the combination of subunit isoforms. Four crotapotin and four PLA_2-CB present in venom collected from numerous snakes were purified and some sequenced (Faure et al., 1991). The crotapotin isoforms consist of three disulfide-linked polypeptide chains (α, β, γ), which result from different proteolytic cleavages of a unique precursor pro-crotapotin that has been identified from its cDNA. Two cDNAs encoding PLA_2-CB isoforms have been cloned and their nucleic acid sequences determined (Bouchier et al., 1991). The PLA_2-IC was first observed by Laure (1975), but the enzyme was not isolated or characterized. Vieira (2009) subsequently isolated and characterized the PLA_2-IC demonstrating that was a new isoform, showing differences in the C-terminal region when compared with PLA_2-CB1 and PLA_2-CB2 (basic chain of crotoxin). The alignment of the PLA_2-CB1, PLA_2-CB2 and PLA_2-IC sequences and phylogenetic analysis showed that PLA_2-CB2 isoform showed higher homology with the PLA_2-IC isoform and, furthermore, this homology is greater than that observed between PLA_2-CB1 and PLA_2-IC and even between PLA_2-CB1 and PLA_2-CB2. The PLA_2-CB2 was originated from the duplication of the PLA_2-CB1 gene (Vieira, 2009). Faure et al. (1994) demonstrated that the complex PLA_2-CB1/CA present in crotoxin, is more stable than PLA_2-CB2/CA association, confirming that PLA_2-IC is an isoform of PLA_2-CB2, which does not make the association with crotapotin but is able to exert its biological activity present in the venom of *C. d. terrificus*.

In this study, we evaluated the antiviral activity of crude venom and isolated toxins from *Crotalus durissus terrificus* and found that phospholipases A_2 showed a high antiviral effect against DENV and YFV.

2. Material and methods

2.1. Cells and viruses

VERO E6 and C6/36 cells were maintained in Leibovitz medium (L-15) with 10% of fetal bovine serum (FBS) at 37 °C and 28 °C, respectively. DENV-2 (strain NGC) and YFV (strain 17D) were used in this study. The viruses were propagated in C6/36 cells, titrated by plaque formation assay in VERO E6 cells and expressed in plaque forming units per milliliters (PFU/mL).

2.2. Venom and isolated toxins

Lyophilized yellow and white crude venom from *C. d. terrificus* and crude venom from *Bothrops jararacussu* were obtained from the serpentarium of the Medical School of Ribeirão Preto, São Paulo University (IBAMA authorization: 1/35/1998/000846-1). Crotapotin, crotamin, crotoxin, convulxin, PLA_2-CB and PLA_2-IC were purified from *C. d. terrificus* and BthTX-I from *B. jararacussu*.

2.3. Toxins purification procedure

White crude venom (500 mg) from *C. d. terrificus* was fractionated by size exclusion chromatography on Sephadex G-75 column (4.0 × 110.0 cm) equilibrated with 50 mM ammonium formate, pH 3.5. Elution was carried out using the same buffer at a flow rate of 30 mL/h. Fractions of 5.0 mL were collected in a fraction collector coupled to an ultraviolet detector. The absorbance of collected fractions was determined at 280 nm on a spectrophotometer Beckman DU-640 (Beckman, USA). The fractions containing crotamin, crotoxin, convulxin and PLA_2-IC were collected, dialyzed and lyophilized. The crotoxin (1 g) was dissolved in 5.0 mL of 50 mM Tris-HCl, pH 7.2, containing 7 M Urea, and incubated for 12 h at room temperature. In order to remove urea from the sample, the solution was concentrated in an ultrafiltration apparatus (MILLIPORE, USA) using a membrane of 10,000 Da. The concentrated sample was applied into a cationic ion exchange DEAE-Sepharose column (2.0 × 24 cm) equilibrated with 50 mM Tris-HCl, pH 7.2 and 7 M Urea. After the appearance of the first peak, the buffer was changed to 50 mM Tris-HCl, pH 7.2, containing 0.4 M NaCl. Thus, two fractions were collected, crotapotin and PLA_2-CB. After dialysis, crotapotin and PLA_2-CB were lyophilized (Hendon and Fraenkel-Conrat, 1971).

The fraction PLA_2-IC was resuspended in 50 mM ammonium formate buffer, pH 3.5 and applied to a Sephadex G-75 column pre-equilibrated with 50 mM ammonium formate buffer, pH 3.5. Finally, the PLA_2-IC was resuspended in 100 mM ammonium formate buffer, pH 3.5 and applied into a CM cellulose column (2.5 × 10.0 cm), previously equilibrated with the same buffer. In this last step of purification, elution was carried out using the same buffer at a flow rate of 20 mL/h and collected fractions of 5.0 mL/tube. The ammonium formate gradient was increased gradually and discontinuously (100 mM–2 M). The proof of the purity of the isolated PLA_2 was obtained by RP-HPLC using a reverse phase C18 column (4.6 × 100 mm) (Shimadzu, Japan), equilibrated with solvent A (5% acetonitrile,

0.1% trifluoroacetic acid) and eluted using a gradient concentration (0–100%) of solvent B (60% acetonitrile, 0.1% trifluoroacetic acid) at a flow rate of 1.0 mL/min, during 110 min. All purification steps were performed at room temperature and monitored by spectrophotometer at 280 nm (Vieira, 2009).

The protein BthTX-I was purified from *B. jararacussu* according to the method described by Homsi-Brandeburgo et al. (1988) and Cintra et al. (1993).

2.4. Determination of purity

The purity of crotamin, crotoxin, crotapotin, PLA_2-IC and PLA_2-CB was analyzed by SDS-PAGE using a 12.5% polyacrylaminde gel. Molecular weight standards were: phosphorylase B (97.000), albumin (66.000), ovalbumin (45.000), carbonic anhydrase (30.000), soybean trypsin inhibitor (20.000) and lactoalbumin (14.400). Gel was stained with Coomassie brilliant blue R-250 (Sigma, Germany).

2.5. Enzymatic activity

2.5.1. Phospholipasic activity

The phospholipasic activity of all compounds was performed following the modified method described by Gutiérrez et al. (1988). A translucent halo formation around the hole where the compounds were added in the gel is indicative of activity.

2.5.2. Myotoxic activity

Crotamin and BthTX-I have showed to present myotoxic activity; therefore, we determined this activity by the method previously described by Gutiérrez et al. (1988). Briefly, 50 μg of the toxins, suspended in PBS (50 μL), were injected into the right gastrocnemius muscle of Swiss male mice (18–22 g). Groups of six Swiss mice were used in this experiment. Control mice group received only PBS. Three hours after injection, the mice blood was collected in heparinized capillaries and immediately centrifuged at $3000 \times g$ for 10 min to obtain the plasma. The activity of the enzyme creatine kinase (CK) was determined in 4 μL of plasma using the kinetic reactive Kit_UV K010 (Bioclin, USA) following protocol recommended by the manufacturer.

2.6. Cytotoxicity of the compounds

The cytotoxicity of the compounds was evaluated in VERO E6 cells. Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich, Germany) method. Confluent VERO E6 cells monolayers contained in 96-well plates were exposed to different concentrations of crude venom and isolated toxins for seven days at 37 °C. Then, 50 μL of L-15 containing MTT (final concentration 1 mg/mL) was added to each well. After 4 h of incubation at 37 °C, the supernatant was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. After shaking, absorbance was measured at 540 nm. The concentration of each compound necessary to reduce cell viability in 50% (CC_{50}) was calculated by comparison with the untreated cells.

2.7. Determination of antiviral activity

The antiviral activity of the venoms and their isolated toxins was evaluated using several methodological strategies as follows.

2.7.1. Treatment of cells with crude venom and isolated toxins before viral infection (pre-treatment)

This assay was carried out to determine whether any crude venom and/or isolated toxin could confer protection against viral infection. VERO E6 cells (2×10^5 cells/mL/well) were seeded in a 24-wells plate and incubated at 37 °C for 24 h. The medium was removed and the cells were incubated with serial two-fold dilutions (from 100 to 0.01 ng/μL) of crude venom or isolated toxin from *C. d. terrificus*. After 3 h of incubation, the supernatant was removed and cells were infected with 25–100 PFU of virus contained in 400 μL of L-15 for 1 h. The supernatant was removed and the cells monolayers were overlaid with 1 mL of L-15 2× supplemented with 2% FBS and 1.8% carboxymethylcellulose. After 7 days at 37 °C, the semisolid overlay medium was removed and the cells were fixed and stained with naphthol blue-black in 5% acetic acid for plaque counting. Each experiment was repeated three times and the concentration that inhibited 50% of plaque formation (Effective Concentration 50%, EC_{50}) was calculated by comparison with the number of plaque observed in the untreated cells. The values of CC_{50} and EC_{50} were used to calculate the selectivity index ($SI = CC_{50}/EC_{50}$), which suggests the potential antiviral activity of the compounds. SI with value of four or higher suggests that a compound have a promising antiviral activity that merit further studies.

2.7.2. Treatment of cells with crude venom and isolated toxins after viral infection (post-treatment)

This assay was performed to determine whether the tested samples inhibited viral replication after cells infection. VERO E6 cells (2×10^5 cells/mL/well) were growth in a 24-well tissue culture plates for 24 h at 37 °C. The cells were infected with 25–100 PFU contained in 400 μL of L-15 and incubated during 1 h at 37 °C. The supernatant was then removed and the cells were covered with 1 mL of medium overlay containing serial two-fold dilutions of crude venom or isolated toxin from *C. d. terrificus*. The plates were incubated at 37 °C for 7 days; then the overlay medium was removed and the plaques were stained and counted. EC_{50} and SI were calculated as described above.

2.7.3. Virucidal activity

The virucidal test evaluates the ability of the crude venom or isolated toxins to act directly on the viral particle. Serial two-fold dilutions of crude venom or isolated toxin from *C. d. terrificus* were incubated with 25–100 PFU of each virus for 1 h at 37 °C. This mixture was added to a monolayer of VERO E6 cells to calculate EC_{50} and SI as mentioned above.

2.7.4. Evaluation of viral adsorption inhibition

To evaluate the capacity of the compounds to inhibit the virus adsorption, the viruses were incubated with the cells

at 4 °C. At this temperature the virus adsorbed on the cells surface but does not internalize. VERO E6 cells (2×10^5 cells/mL/well) were grown in 24-well tissue culture plates to reach confluence (24 h). Then, the plate was incubated at 4 °C during 30 min, the medium was removed and then the cell monolayers were infected with cool viral suspension (25–100 PFU/well) and at the same time, each crude venom or isolated toxins were added at different concentrations. After 2 h of incubation at 4 °C, the supernatant was removed and the cells were washed with PBS. The cells were covered with the overlay medium and incubated at 37 °C for for 7 days. EC_{50} and SI were calculated as described above.

2.7.5. Evaluation of viral internalization inhibition

This assay was performed to evaluate the capacity of the compounds to inhibit the viral internalization. Confluence VERO E6 cells (2×10^5 cells/mL/well) were incubated at 4 °C during 30 min and then the medium was removed and cells were infected with cool viral suspension (25–100 PFU/well). The infected cells were further incubated at 4 °C for 2 h and then washed 3 times with PBS. The cells were incubated at 37 °C for 5 min and immediately treated with crude venom or isolated toxins at different concentrations and incubated at 37 °C during 1 h. After this period, the supernatant was removed to wash the cells with citrate buffer (8.4 g of citric acid, 0.75 g of KCl and 8 g of NaCl in 1 L – pH = 3,0) and then with PBS. The procedure for plaques counting and EC_{50} and SI calculation was carried out as described above.

3. Results

3.1. Purification of toxins from C. d. terrificus and B. jararacussu

The venom of *C. d. terrificus* was subjected to size exclusion chromatography on Sephadex G-75 to obtain the fractions containing convulxin (I), giroxin (II), crotoxin (III), PLA_2-IC (IV), crotamin (V) and other peptides (VI) (Fig. 1A). After the first chromatographic step, crotoxin fraction was subjected to a cationic exchange chromatography to obtain crotapotin (CA) and PLA_2-CB (CB) (Fig. 1B). The purity of the purified proteins was estimated by SDS-PAGE (Fig. 1C).

In Fig. 1C, crotamim, crotoxin and PLA_2-IC ("inter-cro") showed only one electrophoretic band indicating the purity of these proteins. The PLA_2-CB toxin (basic chain of crotoxin) can be viewed with its CB1 and CB2 isoforms. The protein BthTX-I was purified from *B. jararacussu* according

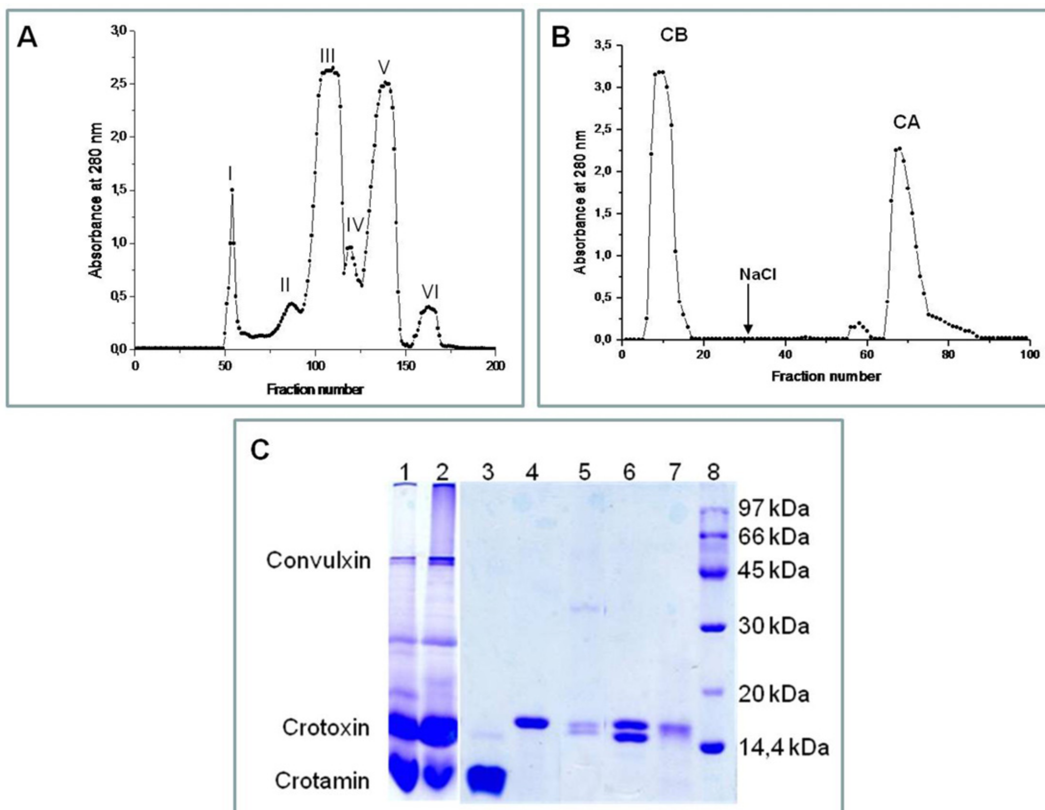

Fig. 1. Isolation of toxins from *Crotalus durissus terrificus* snake venom. Sequential purification steps: (A) Size exclusion chromatography of crude snake venom (500 mg) on Sephadex G-75, in 50 mM ammonium formate, flow rate 30 mL/h, 5 mL/tube; (B) re-chromatography of the active fraction (crotoxin) on cationic exchange in DEAE-sepharose column to purify PLA_2-CB (CB) and crotapotin (CA). (C) electrophoretic analysis in SDS-PAGE: (1) *C. d. terrificus* (yellow venom); (2) *C. d. terrificus* (white venom); (3) crotamin; (4) crotoxin; (5) convulxin; (6) PLA_2-CB; (7) PLA_2–IC; (8) Molecular weight markers.

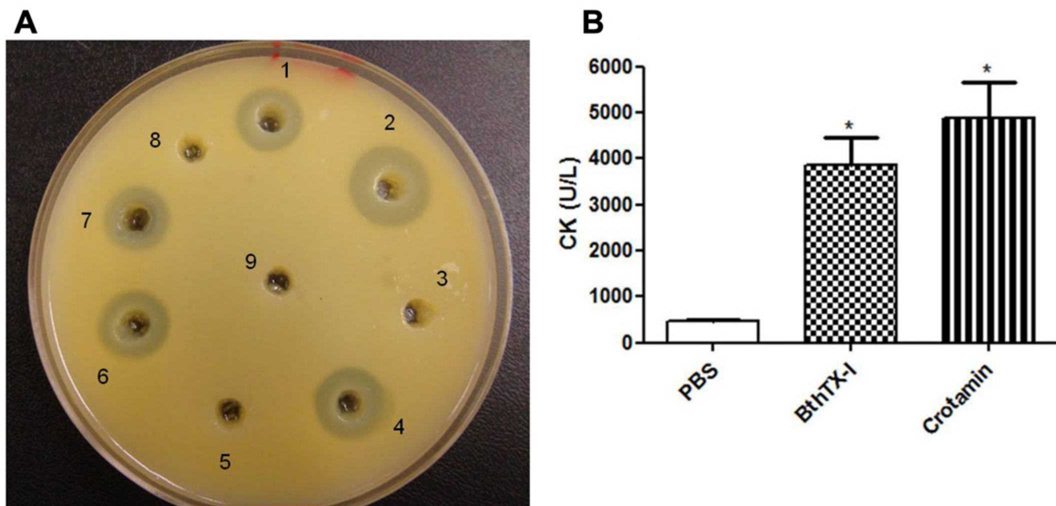

Fig. 2. Evaluation of enzymatic activity. (A) Phospholipasic activity of the *C. d. terrificus* venom and fractions. 1. Crude venom (yellow); 2. Crude venom (white); 3. Convulxin; 4 Crotoxin; 5. Crotapotin; 6. PLA_2-CB; 7. PLA_2–IC; 8. Crotamin; 9 BthTX-I. (B) Myotoxic activity of Crotamin and BthTX-I in mice: the increased release of creatine kinase (CK) in plasma was measured after intramuscular injection of crotamin or BthTX-I (25 μg in 50 μL of PBS) or PBS alone as negative control (50 μL). The results are expressed as mean ± SD ($n = 6$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

to the method described by Homsi-Brandeburgo et al. (1988) and Cintra et al. (1993).

3.2. Enzymatic activity of the isolated toxins

To show that the different compounds were enzymatically active, we have evaluated the phospholipasic and myotoxic activities of them.

The crude (yellow and white) venom of *C. d. terrificus*, crotoxin, PLA_2-CB and PLA_2-IC showed phospholipasic activity (Fig. 2A). Crotamin, crotapotin, convulxin and BthTX-I did not show any phospholipasic activity.

The myotoxic activity of crotamin and BthTX-I was evaluated by measuring the levels of creatine kinase in mice plasma (Fig. 2B). Both compounds showed a significant myotoxic activity in mice.

3.3. Cytotoxicity of toxins

The crude venom and isolated toxins of *C. d. terrificus* did not show cytotoxicity to 50% (CC_{50}) to VERO E6 cell monolayers up to a concentration of 500 ng/μL. Based on the amount of compounds available for the experiments, 100 ng/μL of each compounds were used as maximum concentration in all the antiviral experiments. BthTX-I isolated from *B. jararacussu* venom showed cytotoxicity to VERO E6 cells with a CC_{50} of 108.6 ± 8 ng/μL.

3.4. Antiviral activity of crude venoms and toxins of *C. d. terrificus*

3.4.1. Pre-treatment

To evaluate the ability of crude venoms and toxins of *C. d. terrificus* to confer resistance to VERO E6 cells against DENV and YFV infection, the cells were treated with serial two-fold dilutions (from 100 to 0.01 ng/μL) of the compounds, 3 h before viral infection. Considering that the compounds did not show cytotoxicity for VERO E6 in the maximum concentration tested for cytotoxicity (500 ng/μL), this concentration was considered as the CC_{50} to calculate the SI (SI = CC_{50}/CE_{50}) (Table 1). Treatment of the cells with yellow and white crude venoms, crotoxin, PLA_2-CB and PLA_2-IC conferred resistance to the infection against DENV-2 and YFV. Greater inhibition of viral infection was

Table 1
Antiviral activity of crude venoms and purified toxins from *C. d. terrificus* against YFV and DENV-2 in the pre-treatment assay.

Compounds	YFV			DENV-2		
	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})
Cdt yellow	100	0.1 ± 0.09	5000	100	0.054 ± 0.004	9259
Cdt white	96	0.19 ± 0.09	2631.6	100	0.067 ± 0.003	7462
Crotoxin	93	0.04 ± 0.04	12500	100	0.05 ± 0.002	10000
Crotapotin	43	WA	WA	68	39 ± 2.5	12.8
Crotamin	20	WA	WA	11	WA	WA
Convulxin	22	WA	WA	5	WA	WA
PLA_2-CB	100	0.26 ± 0.1	1923	100	0.06 ± 0.002	8333
PLA_2 – IC	100	1.30 ± 0.6	385	100	0.775 ± 0.09	645

WA: without activity; SD= Standard deviation.

Table 2
Antiviral activity of crude venoms and purified toxins from *C. d. terrificus* against YFV and DENV-2 in the pos-treatment assay.

Compounds	YFV		DENV-2	
	Inhibition at 100 ng/μL (%)	$EC_{50} \pm SD$ (ng/μL)	Inhibition at 100 ng/μL (%)	$EC_{50} \pm SD$ (ng/μL)
Cdt yellow	9.5	WA	47	WA
Cdt white	25	WA	33	WA
Crotoxin	−34	WA	44	WA
Crotapotin	−38	WA	−30	WA
Crotamin	9	WA	40	WA
Convulxin	−19	WA	−36	WA
PLA_2-CB	−23	WA	47	WA
PLA_2-IC	−16	WA	15	WA

WA: without activity; SD= Standard deviation.

observed when cells were treated with crotoxin, which showed an SI of 12500 and 10000, against YFV and DENV-2, respectively. The cells treated with crotapotin showed less than 50% inhibition of YFV infection, but an inhibition of 68% for DENV-2 leading to an SI of 12.8.

3.4.2. Post-treatment

The crude venoms and isolated toxins were evaluated for their ability to inhibit the replication of YFV and DENV-2 after the entry of viruses into cells (Table 2). Therefore, the cells were infected with YFV or DENV-2 and after 1 h the cells were treated with serial two-fold dilutions (from 100 to 12.5 ng/μL) of the compounds. None of the tested material was able to inhibit at least 50% of viral replication. Interestedly, crotoxin, crotapotin, convulxin, PLA_2-CB, PLA_2-IC showed an increase of YFV replication and crotapotin and convulxin of DENV-2.

3.4.3. Virucidal

To verify the capacity of the compounds to inactivate DENV-2 and YFV by direct effect on the viruses, these were incubated at room temperature with serial two-fold dilutions (from 100 to 3×10^{-6} ng/μL) of the compounds for 1 h. The mixture was used to infect confluent monolayers of VERO E6 cells (Table 3). Crude venoms (yellow and white), crotoxin, PLA_2-CB and PLA_2-IC showed extremely high inhibition effects against YFV and DENV-2. These results are similar to those observed in the pre-treatment assay, suggesting that these compounds inhibit the initial steps of the viral replication. Therefore, we evaluated the effect of compounds in the adsorption and internalization of YFV and DENV-2 in VERO E6 cells.

3.4.4. Adsorption

VERO E6 cells were incubated at 4 °C for 30 min and then treated with serial two-fold dilutions (from 100 to 0.006 ng/μL) of the compounds and infected with YFV or DENV-2 at the same time. The EC_{50} and SI were calculated for each compound (Table 4). The crude venoms (yellow and white) of *C. d. terrificus* and the isolated toxins crotoxin, PLA_2-CB and PLA_2-IC showed high inhibition of both YFV and DENV-2 infection. However, the isolated toxins crotamin and convulxin, did not inhibit the replication of DENV-2, but inhibited the YFV replication, showing SI between 8.4 and 38.

3.4.5. Internalization

To evaluate the effect of crude venom and isolated toxins from *C. d. terrificus* in the viral internalization, VERO E6 were incubated at 4 °C and subsequently infected with YFV or DENV-2. After 2 h at 4 °C, the supernatant was removed and the cells were treated with serial two-fold dilutions (from 100 to 0.01 ng/μL) of the compounds. The antiviral activity is shown in Table 5. The crude venoms (yellow and white), PLA_2-CB and crotoxin showed inhibition of the internalization of YFV and DENV-2; however, with SI lower than that observed in the inhibition of viral adsorption. Interestedly, convulxin stimulated the infection of YFV in 70%.

3.5. Evaluation of the phospholipasic enzymatic activity

The results obtained in most of the methodological strategies showed that PLA_2-CB, PLA_2-IC, and crotoxin were the isolated toxins with the highest antiviral activity. Crotoxin is constituted by crotapotin and PLA_2-CB; thus the responsible compounds for the antiviral activity of the *C. d. terrificus* venom were the phospholipases. Thus, aiming to evaluate whether the antiviral action of the phospholipases was related to the catalytic activity, we analyzed the antiviral activity of BthTX-I, a phospholipase without catalytic activity, however with high myotoxic activity isolated from the venom of *B. jararacussu*. Serial two-fold dilutions (from 100 to 1.5 ng/μL) of BthTX-I (CC_{50} = 108.6 ng/μL) were used in all methodological strategies. BthTx-I showed antiviral activity in the virucidal, adsorption and internalization assays (Table 6); however, with SI extremely lower than that observed for crotoxin, PLA_2-CB and PLA_2-IC (Table 1, 3 and 4).

Table 3
Antiviral activity of crude venoms and purified toxins from *C. d. terrificus* against YFV and DENV-2 in the virucidal assay.

Compounds	YFV			DENV-2		
	Inhibition at 100 ng/μL (%)	$EC_{50} \pm SD$ (ng/μL)	SI (CC_{50}/CE_{50})	Inhibition at 100 ng/μL (%)	$EC_{50} \pm SD$ (ng/μL)	SI (CC_{50}/CE_{50})
Cdt yellow	100	0.006 ± 0.0008	83333	100	0.005 ± 0.0006	100000
Cdt white	100	0.0045 ± 0.0007	111111	100	0.004 ± 0.0002	125000
Crotoxin	100	0.00045 ± 0.00007	1111111	100	0.001 ± 0.0003	500000
Crotapotin	100	66 ± 29	7.57	100	0.82 ± 0.08	610
Crotamin	14.34	WA	WA	33	WA	WA
Convulxin	25.5	WA	WA	32	WA	WA
PLA_2-CB	100	0.0037 ± 0.002	135135	100	0.00003 ± 0.000003	16666666
PLA_2-IC	100	0.0054 ± 0.0017	92592	100	0.0137 ± 0.005	36496

WA: without activity; SD= Standard deviation.

Table 4
Antiviral activity of crude venoms and purified toxins from *C. d. terrificus* against YFV and DENV-2 in the adsorption assay.

Compounds	YFV			DENV-2		
	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})
Cdt yellow	100	0.037 ± 0.005	13513.5	100	0.07262 ± 0.005	6885
Cdt white	100	0.03608 ± 0.004	13858	100	0.0174 ± 0.003	28735
Crotoxin	100	0.0365 ± 0.004	13698	100	0.018 ± 0.002	27777
Crotapotin	90	13.1 ± 0.1	38	65	53 ± 17	9.4
Crotamin	66	33.7 ± 4.2	14.8	8	WA	WA
Convulxin	66	59.3 ± 4.3	8.4	12.6	WA	WA
PLA_2-CB	100	0.01647 ± 0.004	30358	100	0.044 ± 0.007	11363
PLA_2-IC	100	0.268 ± 0.04	1865	100	0.133 ± 0.03	3759

WA: without activity; SD= Standard deviation.

4. Discussion

Several studies describe the use of natural or synthetic substances as potential antidengue or antiyellow fever agents (Benarroch et al., 2004; Poh et al., 2009; Talarico et al., 2005; Wang et al., 2009; Zhang et al., 2009). However, currently there is no drug available for treatment of these infections.

Snake venoms have shown to present antibacterial (Samy et al., 2010; Wang et al., 2009), antiparasite (Deolindo et al., 2010), antifungal (Magaldi et al., 2002), and antiviral activities (Fenand et al., 1999; Petricevich and Mendonça, 2003); thus, representing a promising source of antidengue and antiyellow fever compounds.

This study describes the potential antiviral activity against YFV and DENV of toxins isolated from *C. d. terrificus* venom. Crude venoms (yellow and white), crotoxin, PLA_2-CB and PLA_2-IC showed a potent inhibition of YFV and DENV replication in VERO E6 cells and presented high selectivity indexes. The highest inhibitions of viral replication were observed in the virucidal, pre-treatment and adsorption assays indicating that the inhibition occurs at the initial steps of the replication cycle. Our results suggest that the antiviral effect showed by *C. d. terrificus* venom was due, mainly, to the phospholipases A_2, since PLA_2-CB, PLA_2-IC and crotoxin showed a potent inhibitory activity against YFV and DENV.

Based on their function, localization, regulation, catalytic mechanism and structure, the PLA_2 can be divided into five groups: secreted PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$), PAF acetylhydrolases (PAF-AH) and lysosomal PLA_2 ($LPLA_2$s). The phospholipases of snake venoms belong to the secreted PLA_2 (Dennis, 1997; Schaloske and Dennis, 2006). They represent a versatile class of enzymes that occur ubiquitously in nature catalyzing the hydrolysis of the glycerophospholipids at the *sn*-2 position releasing free fatty acids and lysophospholipids, playing a key role in various biological activities, such as lipid digestion, host defense and homeostasis of cellular membranes (Kini, 2003; Schaloske and Dennis, 2006). We have found that phospholipases showed the highest antiviral activity in the virucidal assay, i.e., act directly on the virus particle. This result suggests that the enzymatic activity of the phospholipases is important for the antiviral effect. To test this hypothesis, we have analyzed the effect of a phopholipase A_2 without catalytic activity (BthTX-I) on the replication of YFV and DENV. BthTX-I has a substitution of the aspartate residue at position 49 (Asp49) by a lysine residue (Lys49), turning this protein with a very low or no catalytic activity (Cintra et al., 1993). Although BthTX-I showed antiviral activity in the virucidal, adsorption and internalization strategies, the SI obtained for this toxin was extremely lower when compared to the obtained for crotoxin, PLA_2-CB and PLA_2-IC. Therefore, these results suggest that the enzymatic activity is an important factor for the antiviral activity. These results are in agreement with a previous study, which showed that the catalytic function of a human $sPLA_2$-X was required for antiviral activity against human immune deficiency virus 1 (HIV-1) (Kim et al., 2007). The importance of $sPLA_2$ catalytic activity in the antiviral effect was also observed against adenovirus infection; however, in this case the action was against host cell membrane (Mitsuishi et al., 2006).

In addition to the catalytic activity, the physiological function of $sPLA_2$ can be mediated by ligation to specific receptors on the cell membrane (Lambeau and Lazdunski, 1999). Thus, this mechanism might also be related to the antiviral effect showed by $sPLA_2$. In that sense, previous

Table 5
Antiviral activity of crude venoms and purified toxins from *C. d. terrificus* against YFV and DENV-2 in the internalization assay.

Compounds	YFV			DENV-2		
	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})
Cdt yellow	76	28.7 ± 3.3	17	85	27.7 ± 3.8	18
Cdt white	67	40.6 ± 0.6	12	73	53.9 ± 1.4	9.3
Crotoxin	83	13.7 ± 1.0	36	77	34.4 ± 2.7	14.5
Crotapotin	72	69.9 ± 5.5	7.2	41	WA	WA
Crotamin	32	WA	WA	46	WA	WA
Convulxin	−70	WA	WA	2.3	WA	WA
PLA_2-CB	95	3.3 ± 1.0	151	89	17.2 ± 3.3	29
PLA_2-IC	46	WA	WA	76	21.6 ± 3.4	23

WA: without activity; SD= Standard deviation.

Table 6
Antiviral activity of BthTX-I in the pre-treatment, post-treatment, virucidal, adsorption and internalization assays against YFV and DENV-2.

Strategy	YFV			DENV-2		
	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})	Inhibition at 100 ng/μL (%)	EC_{50} ± SD (ng/μL)	SI (CC_{50}/CE_{50})
Pre-treatment	22	WA	WA	9.2	WA	WA
Post-Treatment	7.8	WA	WA	−11	WA	WA
Virucidal	100	7.063 ± 0.25	15.4	96	4.8 ± 0.36	22.6
Adsorption	77	25 ± 3.6	4.3	79	57.3 ± 0.8	1.89
Internalization	78	23.4 ± 1.8	4.6	64	69.0 ± 5.3	1.57

WA: without activity; SD= Standard deviation.

studies have shown that $sPLA_2$ isolated from bee and snake venom inhibits the entry of HIV-1 in human primary blood leukocytes, and therefore the replication, through a mechanism linked to the binding of $sPLA_2$ to cells (Fenand et al., 1999, 2001). In addition, Petricevich and Mendonça (2003), showed that crude venom of *C. d. terrificus* inhibit the replication of measles virus on VERO cells, and that this inhibition occurred at the initial steps of the replication cycle, independently of the virucidal effect. Although the higher antiviral activity observed in this study was in the virucidal assay, a high antiviral effect in the pre-treatment assay was also observed, suggesting that $sPLA_2$ might confer resistance to VERO E6 cells against infection to DENV-2 and YFV through of a specific interaction of $sPLA_2$ to host cells.

In contrast to the inhibitory effect of *C. d. terrificus* venom observed in this study, we have also observed that crotoxin, crotapotin, convulxin, PLA_2-CB and PLA_2-IC showed an enhancement of viral infection in the post-treatment assay, and convulxin in the internalization assay. More studies are needed to understand the mechanism leading to the enhancement of viral infection.

In summary, we have described the antiviral activity of $sPLA_2$s of *C. d. terrificus* venom, which inhibit the infection of VERO E6 cells by YFV and DENV-2. The inhibition of viral replication, which might depends on the catalytic action of the $sPLA_2$s, occurred at the first steps of the virus replication cycle. The results suggest that the PLA_2s have an action on the cells and on the virus particle, which lead the high antiviral activity seen in this study. Interestedly, most of the isolated toxin induced an enhancement of infection when they were added after the virus internalization. These toxins, specially the $sPLA_2$s, could be new targets to be used as a model for the developing drugs against DENV and YFV, or for the study of the mechanism of viral replication.

Conflict of interest

The authors declare that there are no conflicts of interest.

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