Antimicrobial activity of the toxin VdTX-I from the spider *Vitalius dubius* (Araneae, Theraphosidae)

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ABSTRACT

Background: Currently there is an urgent need to develop new classes of antimicrobial agents with different mechanisms of action from conventionally antibiotics used for the control of pathogenic microorganisms. The acylpolyamine called VdTX-I was isolated from the venom of the tarantula *Vitalius dubius*, and first described with activity as an antagonist of nicotinic cholinergic receptors. The main objective of this study was to investigate the antimicrobial activity found in the venom of the spider, with emphasis on the toxin VdTX-I.
Methods: Antimicrobial assays were performed in 96 well plates culture against 14 micro-organisms (fungi, yeasts and bacteria), which were tested concentrations from 0.19 to 100 μM of VdTX-I. After qualitative analysis, dose-response curve assays were performed in bacterial kill curve using MTT reagent and hemolytic assay.
Results: The antimicrobial activity of the VdTX-I toxin was observed in 12 tested species of *Candida*, *Trichosporiun*, *Staphylococcus* and *Micrococcus*. The toxicity had a dose-response at 3.12 μM – 100 μM in *Candida albicans*, *Candida guillermondii*, *Micrococcus luteus* and *Escherichia coli*. VdTX-I took about 5 min to inhibit bacterial growth, which was faster than streptomycin. The toxin showed no hemolytic activity between 0.19 and 100 μM. At 2.5 μg/mL of toxin it was observed no growth inhibition against a mammalian cell lineage.
Conclusions: The VdTX-I toxin has a significant antimicrobial activity, with broad spectrum, and is experimentally inert to mammalian blood cells.
General Significance: This paper explores the antimicrobial potential of the spider toxin VdTX-I, which can provide a new model to design new antimicrobial drugs.

1. Introduction

The introduction of antibiotics was largely responsible for the dramatic decline in the mortality rate of diseases reported in developed countries. However bacteria are remarkably adaptable to environmental stresses and have evolved at least one mechanism of resistance for all 17 classes of antibiotics developed to date [1].

Many in use active molecules were obtained from organisms. One of the findings are the biogenic amines (polyamines, acylpolyamines), which are particularly powerful and have a wide spectrum of action. Typically its activity is not restricted to a single type of pathogen as Gram-positive and Gram-negative bacteria, fungi, protozoa [2].

Polyamines are found in nearly all living organisms and cells-and can be well studied as putrescine, spermidine and spermine. It contains polycations that can interact with polyanionic macromolecules such as DNA, RNA, ion channels and protein kinases. This interaction promotes the regulation of gene expression, post-translational modifications, and alterations in the cell cycle, cell membrane structure and function. Thus, the poliamines also play an important role in the regulation of growth, development and function of animals, plants and microorganisms. In addition, the concentration and composition may present a great variation according to the cell type and the physiological conditions of the organism [3].

Further present in many organisms, the polyamines can have several applications such neuroprotective effect in animal models of multiple sclerosis [4], antimalarial and antitripanossomal activity [5,6] and have high efficiency and low toxicity against Chagas disease [7]. Synthetic polyamines can act as antimicrobial agents that

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may interact with the membrane of microorganisms, resulting in their breakage. It can also be covalently attached to the surface of medical implants, surfaces of medical materials, in order to prevent adhesion or growth of microorganisms [8].

In the venom of spiders, identification of antimicrobial compounds is constant, as in the case of *Cupiennius salei* [9,10], and *Ornithoctonus hainana* [11]. The antibiotic property is presumed to avoid infection by bacteria, fungi and yeasts on feeding, since these animals carry out extracorporeal digestion [11]. Nevertheless, all cited molecules are peptides.

Spider venoms are known as sources of many natural products, particularly acylpolyamines. Have been characterized in the venom of the spider *Agelenopsis aperta*, *Hololena curta* and *Paracoelotes birulai* [12–14]. Biogenic amines of low molecular weight such as putrescine, cadaverine, spermidine and spermine were characterized in the venom of spiders mygalomorph *Scorda griseipes*, *Atrax robustus*, and species of tarantulas spiders *Dugesiella hentzi*, *Aphonopelma emilia* and two other species *Aphonopelma* [15,16].

Among species of São Paulo state, the venom of tarantula *Vitalius dubius*, a variety of peaks seen in reversed phase chromatography and the range of molecular masses observed after SDS-PAGE and immunoblotting indicated that the venom of this spider contained several components, especially low molecular weight indicating a large amount of peptides. [17]

Rocha-e-Silva et al. purified the polyamine VdTX-I, arising from tarantula spider *V. dubius*, and showed that this toxin was capable of producing reversible blockade in biventer cervices neuromuscular preparations. [18]

In this context, this paper proposes to evaluate the antimicrobial activity of the toxin VdTX-I.

2. Materials and methods

2.1. Spiders

The spiders were obtained at Centro de Animais Peçinhentos René D´ávila, Itu, Brasil.. After identification, the animals were brought to the Butantan Institute, where they were kept in a vivarium of Silva Jr´s Laboratory. The venoms of spiders are extracted by electro stimulation, as described by Rocha-e-Silva [17].

2.2. Fractionation and purification

The venom of *V. dubius* and VdTX-I were obtained following the method proposed by Rocha-e-Silva [18] protocol.

2.3. Antimicrobial activity

For antimicrobial activity test the bacterial growth in liquid medium assay was used [19]. In a 96-well micro plate,10 µL of the toxin or water (control) were applied to wells of 90 µL of the culture medium PB (Poor Broth) used for bacteria and PDB (Potato Dextrose Broth) used for fungi and yeasts in the logarithmic phase of growth. The absorbance of the cultures was measured after 18 h incubation at 30 °C on a micro plate reader IEMS (LABSYSTEM™) and Victor^3 (1420 Multilabel Counter/Victor^3 – Perkin Elmer) at 595 nm.

The VdTX-I toxin was tested against 15 microorganisms at concentrations from 0.19 to 100 µM (*Aspergilus niger*, *Cladosporium sp.*, *Trichosporium sp.* IOC4569, *Micrococus luteus* A270, *Staphilococus aureus* ATCC29213, *Staphilococus epidermides* ATCC12223, *Enterobacter cloacae* β12, *Escherichia coli* ATCC 25922, *E. coli* D31, *Candida albicans* MDM8, *Candida parapsiliosis* IOC4564, *Candida guillermondii* IOC4557, *Candida tropicalis* IOC4560, *Candida glabata*

Table 1
Minimum Inhibitory Concentration (MIC) of the toxin VdTX-I.

Microorganism	MIC
Aspergilus niger (isolated)	ND
Cladosporium sp. (isolated)	6.25–12.5 µM
Trichosporium sp. IOC4569	6.25–12.5 µM
Micrococus luteus A270	6.25–12.5 µM
Staphylococus aureus ATCC29213	6.25–12.5 µM
Staphylococus epidermides ATCC12223	6.25–12.5 µM
Enterobacter cloacae β12	50 µM
Escherichia coli ATCC 25922	6.25–12.5 µM
E. coli D31	6.25–12.5 µM
Candida albicans MDM8	6.25–12.5 µM
Candida parapsiliosis IOC4564	ND
Candida guillermondii IOC4557	6.25–12.5 µM
Candida tropicalis IOC4560	6.25–12.5 µM
Candida glabata IOC4565	6.25–12.5 µM
Candida krussei IOC4559	6.25–12.5 µM

ND=Not Detected in test concentration.

IOC4565 and *Candida krussei* IOC4559).Furthermore, quantitative analysis (dose-response) was performed and the dose-response curve was performed using 4 microorganism (*C. albicans* MDM8, *C. guillermondii* IOC4557, *M. luteus* A270 and *E. coli* ATCC 25922).

Since it is a qualitative test, the Minimum Inhibitory Concentration (MIC) is between the highest value without activity and the lowest active concentration, and thereby presented as a narrow range of concentrations.

2.4. Kill curve assay

To evaluate the minimum time that VdTX-I toxin acts on microorganisms, the kill curve assay was performed according to, the protocol described by Wang [20] with modifications.

For this assay, 50 µM toxin were added 200 µL of suspension of *E. coli* D31 and *M. luteus* A270 at a concentration of 10^6 CFU and were incubated at 37 °C at times 0, 5, 10, 15, 30, 60, 120, 180 and 240 min. After incubation were added 20 µL of a solution at 5 mg/mL of 3-(4,5-imethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated at 37° C for 20 min.

Then, the tubes were centrifuged at 10,000g for 30 s and the supernatant was removed leaving the formazan crystals at the bottom of the tubes. The crystals were dissolved in 1 mL of isopropanol and this volume was transferred to glass tubes, and further added 1.5 mL of isopropanol to obtain a final volume of 2.5 mL. Reading was performed in 96-well plates at 595 nm. As a positive control the same procedure was performed with 20 µL of antibiotic Streptomycin instead toxin, and as negative control 20 µL of ultra-pure H$_2$O was used.

2.5. Hemolytic activity

In this evaluation, human erythrocytes were collected in 0.15 M citrate buffer pH 7.4 and washed 3 times by centrifugation at 700g with PBS pH 7.4. After the last centrifugation the cells were resuspended in PBS pH 7.4. Aliquots at a concentration of 0.19 to 100 µM VdTX-I were added to the 96 well plate with bottom "U", where in each well containing 50 µL of a suspension of erythrocytes to 3%. After that, the samples were incubated at room temperature for 3 h. Hemolysis was determined by reading absorbance at 595 nm of each well in a bed of plates (Titertek Multiskan Elfa B, Finland). As a positive control was used Triton, and as negative control was used PBS only.
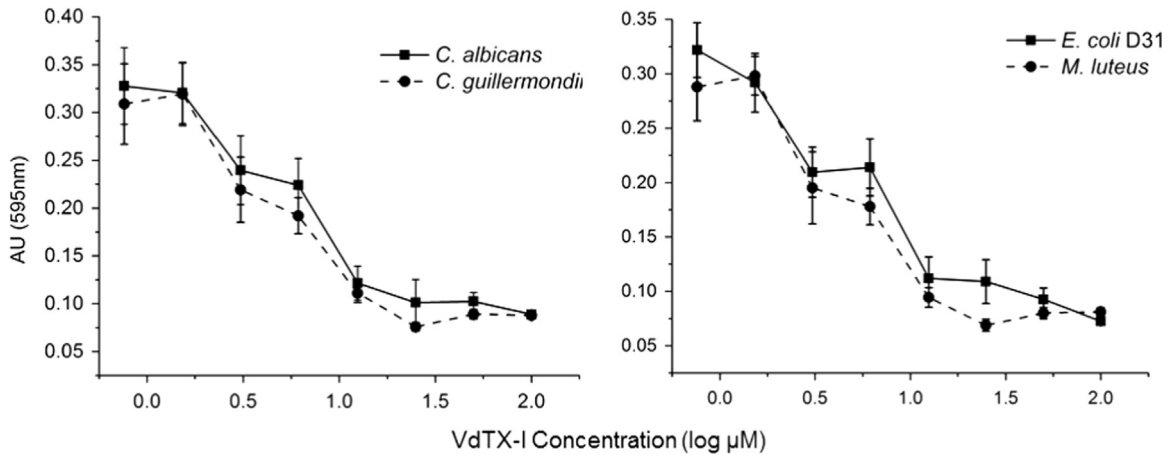
Fig. 1. Dose-response curve of the toxin VdTX-I against four species of microorganisms. (Left)*C. albicans* MDM8 and *C. guillermondii* IOC4557. (Right)*M. luteus* A270 and *E. coli* ATCC 25922. The points represent ± SEM, $n=3$.
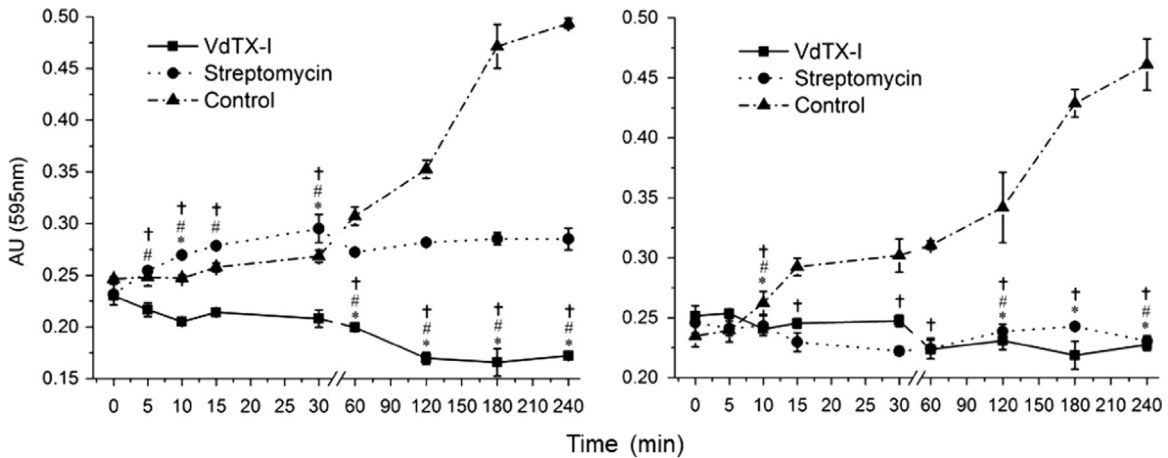

Fig. 2. Kill curve assay testing VdTX-I. (Left) Test of VdTX-I against *M. luteus* A270 (Right) Test of VdTX-I against *E. coli* ATCC 25922. The points represent ± SEM, $n=3$. * Significant difference from the starting point of the same group. ♯ Significant difference compared to streptomycin group at the same point. † Significant difference compared to the control group at the same point. $p < 0.05$.
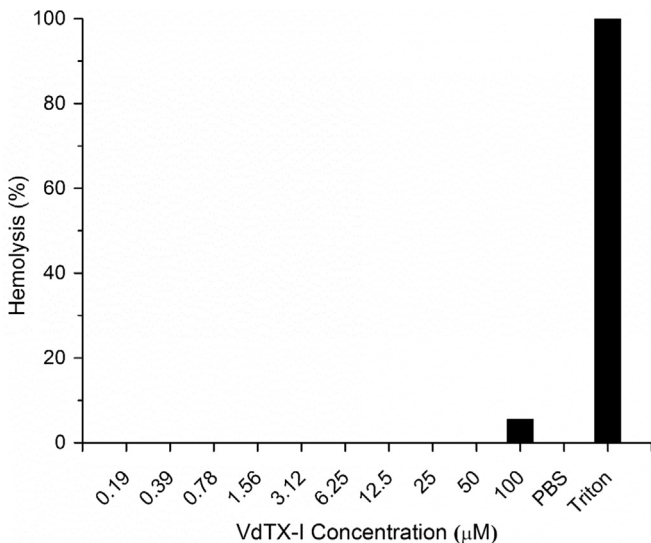

Fig. 3. Hemolytic activity of VdTX-I. It is not observed significant hemolysis at any concentration tested. The points represent ± SEM, $n=3$.

2.6. Cytotoxic activity

The cytotoxicity assay was performed in K562 cells (human erythroleukemia) grown in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, and 1% of solution antibiotic-antimicotic made with 10,000 U of penicillin, 10 mg de streptomycin e 25 μg amphotericin B, in an incubator at 37 °C with 5% CO_2. The number of K562 cells the culture was set to 2×10^6 viable with 96% in three bottles (12.5 cm^2). Two concentrations of VdTX-I (2.5 μg/ml and 20 μg/ml) and the controls were applied. The counting was performed by hemocytometer method by exclusion of trypan blue and the cytotoxic potential, defined based on the control curve at time 24 and 48 h [21].

2.7. Statistical analysis

The results were expressed as mean ± error of mean (SEM), and statistical comparisons were made using analysis of variance (ANOVA) by Tukey test or Student t test to compare means. A value of $p < 0.05$ indicated significance.
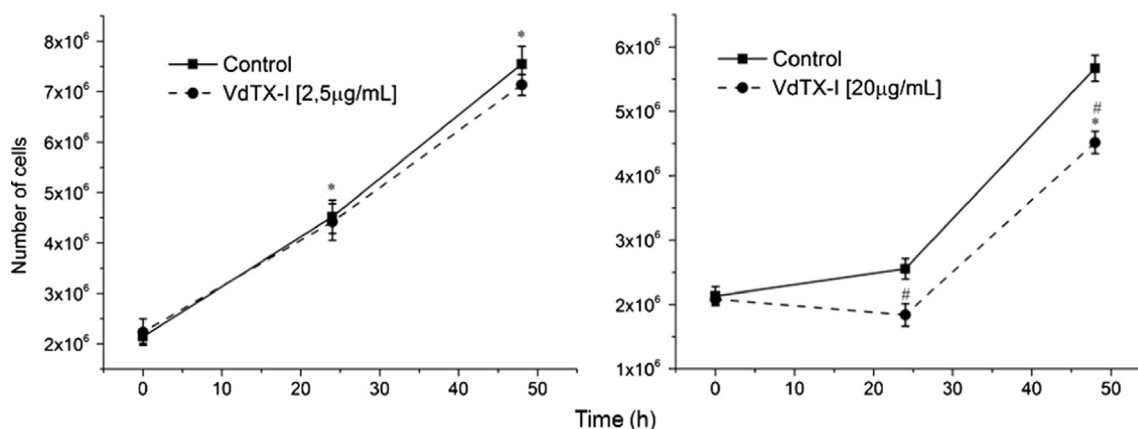
Fig. 4. Cytotoxic activity of the toxin VdTX-I against K562 cultured cells. (Left) Concentration of 2.5μg/mL VdTX-I. (Right) Concentration of 20 μg/mL VdTX-I. * Significant difference from the starting point of the same group. ♯ Significant difference compared to streptomycin group at the same point.

3. Results

The toxin VdTX-I showed activity against 13 microorganisms tested, mostly in the concentration of 12.5 to 100 μM (Table 1). Against the bacterium *E. clocae* β12 the toxin showed activity at 50 μM. VdTX-I was not active against two microorganisms tested, *Candida parapsiliosis* and *Aspergillus niger*.

A similar activity was observed when the toxin was tested in dose-response curve against *C. albicans* MDM8, *C. guillermondii* IOC4557, *M. luteus* A270 and *E. coli* ATCC 25922. It was observed an inhibition of the growth of these microorganisms at concentrations of 3.12 μM, reaching full growth inhibition at 100 μM of toxin. (Fig. 1)

The incubation time that VdTX-I needed to act on microorganisms was determined by killing curve protocol against *M. luteus* (gran positive) and *E. coli* D31 (gran negative). Our results have shown that the toxin inhibits the growth of the bacteria *M. luteus* after 5 min of exposure and *E. coli* D31 has its growth inhibited after 10 min exposure. For comparison, streptomycin takes 30 and 10 min against the same microorganisms, respectively (Fig. 2).

The hemolysis test showed that VdTX-I was not able to cause significant hemolysis at concentrations from 0.19 to 100 μM, with approximately 6% of hemolysis only at the highest concentration. (Fig. 3)

The cytotoxic activity of VdTX-I was tested using the concentrations of 2.5 and 20 μg/mL, and only in the concentration of 20 μg/mL a low inhibitory activity has been found in K562 eritroleukemia cells. (Fig. 4)

4. Discussion

Since antibiotics were introduced into clinical practice, bacterial pathogens have developed resistance that might reduce their effectiveness. Moreover, with opportunistic pathogens innate resistance to antibiotics has become particularly problems arising in hospital environments [22]. Thus, the research of new molecules may provide an alternative for this scenario.

In general polyamines such as poly (ethyleneimines) (PEIs) show strong antimicrobial activity against Gram-Positive bacteria, *Staphylococcus aureus*, and Gram-Negative Bacteria, *E. coli*. However higher concentrations were required to destroy *E. coli* compared to *S. aureus*[23].

This can be explained by differences in cell wall structure of Gram-positive and Gram-negative bacteria. Gram-Positive microorganisms such as *Staphylococcus aureus*, has a relatively thick wall, but porous, as Gram-Negative bacteria such as *E. coli*, have an outer membrane structure, forming a barrier to foreign molecules. [23]

The VdTX-I toxin has its antimicrobial activity at a relatively high MIC of 12.5 to 100 μM, but shows a wide-spectrum, being active against gran negative and positive bacteria, fungi and yeasts. Compared to antimicrobial peptides from other animal venoms, they used to present a low MIC but an activity against few microorganisms, such as the Cupienin 1 (purified venom *C. salei*) with MIC between 0.31 0.62 μM and active against four bacterial species [24]. Furthermore, the Latarcins (1, 2a, 3a, 3b, 4a and 5) purified from the venom of the spider *Lachesana tarabaevi* (Zodariidae) also have a MIC between 0.5 and 2.9 μM. In this same venom we can find two other Latarcins (6a and 7) having antimicrobial activity with MIC greater than 70 μM [25], showing a similar activity to VdTX-I. Mygalin, an acylpolyamine from the hemocytes of the spider *Acanthoscurria gomesiana*, was active only against *E. coli* (MIC 85 μM) [26].

Antimicrobial peptides can be found in other animal venoms, such as from Migainin-2 [24], derived from the venom of the skin of african frog *Xenopus laevis* having MIC larger than 60 μM. High activity antimicrobial peptides can also be found in the venoms of scorpions. For example, BmKb1 (*Mesobuthus martensii*) with MIC between 16 and 90 μM, and IsCT (*Opisthacanthus madascarensis*) with MIC between 0.7 and 150 μM. *Tityus serrulatus* peptide that may be greater than 150 μM, which have high MIC but a broad spectrum of activity as VdTX-I, revealing that its activity is relatively low [27].

The time that VdTX-I inhibits the growth of the bacteria *E. coli* and *M. luteus* was 5 and 10 min, respectively, which reflects an effect relatively slow when compared with La47, peptide isolated from the venom of spider *Lachesana sp*, which has its action after 2 min of incubation against *E. coli*. The purified peptide from the venom of the scorpion *Centruroides suffusus suffusus*, the Css54, has its activity soon after the start of incubation time [28].

The low hemolytic activity displayed by VdTX-I, with 6% of hemolysis with 100 μM toxin, is comparable to the one caused by the peptide O-defensin, with 7% hemolysis after incubation under 36 μM [11]. On the other hand, the toxin LyeTx-I (purified from the spider *Lycosa erythrognatha*) has a high hemolytic activity, with 40% hemolysis at 0.13 μM [29] and Css54 with approximately 65% at 20 μM [28].

We did not observe the cytotoxicity of VdTX-I against K562 cells at the tested concentrations of 2.5 μg/mL, but at the concentration of 20 μg/mL, were observed a small activity. This may be due to the fact that some polyamines such as polyhexamethylene biguanide (PHMB) did not affect eukaryotic cells.

Since these cells have neutral phospholipids in the composition of the membrane and polyamines have increased activity on phospholipids acids present in the membranes of microorganisms [23]. Just as PHMB, VdTX-I also has selective action, making their cytotoxicity and irritation potential smaller than other antiseptics [23]

5. Conclusions

We can conclude that VdTX-I toxin has a significant antimicrobial activity and broad spectrum. Also is a toxin that acts relatively fast against the tested bacteria. The VdTX-I toxin has low hemolytic activity but is not active against tumor cells of a particular lineage.

Conflicts of interest

The authors declare no conflicts of interest

Acknowledgment

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