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Structural characterization of a novel peptide with antimicrobial activity from the venom gland of the scorpion *Tityus stigmurus*: Stigmurin

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ABSTRACT

A new antimicrobial peptide, herein named Stigmurin, was selected based on a transcriptomic analysis of the Brazilian yellow scorpion *Tityus stigmurus* venom gland, an underexplored source for toxic peptides with possible biotechnological applications. Stigmurin was investigated *in silico*, by circular dichroism (CD) spectroscopy, and *in vitro*. The CD spectra suggested that this peptide interacts with membranes, changing its conformation in the presence of an amphipathic environment, with predominance of random coil and beta-sheet structures. Stigmurin exhibited antibacterial and antifungal activity, with minimal inhibitory concentrations ranging from 8.7 to 69.5 μM. It was also showed that Stigmurin is toxic against SiHa and Vero E6 cell lines. The results suggest that Stigmurin can be considered a potential anti-infective drug.

Introduction

Antimicrobial peptides (AMPs) are important components of the innate immune system, showing a broad spectrum of action against microbes [33,40]. It is believed that the mechanism of action of these peptides is not dependent on interaction with a specific receptor, making them interesting therapeutic alternatives when compared to available antibiotics; the latter act mainly by inhibiting biosynthetic pathways and are more prone to induce microbial resistance [32,54,85]. AMPs are distributed across a wide variety of species, including unicellular microorganisms, invertebrates and vertebrates, which strongly suggests the importance of these molecules in the process of survival [48,66].

The presence of AMPs in the venom of scorpions is frequently reported in the literature as an early evolved primitive component of the immune system [1,56]. Some examples of antimicrobial peptides found in scorpion venom include Hadrurin from *Hadrurus aztecus* [75], Opistoporins from *Opistophtalmus carinatus* [50], Parabutoporin from *Parabuthus schlechteri* [76], IsCTs from *Opisthacanthus madagascariensis* [18,19], Pandinins from *Pandinus imperator* [15], Mucroporin from *Lychas mucronatus* [17], Imcroporin from *Isometrus maculatus* [88], VmCt1, VmCt2 and Vejovine from *Vaejovis mexicanus* [36,63], BmKn2 and BmKb1 from *Mesobuthus martensii* [87], Ctriporin from *Chaerilus tricostatus* [27], Meucin 13 and Meucin 18 from *Mesobuthus eupeus* [29], StCT2 from

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Scorpiops tibetanus [10], TsAP-1 and TsAP-2 both from *Tityus serrulatus* [31]. A recent comprehensive review on the different classes of AMP found in the venom of several scorpion species can be found elsewhere [35]. Taken together, these examples indicate that scorpion venoms are rich resources for the study of antimicrobial peptides.

The members of Buthidae scorpions are dangerous to humans; to date, approximately 500 species have been identified, of which 25 can cause lethal accidents [60]. These animals have a wide distribution around the globe: species of the genera *Androctonus*, *Leiurus* and *Buthus* are found in North Africa, Middle East and India, genus *Parabuthus* is found in South Africa and genus *Tityus* in South America, especially Brazil [73]. In Brazil, studies on the chemical properties of scorpion venoms have been performed more frequently in *T. serrulatus*, *T. bahiensis* and *T. fasciolatus* [5,39]. In contrast, the venom of *Tityus stigmurus* was not yet explored as an arsenal of toxins with potential biotechnological applications. Our group has previously reported the identification of a transcript encoding an antimicrobial peptide (AMP) from *T. stigmurus*, which was the most abundant transcript in the venom gland [3]. In the present study, this peptide (herein named Stigmurin) had its structure evaluated *in silico* and by circular dichroism, and its biological activities investigated against different microorganisms and in different mammal cell lines *in vitro*.

Materials and methods

Bioinformatic and phylogenetic analyses

The complete nucleotide sequence TSTI0001C selected from the full length cDNA library [3] was analyzed by ORF-Finder [70]. Multiple alignments were performed with CLUSTAL X [44]. Putative signal peptide, propeptide and mature peptide were predicted using SignalP v4.0 [57] and ProP 1.0 [24]. The amino acid sequence was subjected to a similarity search in the database of the National Center for Biotechnology Information (NCBI) using BLASTp [4].

The phylogenetic analysis was performed using AMP sequences with known three-dimensional structure, obtained from the Antimicrobial Peptide Database (APD; http://aps.unmc.edu/AP/main.php) [78]. Selected sequences were aligned using the programs MAFFT [41] and MUSCLE [25]. A best-fit adjustment of the amino acids substitution model was performed using the ProtTest tool [20]. The dendrogram was calculated based on a Bayesian analysis using the BEAST tool [23]. Inferences were conducted in two independent runs with four simultaneous chains each, and fixed WAG model, allowing gamma distributed rates among sites. Each Markov Chain was initiated with a random tree, ran for 10^6 generations, and sampled every 100 generations. Consensus tree was estimated following a discarded burn-in of 1,000,000 trees. The convergence of the simultaneous runs was assessed using the Tracer tool v.1.5 [22], to evaluate the statistic support and robustness of the Bayesian analysis. Generated trees were edited in the software FigTree (http://tree.bio.ed.ac.uk/software/figtree/) [62].

Peptide modeling

Stigmurin modeling was performed using I-TASSER [68], and 5 models were generated. The structures were validated by checking Ramachandran plots, Cβ derivation, clash scores, rotamers, backbone bonds and backbone angles [12]. The models were analyzed and sorted by score values, and pdb files were visualized using USFC Chimera software [83]. The top three models were submitted to a molecular dynamics analysis.

Models refinement

Molecular dynamics simulations were performed to optimize the obtained models. All simulations were performed in explicit water (TIP3P) or 2,2,2-trifluoroethanol (TFE) 70%, using GROMACS [71]. Simulation package and AMBER 99SB-ILDN force field were used [45]. The protonation state of the protein residues at pH 7.0 was determined by PROPKA web server [53]. The simulation time step was 0.5 μs; the minimum distance between any atom of the protein and the box wall was 1.2 nm. Coulomb and van der Waals interactions within a shorter-range cutoff of 1.0 nm were computed every time step. Particle Mesh Ewald was employed to minimize the effects of truncating the electrostatic interactions beyond the 1.2 nm long-range cutoff. Covalent bonds in the protein were constrained using the LINCS algorithm [37].

Circular dichroism (CD)

Far-UV spectra of the peptide were recorded at 25 °C in water, TFE 20%, 50% or 70% (v/v) or sodium dodecyl sulfate (SDS), using 0.1 cm pathlength quartz cuvette in a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) with Peltier-type temperature controller. Each spectrum corresponds to an average of five scans taken from 182 to 260 nm, at a scan rate of 50 nm/min [77,80]. Secondary structure of Stigmurin was determined by deconvoluting CD data using CONTIN/LL [72], CDSSTR [14] and K2D [6] algorithms. We have also used CAPITO web-server [81] to verify the results.

Peptide synthesis

Mature peptide (without both, signal peptide and propeptide), herein named Stigmurin, was synthesized in its amidated form by Invitrogen Life Technologies, USA, using FMOC solid-phase technology [11,49]. C-terminal amidation was selected based on the presence, in the complete translated sequence, of a highly conserved post-translational processing signal (Gly-Arg-Arg-Lys), of which the proteolytic cleavage typically precedes the amidation of the adjacent residue in the mature peptide, as described previously for several similar scorpion peptides [17,31,42,86,88]. The synthetic peptide was purified by reverse-phase HPLC (>90% purity) and its molecular weight was confirmed by mass spectrometry performed by the supplier (Supplementary Fig. 1).

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2015.03.003.

Microorganism strains and in vitro *antimicrobial assay*

For antimicrobial assays, the following strains were used: *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), methicillin-resistant *Staphylococcus aureus* MRSA (ATCC 33591), *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258) and *Candida glabrata* (ATCC 90030).

Minimal inhibitory concentration (MIC) was determined by broth microdilution method in Mueller Hinton Broth (MHB), following the guidelines of the Clinical and Laboratory Standards Institute CLSI [13], with small variations as indicated below. Inocules were prepared at 1×10^5 colony forming units per milliliter (CFU/mL) for bacteria and to 1×10^4 CFU/mL for yeasts. One hundred microliters of the bacterial suspension and 100 μl of serially diluted Stigmurin were added to each well in microtiter plates, to a final peptide concentration of 1.1–139.5 μM. The plates were incubated at 35 °C for 24 h (bacteria) or at 30 °C for 48 h (yeasts) with continuous shaking in a humidified atmosphere. Positive growth controls were inoculated as describe above, without addition of peptide. Sterile MHB was used as negative control. The

MIC was defined as the lowest concentration able to prevent microbial growth, based on optical density (absorbance at 550 nm) [82].

Hemolysis assay

Hemolytic activity was assessed by incubating a suspension of healthy human donor erythrocytes with increasing concentrations of synthetic peptide. Cells were first washed three times by centrifugation at 8000 × *g* for 5 min in 1X PBS, pH 7.4 [52,58], then incubated with Stigmurin (1.1–139.5 μM) at 37 °C for 180 min. Optical density (absorbance at 540 nm) of supernatants was recorded using a plate reader (Epoch-Biotek, Winooski, VT, USA).

Cytotoxicity assay

The cytotoxicity of the synthetic peptide was evaluated in SiHa cells (human cervical cancer cell line) and Vero E6 cells (African green monkey kidney epithelial cell line). Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma–Aldrich, Germany). Confluent cell-monolayers contained in 96-well plates were incubated with serially diluted Stigmurin (2.17–279 μM) in RPMI-1640 for SiHa cells, or Leibovitz (L-15) for Vero E6 cells, at 37 °C for 24 h. Then, 50 μL of MTT (1 mg/mL) was added to each well and further incubated for 4 h at 37 °C. Supernatants were removed and replaced by 100 μL of dimethyl sulfoxide (DMSO) to solubilize formazan crystals [51], and absorbance was measured at 540 nm. Peptide concentration necessary to reduce cell viability in 50% (IC_{50}) was calculated by comparison with untreated cells.

Statistical analysis

For the interpretation of bioassays, multiple-group comparisons were evaluated by analysis of variance (ANOVA) using Graph Pad Prism software (Version 5.00, Graph Pad, San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

Results

Bioinformatic and phylogenetic analyses

From the cDNA library obtained from *T. stigmurus* venom glands [3], the clone TSTI0001C was isolated and identified, coding for a mature protein with 73 amino acid residues [GenBank: JK483709], named Stigmurin. The protein sequence shows a 22 amino acids-long signal peptide, and propeptide starting with the highly conserved post-translational processing signal: Gly-Arg-Arg-Lys-Arg (GRRKR), and 29 amino acids preceding the mature protein of 17 amino acids (Fig. 1).

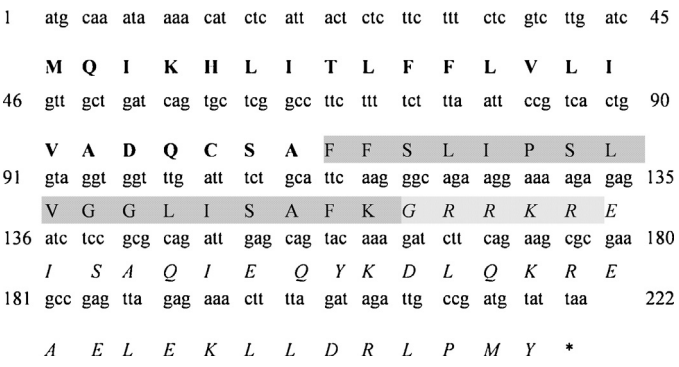


Fig. 1. Nucleotide and translated amino acid sequences of the cDNA clone encoding Stigmurin precursor, as analyzed by ORF-Finder. Predicted amino acid sequence is shown below corresponding codons. Putative signal peptide (bold), propeptide (italics) and mature peptide (dark gray box) were predicted using SignalP v4.0 and ProP 1.0. The conserved propetide convertase cleavage site is indicated in light gray box.

Multiple sequence alignment with peptides retrieved from GenBank (Fig. 2) showed high similarity between Stigmurin and PAMs of different scorpion species, all exhibiting experimentally proved *in vitro* antimicrobial activity [2,8,17,21,31,47,88]. Several amino acid residues are observed in conserved positions of these peptides, especially in mature toxin region. Probably all aligned sequences undergo post-translational modification, given the presence of the signal peptide, propeptide and cleavage site [31]. Interestingly, the phylogenetic analysis (Fig. 3) demonstrated that Stigmurin, along with the scorpion peptides Mucroporin and Bmkb1, exhibited significant sequence similarity (as well as an evolutive proximity) to phylloseptins, a family of antimicrobial peptides from South/Central American *Phyllomedusinae* frog skin [43,61].

According to structural modeling, it was observed that Stigmurin shows alpha-helical conformation (Fig. 4A). The stability of the secondary structure depends on the environment by which it is surrounded; this is supported by two simulation experiments where the peptide changed from alpha-helix structure to a beta-sheet conformation when in water, whereas it assumed a random coil structure when the simulation was performed in TFE 70% (Fig. 4B and C, and Supplementary videos 1 and 2).

Supplementary Videos 1 and 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2015.03.003.

Secondary structure evaluated by CD spectroscopy

The CD analysis of synthetic Stigmurin in aqueous solution showed a typical spectrum of disordered structure. When 20% TFE was added to the system, the dichroic spectrum changed; in

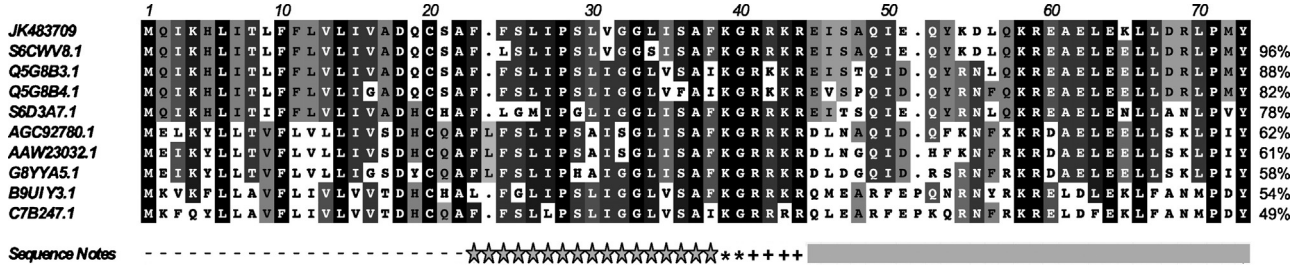


Fig. 2. Alignment of Stigmurin (GenBank: JK483709) with that of known antimicrobial peptides from scorpions by CLUSTAL X, indicating different peptide regions. Gray scale indicates the residues' degree of conservation. Dashed line: signal peptide; stars: probable active peptide; asterisks: C-terminal amidated lysine and adjacent amide donor glycine residues; crosses: cleavage site for proprotein convertase; rectangle: propeptide. GenBank accession numbers are: S6CWV8.1: TsAP-1 from *Tityus serrulatus*; Q5G8B3.1: antimicrobial peptide clone 6 from *T. costatus*; Q5G8B4.1: antimicrobial peptide clone 5 from *T. costatus*; S6D3A7.1: TsAP-2 from *T. serrulatus*; AGC92780.1: caerin-like peptide from *Mesobuthus eupeus*; AAW23032.1: toxin peptide from *M. martensii*; G8YYA5.1: AamAP1 from *Androctonus amoreuxi*; B9UIY3.1: Mucroporin, from *Lychas mucronatus*; C7B247.1: Imcroporin from *Isometrus maculatus*.

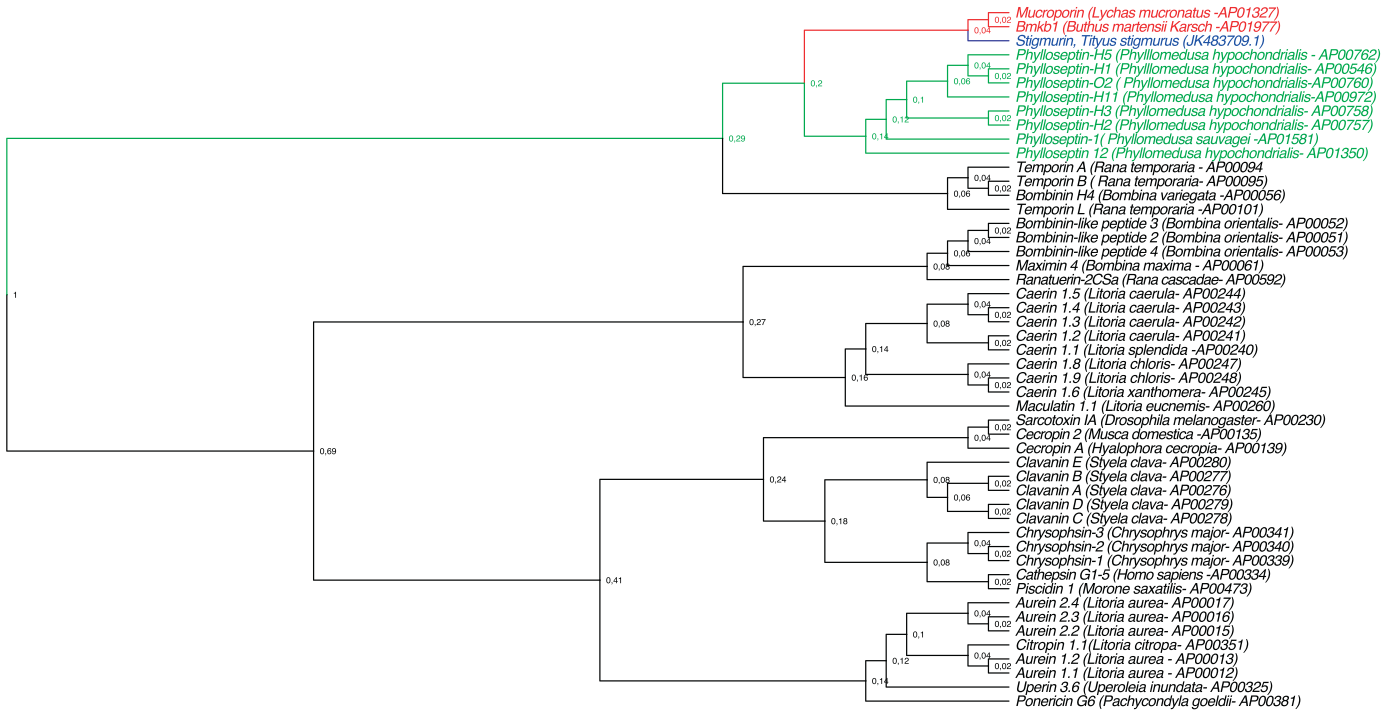


Fig. 3. Phylogenetic analysis of Stigmurin and antimicrobial peptides with known secondary structure, obtained from multiple alignment using MAFFT program. Bayesian analysis was performed in BEAST tool using fixed WAG model. Posterior predictive p-values are shown in the nodes. Color code represent: red = scorpion AMPs; blue = Stigmurin; green = phylloseptins, black = peptides from hylids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

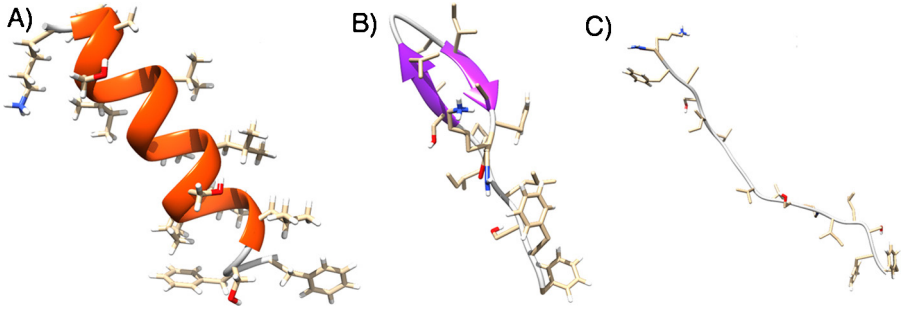


Fig. 4. Modeled patterns of Stigmurin's secondary structure (A) Stigmurin modeled *ab initio* by I-TASSER, visualized using USFC Chimera software. Hydrophobic residues and hydrophilic residues are shown in red and blue, respectively. Also, representative snapshots from the dynamics simulations are shown in (B) in explicit water and (C) in 70% (v/v) TFE solution. Simulations were performed in GROMACS with 0.5-μs time steps, starting from full α-helices and using AMBER 99SB-ILDN force field.

50% TFE, the deviation of the spectra from zero-value of ellipticity became more prominent, and the results were similar in 70% TFE, as well as in SDS above critical micelle concentration (CMC), a membrane–mimetic environment (Fig. 5A). The spectra were analyzed by deconvolution using four different approaches [6,14,72,81]. Fig. 5B presents the proportion of the sequence assuming alpha-helical, beta-sheet or random coil secondary structure in the different environments, calculated as the mean of the values found by the four methods. The results show a general predominance of random coil and beta-sheet conformation.

Antimicrobial activity

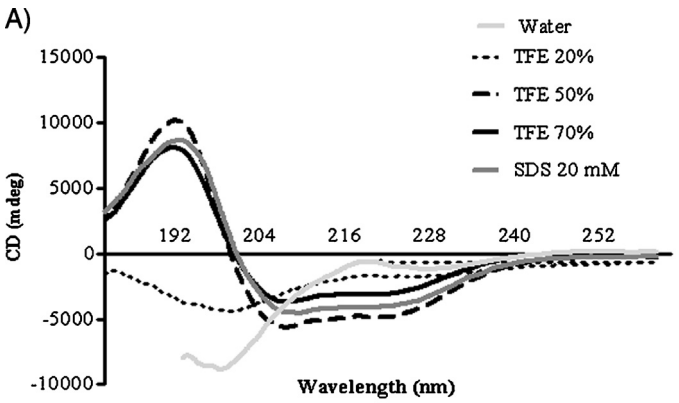
Stigmurin was active against Gram-positive bacterial strains *Staphylococcus aureus* and Methicillin-resistant *S. aureus* MRSA, and the yeasts *C. albicans*, *C. krusei*, and *C. glabrata*, but it was ineffective against Gram-negative *E. coli* (Table 1). Interestingly, Stigmurin was found to possess low hemolytic activity (22%) at the highest concentration tested (139.5 μM), as shown in Fig. 6.

In vitro *cytotoxicity*

Stigmurin showed toxicity against SiHa cells, leading to 36%, 69% and 92% of non-viable cells after exposition to the peptide

Table 1
Minimum inhibitory concentrations (MIC) of synthetic Stigmurin peptide against standard bacterial strains. Values represent mean ± SD ($n = 3$). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to positive control.

Strains	MIC (μM ± SD)
Gram-positive	
Staphylococcus aureus (ATCC 29213)	$8.68 \pm 1.1^{***}$
Methicillin-resistant *S. aureus* MRSA (ATCC 33591)	$17.37 \pm 1.2^{***}$
Gram-negative	
Escherichia coli (ATCC 25922)	>139
Yeasts	
Candida albicans (ATCC 90028)	$34.75 \pm 1.4^{***}$
C. krusei (ATCC 6258)	$69.5 \pm 4.02^{**}$
C. glabrata (ATCC 90030)	$69.5 \pm 2.83^{***}$



B)

Solvent	Alpha-helical %	Beta-sheet %	Random coil %
H_2O	0.0	40.0	60.0
TFE 20%	6.3 ± 3.7	42.4 ± 0.5	51.3 ± 4.1
TFE 50%	13.5 ± 2.5	34.2 ± 3.6	52.3 ± 2.8
TFE 70%	6.6 ± 2.0	37.3 ± 8.1	56.1 ± 9.1
SDS 20 mM	10.3 ±6.6	36.4 ± 5.6	53.3 ± 1.5

Fig. 5. Stigmurin evaluation by Far-UV circular dichroism. (A) CD spectra in water (light gray), TFE 20% (dotted), 50% (dashed), and 70% (solid black), 20 mM SDS (dark gray). (B) Predominance of secondary structures according to deconvolution data of CD spectra using different algorithms (CONTIN/LL, SELCON and K2D) and CAPITO server (CD Analysis and Plotting Tool). Values presented are mean ± SD of the percentages obtained by the four different methods, except in water, which was performed exclusively using CAPITO.

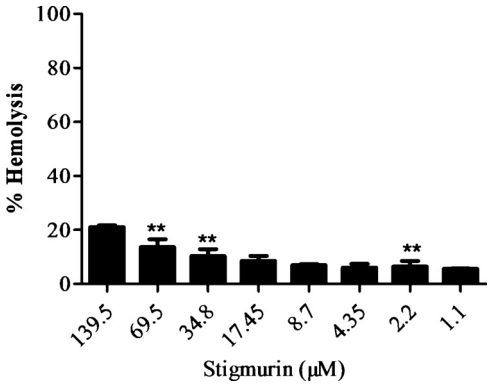


Fig. 6. Hemolytic activity of Stigmurin. Human red blood-cells were incubated with serially diluted Stigmurin (1.1–139.5 μM), at 37 °C for 180 min. The activity was determined by measuring the absorbance at 540 nm, using PBS and distilled water as negative and positive lysis control, respectively. Percentage hemolysis was calculated relative to the positive control (100%). Values represent mean ± SD ($n = 3$). $^{**}p < 0.01$ compared to positive control.

at concentrations of 69.8 μM, 139.5 μM and 279 μM, respectively (Fig. 7A). Cytotoxic effect it was also observed in Vero E6 cells, where 57% and 89% of the cells were non-viable after incubation with Stigmurin at concentrations of 139.5 μM and 279 μM, respectively (Fig. 7B). The IC_{50} parameter verifies that viability reduction was more prominent on human carcinoma lineage SiHa (IC_{50} 118 μM), than on monkey epithelial cells Vero E6 (IC_{50} 150 μM).

Discussion

The identification and analysis of Stigmurin demonstrated that this peptide has high sequence similarity to other non-disulfide-bridged antimicrobial peptides [2,8,17,21,31,47,88]. Through multiple sequence alignment and phylogenetic analysis, it could be noticed a significant conservation of key amino acid residues that are characteristic of antimicrobial peptides [73]. This observation is consistent with the hypothesis that many AMPs are evolutionarily related within the Scorpionidae taxon, having arisen from multiple duplications of an ancestral gene [73,86]. The diversification of AMPs could then be part of an evolutionary strategy driven by the rapid evolution of pathogens [67]. Furthermore, bioinformatic analysis showed evidence that Stigmurin is a new AMP.

Structural analysis of Stigmurin by molecular dynamics and CD showed that its structure differs when in the presence of water, SDS or TFE. These results suggest that it may assume different conformations depending on the environment, which is a common behavior in AMPs [74]. It is essential to highlight that, according to CD analysis, Stigmurin exhibited a more regular secondary structure when in contact with TFE or SDS. TFE diminishes the peptide interaction with the solvent and favors intramolecular interactions, thus increasing the chance of secondary structure conformation (in this work, mainly β-sheet) [65]. Stigmurin presented a different structure in the presence of water, mostly random coil. Furthermore, the contact with a micellar solution led to a conformational shift in Stigmurin's structure, indicating that it may interact with micelles, which mimic membranes [74]. This suggests that the mechanism of action of Stigmurin is through interaction with membranes, which is very common among other AMPs already characterized [33,40,65,74,85].

It is noticed a discrepancy between the homology models (all alpha-helical structure, Fig. 4A) with the findings from dynamics

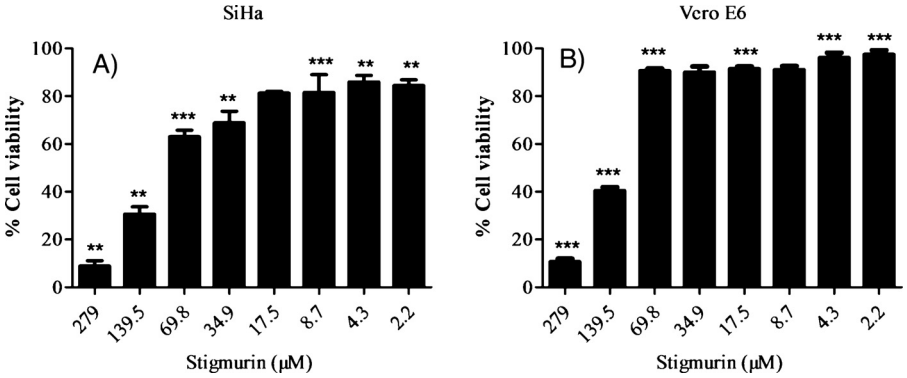


Fig. 7. Citotoxicity of Stigmurin. Dose–response curves of cell viability as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in cell lines SiHa (A) and Vero E6 (B). Cells were cultured in 96-well microtiter plates using RPMI-1640 or L-15 media in the presence of serially diluted Stigmurin (2.17–279 μM) for 24 h. The activity was determined by measuring the absorbance at 540 nm after addition of MTT for 4 h and subsequent solubilization with DMSO. Values represent mean ± SD ($n = 3$). Statistical significance was analyzed using a paired t-test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to positive control.

simulation (Fig. 4B and C) and experimental CD results (Fig. 5A and B). Altogether, the present study revealed that Stigmurin alters its secondary structure according to the medium, which shows that the residues can be structured or flexible in specific conditions, possibly having a functional significance [33,34,74]. Another point to be discussed is that the molecular dynamics simulation agrees with deconvoluted CD results regarding the type of predominant structures, but not the tendency of variation in different media. This could derive from the deconvolution algorithms, which are optimized for larger proteins rather than for small peptides [6,14,72], or from issues to accurately represent the dielectric properties with the force field employed in the simulations [38]. Nevertheless, it can be concluded that Stigmurin has an environment-dependent conformation.

In this work we have demonstrated that Stigmurin, a novel antimicrobial peptide, possesses antimicrobial activity against Gram-positive bacteria, including antibiotic-resistant pathogens (MRSA), and fungi. Peptide TsAP-1 from *T. serrulatus* venom, a highly similar scorpion AMP (96% identity) from a closely related species, presented poor antimicrobial activity (MIC > 120 μM for *S. aureus*, *E. coli* and *C. albicans*) when compared to Stigmurin [31]. Both peptides have the same net charge (+2 considering the C-terminal amidation), and there are only two residues which differ in the mature peptide sequences, exchanging Phe24 and Leu34 in Stigmurin for a Leu and a Ser, respectively, in TsAP-1 [31]. It is possible that the different activity is a result of a decreased hydrophobicity conferred by Ser to TsAP-1. Peptide hydrophobicity, together with a positive net charge in an amphipathic arrangement, is considered a major requirement for their antimicrobial activity [59]. In contrast, despite a lower sequence similarity to Stigmurin (78%), peptide TsAP-2 from *T. serrulatus* has identical proportion of hydrophobic residues (10 out of 17) and presented a more similar activity, causing deleterious effects in Gram-positive bacterium *S. aureus* development, and in *C. albicans*, but being less effective against Gram-negative *E. coli* [31].

In fact, activity against Gram-positive bacteria with a weaker activity against Gram-negative bacteria was also described for other non-disulfide-bridged peptides from scorpions such as Pandinin1 and -2, IsCT and IsCT2 [15,18,19]. Among these peptides, IsCT, IsCT2 and TsAP-2 present a conserved consensus sequence ($X_{(0-5)}IX_3IX_3IX_4$) where the three Ile residues seem to play an important functional role [86]. This consensus is partially present in Stigmurin and Pandinin2, where the second Ile residue is substituted by other hydrophobic residues (Val and Phe, respectively). Here again, hydrophobicity seems to be a key aspect in the antimicrobial activity of cationic peptides. The initial electrostatic interaction with negatively charged surface molecules (*e.g.* teichoic acids in the Gram-positive cell wall), and subsequent membrane insertion are important steps in the process of killing; however, the mechanisms of action and the specific cellular targets can vary among peptides and among organisms for a given peptide [28]. Furthermore, it is important to note that the differences in the cell surface, or even differences in the absorption of the agent by each type of microorganism are other factors that can influence the MIC values [69].

Infections by multi-drug resistant pathogens such as MRSA are reaching alarming numbers worldwide, frequently causing the death of hospital patients [55]. It is appreciated that the mechanism of action of AMPs, in contrast to most conventional antibiotics, is less prone to induce microbial resistance, and peptide antibiotics have been considered valuable alternatives in the treatment of such infections [55]. Our results suggest that Stigmurin can be a candidate molecule for use in antimicrobial therapy. Furthermore, at the concentration which Stigmurin has prominent antimicrobial activity, its hemolytic activity is only mild, supporting the potential use of this molecule for therapeutic purposes [88]. Interestingly, Stigmurin combines a good antimicrobial activity with a fairly low hemolytic activity, in contrast to the closely related peptides from *T. serrulatus* which are either poorly antimicrobial (TsAP-1) or highly hemolytic (TsAP-2) [31].

There are fewer studies reporting AMPs activity against *Candida* species, but it is believed that the mechanism of action is similar to that observed in bacteria [40]. The cell wall of yeasts is an anionic surface characterized by its rigidity, being glucans (1,3-β and 1,6-β) the most abundant polysaccharides (around 60%), followed by mannans. Chitin, the less abundant component, corresponds to approximately 1% of all saccharides present in the cell wall [46]. Some scorpions AMPs tested against *C. albicans* yielded similar results to those we found for Stigmurin, including: TSAP-2 (MIC 10 μM) [31], Meucin-18 (MIC 24.1 μM) [29], and Ctriporin (MIC 20 μg/mL) [27]. This data strengthens our results suggesting that Stigmurin could be considered for the treatment of candidiasis.

The results on the cytotoxic activity of Stigmurin indicate a possible differential toxicity toward tumor cells. This is in accordance with observations by other researchers reporting variations in sensitivity of different cell cultures to antitumor AMPs [7,79]. For instance, peptides present in the venom of the scorpion *Tityus discrepans* were active against SKBR3 breast cancer cell line, having negligible effect upon monkey kidney cells (MA104) [16]. The exact mechanism of toxicity which differs in different cell types is not yet fully elucidated, but may be related to the negative charge of cancer cell membranes, due to increased expression of molecules with anionic character, such as glycosylated mucins, sialylated gangliosides, and heparin sulfate [30]. Furthermore, it has been reported that cell intrinsic factors influence their sensitivity to toxic substances, including chemical biotransformation and binding forms, characteristics of membrane permeability and surface determinants, intracellular synthesis and mechanisms of adaptation and recovery [9,64,84].

From *T. serrulatus*, peptide TsAP-2 showed cytotoxicity against five different human cancer cell lines, with IC_{50} ranging from 4 to 15 μM, whereas TsAP-1 had a less effective antitumoral action [31]. This was in line with their antimicrobial activity; it is thus possible that, like the effect upon microorganisms, Stigmurin's antitumoral potential is more similar to TsAP-2, despite a lower sequence similarity, as discussed above. The IC_{50} values obtained in the high μM range for Stigmurin upon the two cell lines tested in the present study are not *per se* indicators of an antitumoral activity; besides potency, other parameters such as maximum effect and the slope of the dose–response curve, should also be taken into account when analyzing putative novel antitumoral drugs [26]. Further studies on a wider panel of tumor cell lineages will be required to suggest a possible anticancer potential for Stigmurin. Nevertheless, this is to our knowledge the first scientific evidence demonstrating the effect of a component of *T. stigmurus* venom on cell viability of a human cancer cell line.

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References

[1] Almaaytah A, Albalas Q. Scorpion venom peptides with no disulfide bridges: a review. Peptides 2014;51:35–45, http://dx.doi.org/10.1016/j.peptides.2013.10.021.

[2] Almaaytah A, Zhou M, Wang L, Chen T, Walker B, Shaw C. Antimicrobial/cytolytic peptides from the venom of the North African scorpion, *Androctonus amoreuxi*: biochemical and functional characterization of natural peptides and a single site-substituted analog. Peptides 2012;35:291–9, http://dx.doi.org/10.1016/j.peptides.2012.03.016.

[3] Almeida DD, Scortecci KC, Kobashi LS, Agnez-Lima LF, Medeiros SRB, Silva-Junior AA, et al. Profiling the resting venom gland of the scorpion *Tityus stigmurus* through a transcriptomic survey. BMC Genom 2012;13:362, http://dx.doi.org/10.1186/1471-2164-13-362.

[4] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402, http://dx.doi.org/10.1093/nar/25.17.3389.

[5] Alvarenga ER. Transcriptome analysis of the *Tityus serrulatus* scorpion venom gland. Open J Genet 2012;02:210–20, http://dx.doi.org/10.4236/ojgen.2012.24027.

[6] Andrade MA, Chacón P, Merelo JJ, Morán F. Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network. Protein Eng 1993;6:383–90.

[7] Arnusch CJ, Pieters RJ, Breukink E. Enhanced membrane pore formation through high-affinity targeted antimicrobial peptides. PLoS One 2012;7:e39768, http://dx.doi.org/10.1371/journal.pone.0039768.

[8] Baradaran M, Jalali A, Jolodar A, Ghasemian S. A new caerin-like antibacterial peptide from the venom gland of the Iranian scorpion *Mesobuthus eupeus*: cDNA amplification and sequence analysis. Afr J Biotechnol 2012;11:10176–81, http://dx.doi.org/10.5897/AJB11.3373.

[9] Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 2005;3:238–50, http://dx.doi.org/10.1038/nrmicro1098.

[10] Cao L, Li Z, Zhang R, Wu Y, Li W, Cao Z. StCT2, a new antibacterial peptide characterized from the venom of the scorpion *Scorpiops tibetanus*. Peptides 2012;36:213–20, http://dx.doi.org/10.1016/j.peptides.2012.04.010.

[11] Carpino LA, Han GY. 9-Fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. J Am Chem Soc 1970;92:5748–9, http://dx.doi.org/10.1021/ja00722a043.

[12] Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr D: Biol Crystallogr 2010;66:12–21, http://dx.doi.org/10.1107/S0907444909042073.

[13] Cockerill FR, Wikler MA, Alder J, Dudley MN, Eliopoulos GM, Ferraro MJ, et al. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. eth ed; 2012. p. 32.

[14] Compton LA, Johnson WC. Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication. Anal Biochem 1986;155:155–67, http://dx.doi.org/10.1016/0003-2697(86)90241-1.

[15] Corzo G, Escoubas P, Villegas E, Barnham KJ, He W, Norton RS, et al. Characterization of unique amphipathic antimicrobial peptides from venom of the scorpion *Pandinus imperator*. Biochem J 2001;359:35–45, http://dx.doi.org/10.1042/0264-6021:3590035.

[16] D'Suze G, Rosales A, Salazar V, Sevcik C. Apoptogenic peptides from *Tityus discrepans* scorpion venom acting against the SKBR3 breast cancer cell line. Toxicon 2010;56:1497–505, http://dx.doi.org/10.1016/j.toxicon.2010.09.008.

[17] Dai C, Ma Y, Zhao Z, Zhao R, Wang Q, Wu Y, et al. Mucroporin, the first cationic host defense peptide from the venom of *Lychas mucronatus*. Antimicrob Agents Chemother 2008;52:3967–72, http://dx.doi.org/10.1128/AAC.00542-08.

[18] Dai L, Yasuda A, Naoki H, Corzo G, Andriantsiferana M, Nakajima T. IsCT, a novel cytotoxic linear peptide from scorpion *Opisthacanthus madagascariensis*. Biochem Biophys Res Commun 2001;286:820–5, http://dx.doi.org/10.1006/bbrc.2001.5472.

[19] Dai L, Corzo G, Naoki H, Andriantsiferana M, Nakajimaa T. Purification, structure–function analysis, and molecular characterization of novel linear peptides from scorpion *Opisthacanthus madagascariensis*. Biochem Biophys Res Commun 2002;293:1514–22, http://dx.doi.org/10.1016/S0006-291X(02)00423-0.

[20] Darriba D, Taboada GL, Doallo R, Posada D. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 2011;27:1164–5, http://dx.doi.org/10.1093/bioinformatics/btr088.

[21] Diego-García E, Batista CVF, García-Gómez BI, Lucas S, Candido DM, Gómez-Lagunas F, et al. The Brazilian scorpion *Tityus costatus* Karsch: genes, peptides and function. Toxicon 2005;45:273–83, http://dx.doi.org/10.1016/j.toxicon.2004.10.014.

[22] Drummond AJ, Rambaut ABEAST. Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 2007;7:214, http://dx.doi.org/10.1186/1471-2148-7-214.

[23] Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 2012;29:1969–73, http://dx.doi.org/10.1093/molbev/mss075.

[24] Duckert P, Brunak S, Blom N. Prediction of proprotein convertase cleavage sites. Protein Eng Des Sel 2004;17:107–12, http://dx.doi.org/10.1093/protein/gzh013.

[25] Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792–7, http://dx.doi.org/10.1093/nar/gkh340.

[26] Fallahi-Sichani M, Honarnejad S, Heiser LM, Gray JW, Sorger PK. Metrics other than potency reveal systematic variation in responses to cancer drugs. Nat Chem Biol 2013;9:708–14, http://dx.doi.org/10.1038/nchembio.1337.

[27] Fan Z, Cao L, He Y, Hu J, Di Z, Wu Y, et al. Ctriporin, a new anti-methicillin-resistant *Staphylococcus aureus* peptide from the venom of the scorpion *Chaerilus tricostatus*. Antimicrob Agents Chemother 2011;55:5220–9, http://dx.doi.org/10.1128/AAC.00369-11.

[28] Friedrich CL, Moyles D, Beveridge TJ, Hancock REW. Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. Antimicrob Agents Chemother 2000;44:2086–92, http://dx.doi.org/10.1128/AAC.44.8.2086-2092.2000.

[29] Gao B, Sherman P, Luo L, Bowie J, Zhu S. Structural and functional characterization of two genetically related meucin peptides highlights evolutionary divergence and convergence in antimicrobial peptides. FASEB J 2009;23:1230–45, http://dx.doi.org/10.1096/fj.08-122317.

[30] Gaspar D, Veiga AS, Castanho MARB. From antimicrobial to anticancer peptides. A review. Front Microbiol 2013;4:294, http://dx.doi.org/10.3389/fmicb.2013.00294.

[31] Guo X, Ma C, Du Q, Wei R, Wang L, Zhou M, et al. Two peptides, TsAP-1 and TsAP-2, from the venom of the Brazilian yellow scorpion, *Tityus serrulatus*: evaluation of their antimicrobial and anticancer activities. Biochimie 2013;95:1784–94, http://dx.doi.org/10.1016/j.biochi.2013.06.003.

[32] Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 2000;8:402–10, http://dx.doi.org/10.1016/S0966-842X(00)01823-0.

[33] Hancock REW, Sahl H-G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 2006;24:1551–7, http://dx.doi.org/10.1038/nbt1267.

[34] Hansson T, Oostenbrink C, van Gunsteren W. Molecular dynamics simulations. Curr Opin Struct Biol 2002;12:190–6, http://dx.doi.org/10.1016/S0959-440X(02)00308-1.

[35] Harrison PL, Abdel-Rahman Ma, Miller K, Strong PN. Antimicrobial peptides from scorpion venoms. Toxicon 2014;88:115–37, http://dx.doi.org/10.1016/j.toxicon.2014.06.006.

[36] Hernandez-Aponte CA, Silva-Sanchez J, Quintero-Hernandez V, Rodriguez-Romero A, Balderas C, Possani LD, et al. Vejovine, a new antibiotic from the scorpion venom of *Vaejovis mexicanus*. Toxicon 2011;57:84–92, http://dx.doi.org/10.1016/j.toxicon.2010.10.008.

[37] Hess B, Bekker H, Berendsen HJC, Fraaije JGEM. LINCS: a linear constraint solver for molecular simulations. J Comput Chem 1997;18:1463–72, http://dx.doi.org/10.1002/(SICI)1096-987X(199709)18:12<1463::AID-JCC4>3.0.CO;2-H.

[38] Hornak V, Abel R, Okur A, Strockbine B, Roitberg A, Simmerling C. Comparison of multiple Amber force fields and development of improved protein backbone parameters. Proteins 2006;65:712–25, http://dx.doi.org/10.1002/prot.21123.

[39] Horta CCR, de Magalhães BF, Oliveira-Mendes BBR, Carmo AOdo, Duarte CG, Felicori LF, et al. Molecular, immunological, and biological characterization of *Tityus serrulatus* venom Hyaluronidase: new insights into its role in envenomation. PLoS Negl Trop Dis 2014:8, http://dx.doi.org/10.1371/journal.pntd.0002693.

[40] Jenssen H, Hamill P, Hancock REW. Peptide antimicrobial agents. Clin Microbiol Rev 2006;19:491–511, http://dx.doi.org/10.1128/CMR.00056-05.

[41] Katoh K, Toh H. Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics 2010;26:1899–900, http://dx.doi.org/10.1093/bioinformatics/btq224.

[42] Van Kesteren RE, Smit AB, Dirks RW, de With ND, Geraerts WP, Joosse J. Evolution of the vasopressin/oxytocin superfamily: characterization of a cDNA encoding a vasopressin-related precursor, preproconopressin, from the mollusc *Lymnaea stagnalis*. Proc Natl Acad Sci U S A 1992;89:4593–7.

[43] König E, Bininda-Emonds ORP. Evidence for convergent evolution in the antimicrobial peptide system in anuran amphibians. Peptides 2011;32:20–5, http://dx.doi.org/10.1016/j.peptides.2010.10.009.

[44] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007;23:2947–8.

[45] Lindorff-Larsen K, Piana S, Palmo K, Maragakis P, Klepeis JL, Dror RO, et al. Improved side-chain torsion potentials for the Amber ff99SB protein force field. Proteins Struct Funct Bioinforma 2010;78:1950–8, http://dx.doi.org/10.1002/prot.22711.

[46] Lipke PN, Ovalle R. Cell wall architecture in yeast: new structure and new challenges. J Bacteriol 1998;180:3735–40.

[47] Luo F, Zeng XC, Hahin R, Cao ZJ, Liu H, Li WX. Genomic organization of four novel nondisulfide-bridged peptides from scorpion *Mesobuthus martensii* Karsch: gaining insight into evolutionary mechanism. Peptides 2005;26:2427–33, http://dx.doi.org/10.1016/j.peptides.2005.06.008.

[48] Melo MN, Ferre R, Castanho MARB. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. Nat Rev Microbiol 2009;7:245–50, http://dx.doi.org/10.1038/nrmicro2095.

[49] Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 1963;85:2149–54, http://dx.doi.org/10.1021/ja00897a025.

[50] Moerman L, Bosteels S, Noppe W, Willems J, Clynen E, Schoofs L, et al. Antibacterial and antifungal properties of alpha-helical, cationic peptides in the

venom of scorpions from southern Africa. Eur J Biochem 2002;269:4799–810, http://dx.doi.org/10.1046/j.1432-1033.2002.03177.x.

[51] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63, http://dx.doi.org/10.1016/0022-1759(83)90303-4.

[52] Oguiura N, Boni-Mitake M, Affonso R, Zhang G. In vitro antibacterial and hemolytic activities of crotamine, a small basic myotoxin from rattlesnake *Crotalus durissus*. J Antibiot 2011;64:327–31, http://dx.doi.org/10.1038/ja.2011.10.

[53] Olsson MHM, Søndergaard CR, Rostkowski M, Jensen JH. PROPKA3; consistent treatment of internal and surface residues in empirical p K a predictions. J Chem Theory Comput 2011;7:525–37, http://dx.doi.org/10.1021/ct100578z.

[54] Oyston PCF, Fox MA, Richards SJ, Clark GC. Novel peptide therapeutics for treatment of infections. J Med Microbiol 2009;58:977–87, http://dx.doi.org/10.1099/jmm.0.011122-0.

[55] Park SC, Park Y, Hahm KS. The role of antimicrobial peptides in preventing multidrug-resistant bacterial infections and biofilm formation. Int J Mol Sci 2011;12:5971–92.

[56] Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. Crit Rev Biotechnol 2012;32:143–71, http://dx.doi.org/10.3109/07388551.2011.594423.

[57] Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 2011;8:785–6, http://dx.doi.org/10.1038/nmeth.1701.

[58] Polyansky AA, Vassilevski AA, Volynsky PE, Vorontsova OV, Samsonova OV, Egorova NS, et al. N-terminal amphipathic helix as a trigger of hemolytic activity in antimicrobial peptides: a case study in latarcins. FEBS Lett 2009;583:2425–8, http://dx.doi.org/10.1016/j.febslet.2009.06.044.

[59] Powers J-PS, Hancock REW. The relationship between peptide structure and antibacterial activity. Peptides 2003;24:1681–91, http://dx.doi.org/10.1016/j.peptides.2003.08.023.

[60] Prendini L, Wheeler WC. Scorpion higher phylogeny and classification, taxonomic anarchy, and standards for peer review in online publishing. Cladistics 2005;21:446–94, http://dx.doi.org/10.1111/j.1096-0031.2005.00073.x.

[61] Raja Z, André S, Piesse C, Sereno D, Nicolas P, Foulon T, et al. Correction: structure, antimicrobial activities and mode of interaction with membranes of bovel phylloseptins from the painted-belly leaf frog, *Phyllomedusa sauvagii*. PLoS One 2013;8, http://dx.doi.org/10.1371/annotation/dbb3e614-dc4c-40dd-b9e0-37787ae6b150.

[62] Rambaut A. FigTree, a graphical viewer of phylogenetic trees. Inst Evol Biol Univ Edinburgh; 2009.

[63] Ramírez-Carreto S, Quintero-Hernández V, Jiménez-Vargas JM, Corzo G, Possani LD, Becerril B, et al. Gene cloning and functional characterization of four novel antimicrobial-like peptides from scorpions of the family Vaejovidae. Peptides 2012;34:290–5, http://dx.doi.org/10.1016/j.peptides.2012.02.002.

[64] Ran S, He J, Huang X, Soares M, Scothorn D, Thorpe PE. Antitumor effects of a monoclonal antibody that binds anionic phospholipids on the surface of tumor blood vessels in mice. Clin Cancer Res 2005;11:1551–62, http://dx.doi.org/10.1158/1078-0432.CCR-04-1645.

[65] Roccatano D, Colombo G, Fioroni M, Mark AE. Mechanism by which 2,2,2-trifluoroethanol/water mixtures stabilize secondary-structure formation in peptides: a molecular dynamics study. Proc Natl Acad Sci U S A 2002;99:12179–84, http://dx.doi.org/10.1073/pnas.182199699.

[66] Rodríguez de la Vega RC, Schwartz EF, Possani LD. Mining on scorpion venom biodiversity. Toxicon 2010;56:1155–61, http://dx.doi.org/10.1016/j.toxicon.2009.11.010.

[67] Roelants K, Fry BG, Ye L, Stijlemans B, Brys L, Kok P, et al. Origin and functional diversification of an amphibian defense peptide arsenal. PLoS Genet 2013;9:e100, http://dx.doi.org/10.1371/journal.pgen.1003662.

[68] Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 2010;5:725–38, http://dx.doi.org/10.1038/nprot.2010.5.

[69] Russell AD. Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother 2003;52:750–63, http://dx.doi.org/10.1093/jac/dkg422.

[70] Sayers EW, Barrett T, Benson DA, Bolton E, Bryant SH, Canese K, et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 2010;38:5–16, http://dx.doi.org/10.1093/nar/gkh073.

[71] Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJC. GROMACS: fast, flexible, and free. J Comput Chem 2005;26:1701–18, http://dx.doi.org/10.1002/jcc.20291.

[72] Van Stokkum IH, Spoelder HJ, Bloemendal M, van Grondelle R, Groen FC. Estimation of protein secondary structure and error analysis from circular dichroism spectra. Anal Biochem 1990;191:110–8, http://dx.doi.org/10.1016/0003-2697(90)90396-Q.

[73] Sunagar K, Undheim EAB, Chan AHC, Koludarov I, Muñoz-Gómez SA, Antunes A, et al. Evolution stings: the origin and diversification of scorpion toxin peptide scaffolds. Toxins (Basel) 2013;5:2456–87, http://dx.doi.org/10.3390/toxins5122456.

[74] Takahashi T, Nomura F, Yokoyama Y, Tanaka-Takiguchi Y, Homma M, Takiguchi K. Multiple membrane interactions and versatile vesicle deformations elicited by melittin. Toxins (Basel) 2013;5:637–64, http://dx.doi.org/10.3390/toxins5040637.

[75] Torres-Larios A, Gurrola GB, Zamudio FZ, Possani LD. Hadrurin, a new antimicrobial peptide from the venom of the scorpion *Hadrurus aztecus*. Eur J Biochem 2000;267:5023–31, http://dx.doi.org/10.1046/j.1432-1327.2000.01556.x.

[76] Verdonck F, Bosteels S, Desmet J, Moerman L, Noppe W, Willems J, et al. A novel class of pore-forming peptides in the venom of *Parabuthus schlechteri* Purcell (scorpions: buthidae). Cimbebasia 2000;16:247–60.

[77] Wallace BA, Lees JG, Orry AJW, Lobley A, Janes RW. Analyses of circular dichroism spectra of membrane proteins. Protein Sci 2003;12:875–84, http://dx.doi.org/10.1110/ps.0229603.

[78] Wang G, Li X, Wang ZAPD2. The updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 2009;3:7, http://dx.doi.org/10.1093/nar/gkn823.

[79] Wang WX, Ji YH. Scorpion venom induces glioma cell apoptosis *in vivo* and inhibits glioma tumor growth *in vitro*. J Neurooncol 2005;73:1–7, http://dx.doi.org/10.1007/s11060-004-4205-6.

[80] Whitmore L, Wallace BA. Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. Biopolymers 2008;89:392–400, http://dx.doi.org/10.1002/bip.20853.

[81] Wiedemann C, Bellstedt P, Görlach M. CAPITO—a web server-based analysis and plotting tool for circular dichroism data. Bioinformatics 2013;29:1750–7, http://dx.doi.org/10.1093/bioinformatics/btt278.

[82] Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008;3:163–75, http://dx.doi.org/10.1038/nprot.2007.521.

[83] Yang Z, Lasker K, Schneidman-Duhovny D, Webb B, Huang CC, Pettersen EF, et al. UCSF chimera, MODELLER, and IMP: an integrated modeling system. J Struct Biol 2012;179:269–78, http://dx.doi.org/10.1016/j.jsb.2011.09.006.

[84] Yount NY, Yeaman MR. Peptide antimicrobials: cell wall as a bacterial target. Ann N Y Acad Sci 2013;1277:127–38, http://dx.doi.org/10.1111/nyas.12005.

[85] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;415:389–95, http://dx.doi.org/10.1038/415389a.

[86] Zeng X-C, Corzo G, Hahin R. Scorpion venom peptides without disulfide bridges. IUBMB Life 2005;57:13–21, http://dx.doi.org/10.1080/15216540500058899.

[87] Zeng X-C, Wang S-X, Zhu Y, Zhu S-Y, Li W-X. Identification and functional characterization of novel scorpion venom peptides with no disulfide bridge from *Buthus martensii* Karsch. Peptides 2004;25:143–50, http://dx.doi.org/10.1016/j.peptides.2003.12.003.

[88] Zhao Z, Ma Y, Dai C, Zhao R, Li S, Wu Y, et al. Imcroporin, a new cationic antimicrobial peptide from the venom of the scorpion *Isometrus maculates*. Antimicrob Agents Chemother 2009;53:3472–7, http://dx.doi.org/10.1128/AAC.01436-08.