

RESEARCH REPORT

Evidence of cytotoxic activity against mammalian red blood cell of Na⁺ channel neurotoxin (Ae1) from sea anemone (*Actinia equina*)**MG Parisi, MR Trapani, L Cardinale, M Cammarata***Marine Immunobiology Laboratory, Department of Biological, Chemical and Pharmaceutical Science and Technology, University of Palermo, Palermo, Italy*

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Abstract

The diversification of anthozoan toxins played an important role in the ability to colonize various ecological niches. In this study we evaluated the hemolytic activity of HPLC separated fraction of tentacle extracts of sea anemone *Actinia equina*. Toxic components from acid tissue tentacle extracts were investigated by size exclusion and reverse phase HPLC to characterize cytolytic molecules. A novel low molecular weight active fraction was sequenced by MALDI TOF analysis and a protein correspondent to 5.4 kDa Sodium channel neurotoxin (Ae1) from *A. equina* was identified. Synthetic Ae1 was assayed and it showed an hemolytic activity against mammalian erythrocytes in a dose dependent manner. Cytolytic activity in addition to neuro-inhibitory function could be a further property of Ae1 toxin repertoire.

Key Words: *Actinia equina*; hemolysin; Na⁺ channel neurotoxin; sea anemone; Ae1 neurotoxin**Introduction**

Sea anemones are a group of water-dwelling predators (Cnidaria: Anthozoa: Actiniaria). Like sessile benthic organisms, sea anemones produce venoms to immobilize prey and protect themselves. Furthermore their adaptive radiation has led to a high specific richness over the course of million years.

Cnidarians are a rich source of bioactive molecules like antimicrobial peptides, cytolytic proteins and neurotoxins (Parisi *et al.*, 2014).

The presence, diversification and multifunctionality of the toxins may have played an important role in the ability of colonization and adaptation to ecological niches that change over time. The major fraction of the venom is represented by toxins, which are able to extend the action potential kinetics during the depolarization to the extracellular site on Sodium (Nav) channels in a membrane potential-dependent manner (Stevens *et al.*, 2011). Type 1 and 2 Na⁺ channel toxins are composed of 46 - 49 amino acid residues, except for *A. equina* toxin Ae1 of 54 residues (Lin *et al.*, 1996), and cross-linked by three disulfide bridges. They have an anti-parallel β -sheet with four β -strands

and a highly flexible loop (Arg-14 loop) after their most conserved residue, lacking of any α -helix (Frazão *et al.*, 2012). The abundance of toxic proteins extracted from different cnidarian venoms shows its importance in defense and predation actions by sea anemones. The presence of type 1 and 2 toxins seems to be taxonomical related; infact, Actiniidae family shows only type 1 toxin, while either type 1 or 2 toxins are present in Stichodactylidae family.

Type 3 Na⁺ channel toxin is only identified in a few species and it is composed of 27- 32 amino acid residues (Moran *et al.*, 2009).

Na⁺ and K⁺ channel peptide toxins are able to paralyze preys immediately. Thus, important targets for neurotoxins are voltage gated ion channels in excitable cell membranes (Messerli *et al.*, 2006).

Sea anemone peptides active on Nav1 channels are able to produce these effects by acting from the extracellular side of plasma membrane. Instead, cytolytic actinoporins permeate cell membranes and form transmembrane pores leading to cell lysis.

The Actinidae *A. equina* is commonly found in temperate coastal waters and it's well known to possess hemolysins such as equinatoxins (Anderluh and Macek, 2002) and sodium and potassium channel peptide toxins (Minagawa *et al.*, 1998). Its peptide toxins resulted from the whole body, tentacles or secreted mucus. Moreover toxins have been found in specific organs located in a ring

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around tentacles base (acrorhagi) and in cellular components of gastrodermal fluid (Parisi *et al.*, 2014).

In the paper of Lin *et al.* (1996) the neurotoxic lethal activity to crab Na⁺ channel toxin (Ae1) was distinguished from cellular lytic activity suggesting that the studied fraction have cytotoxic activity against erythrocytes because was partial overlapped with the 20 kDa equinotoxin.

In this study a low molecular weight hemolysin from acid tissue tentacle extracts of sea anemone *Actinia equina* was separated by HPLC. Sodium channel neurotoxin (Ae1) was identified by direct sequencing and its cytolytic activity against mammalian erythrocytes was confirmed by using a synthetic Ae1.

The hemolytic activity of the isolated molecule against eukaryotic cells has been detected highlighting its multi-functional role, known until now only for its interaction with sodium channels.

Materials and Methods

Samples preparation

The sea anemone *Actinia equina* was collected from a rocky shore of Capo Gallo coast (Palermo, Italy). Specimens were maintained in a closed-circuit aquarium at 20 °C with aerated sea water (150 L aquaria) and fed every second day with a marine invertebrates filter feeding diet (Kent Marine Inc. WI USA). Tentacles were dissected and suspended in TBS (150 mM NaCl, 10 mM Tris-HCl) in the presence of 10 % acetic acid (acid extract) (Payne and Ames, 1982). After homogenization in ice with Ultra-Turrax for 5 min, sample was centrifuged at 21,000g for 20 min at 4 °C and finally supernatant was recovered and stored at -20 °C.

Fractionation of tentacles crude extract

Membrane filtration was used for the fractionation of proteins that differ significantly in size. 500 µL of tentacle sample was added to the 10 kDa Nanosep devices and after 20 min of centrifugation at 6000g the filtrate was transferred from the bottom receiver to a new tube for storage. The sample with low molecular mass protein was filtered through the membrane, collected and stored at -20 °C as Down Fraction Tentacle Extract (DFTE)

Separation by HPLC chromatography

DFTE was subjected to size exclusion chromatography using BioSuite 250, 10 µm SEC, 7.5×300 mm column (Waters) on a HPLC system (Shimadzu Scientific Instruments, North America). The column was washed two times with 30 ml of TBS. 200 µL of DFTE was injected into the column and elution was carried out with TBS at a flow rate of 1 mL/min for 30 min. The chromatogram was recorded with a UV detector at 280 nm (mAU). The collected fractions were concentrated by a micro-concentrators (3K Omega Centrifugal Devices Nanosep). The recovered sample was analyzed

using Reverse Phase C18 column on HPLC and showed a single peak named Reverse Phase Tentacle Extract (RPTE).

SDS PAGE for separating small peptides

Crude extract from tentacles, DFTE and RPTE were analyzed in SDS-PAGE (16 % acrylamide) following a procedure for separating small peptides on polyacrylamide gels according to BioRad Mini-protean II protocol. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 (Himedia Laboratories, India) and destained in a solution of 50 % (v/v) methanol and 10 % (v/v) acetic acid. Samples were subjected to electrophoresis using a Running buffer (0.025 M Tris-HCl, 0.2 M glycine and 0.1 % SDS at pH 8.4) at 80 V at room temperature for 1.5 h. At 80 V approximately 10 mA current per gel was generated at the beginning of the run that dropped slowly to about 3 mA. Using these conditions, it was possible to maintain the running buffer and gels at room temperature during the entire migration.

Sequence determination

After Coomassie Blue dye staining, acrylamide gel bands were excised and send to BMS Mass Spectrometry and Proteomics Facility of St. Andrews University (UK) to perform sequencing.

N-gel digestion

The gel bands were excised and cut into 1 mm cubes. Then these were subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Digilab) following standard protocols (Shevchenko *et al.*, 1996). Briefly gel cubes were destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37 °C. The peptides were extracted with 10 % formic acid.

MALDI TOF/TOF analysis

The digest solution (0.5 µL) was applied to MALDI target along with alpha-cyano-4-hydroxycinnamic acid matrix (0.5 µL, 10 mg/mL in 50:50 acetonitrile:0.1 % TFA) and allowed to dry. MALDI MS was acquired using a 4800 MALDI TOF/TOF Analyser (ABSciex) equipped with aNd: YAG 355 nm laser and calibrated using a mixture of peptides. The obtained sequence was analyzed in the Swiss-Prot or NCBI nr databases.

Peptide synthesis and hemolysis assay

After sequence identification the peptide Ael (Accession: AAB46904.1) was synthesized from GenScriptservice and hemolytic activity was tested toward sheep erythrocytes (RBCs) obtained by Istituto Zooprofilattico della Sicilia in Alsever solution (0.42 % NaCl; 0.08 % C₆H₅Na₃O₇; 0.045 % C₆H₈O₇; 2.05 % D-C₆H₁₂O₆; pH 7.2). Erythrocytes were washed with PBS (KH₂PO₄ 6mM; Na₂HPO₄ 0.11 M; NaCl 30 mM; pH 7.4) and centrifuged at 400g for 10 min at 4 °C. Supernatant was removed and the cells were re-suspended in TBS gel 0.1 % to make a 1 % suspension.

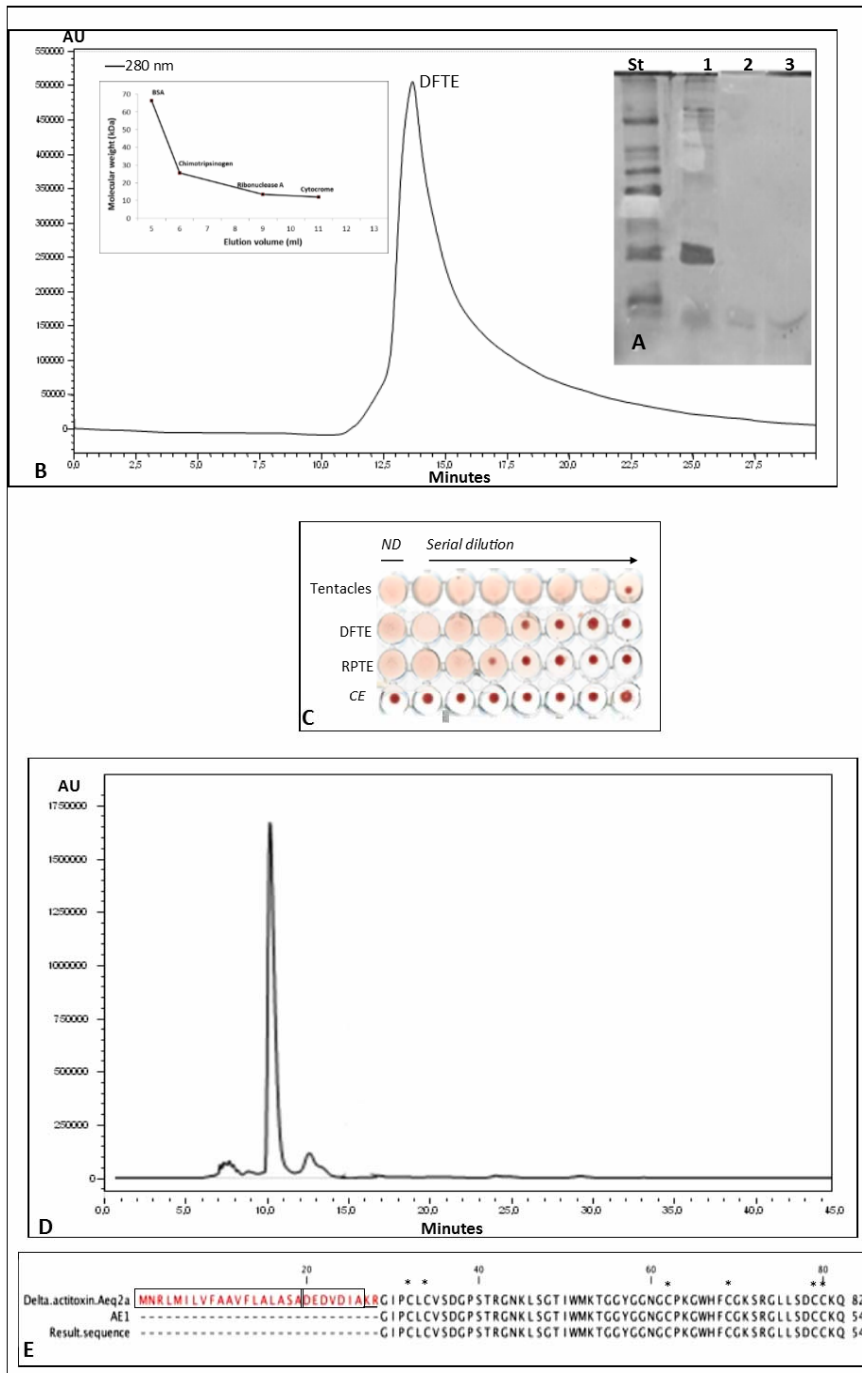


Fig. 1 A) Coomassie stained SDS-PAGE gel for separating small peptides of *A. equina* tentacles tissue acid extract (Lane 1), DFTE extract fractionated by 10 kDa Centrifugal Nanosep (Lane 2) and purified fraction RPTE (Lane 3); B) Chromatogram of the DFTE sample separated on the size exclusion column (BioSuite 250). Elution with TBS at a flow rate of 1 mL/min for 30 min. Absorbance was monitored at 280 nm. BSA, Chymotrypsinogen, Ribonuclease A and Cytochrome have been used as standard; C) Microplate lysis assay toward Sheep erythrocytes. Total tentacles extract (tentacles), DFTE and RPTE showed lysis at the concentration of 0.900 $\mu\text{g/ml}$, 0.510 $\mu\text{g/ml}$ and 0.430 $\mu\text{g/ml}$ respectively. Ce: Control experiment carried out with RBCs and buffer in TBS gel 0.1 % lysates are serially diluted twofold and incubated with an equal volume of 1 % sheep erythrocyte suspension for 1 h at 37 °C. After centrifugation the absorbance of the supernatants was determined at 545 nm. The values were at least the mean of three experiments, each performed in duplicate; D) HPLC chromatograms of *A. equina* DFTE using Reverse Phase C18 column on HPLC that show a single peak (RPTE); E) Sequencing of purified sample by reversed phase HPLC RPTE by N-gel digestion/ MALDI ToF analysis. Blast searching showed a correspondence with the Ae1 toxin (AAB46904) and Delta-actitoxin-Aeq2a (Q9NJQ2.1). Aminoacid in boxes are referred to signal peptide and pro-peptide. Mature peptide extending from position 29 to 82 is mature peptide. Asterisks indicate position of cysteines involved in three disulphide bridges.

For the microplate assay, 25 μ L of fraction purified by HPLC or serial (two-fold) dilution was mixed with an equal volume of RBCs suspension in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark). After 1 h incubation at 37 °C the lytic activity was recorded as the reciprocal of the highest dilution showing complete RBCs lysis. For the quantitative hemoglobin release evaluation, 500 μ L of synthetic peptide (0.5 mg/ml) in TBS was mixed with an equal volume of freshly RBCs suspension (8×10^6 cells) in PBS. The mixture was incubated with continuous and moderate shaking at 37 °C for 2 h and centrifuged at 1500g for 5 min at 4 °C. The supernatant was separated and the amount of released hemoglobin (Hb) was estimated by reading the absorbance at 545 nm in spectrophotometer. The degree of hemolysis was determined according to the equation:

$$\% \text{ hemolysis} = \frac{\text{Measured release} - \text{spontaneous release}}{\text{Complete release} - \text{spontaneous release}} \times 100$$

Complete hemolysis was estimated suspending erythrocytes in distilled water. For each experiment three samples were assayed. In addition, after carrying out cytotoxicity assay, erythrocytes were recovered by centrifugation at 112g for 10 min at 4 °C and lying on glass slides to be observed with phase contrast microscope.

Results

Electrophoretic analysis

Crude extract components from *A. equina* tentacles and the DFTE were analyzed in SDS page and reported in Fig 1A (lane 1 and 2). A procedure for separating small peptides on polyacrylamide gels has allowed the identification of several bands in lane 1 and a band with a low molecular mass (<6kDa) in lane 2.

Separation by HPLC of low weight protein

The DFTE was analyzed by size exclusion chromatograph yielding the chromatogram shown in Figure 1B. The chromatogram showed a single peak identified at 11 min of the elution time.

The recovered sample was analyzed using Reverse Phase C18 column on HPLC and showed a single peak (RPTE) reported in Fig. 1D corresponding to a 6 kDa band approximately (Fig. 1 A, lane 3).

Hemolytic activity

The results of microplate assays (Fig. 1C) showed the presence of hemolytic activity toward RBCs up to 1:64 serial dilution for crude extract of tentacles and to 1:8 for the DFTE (0.572 Abs) and RPTE (0.482 Abs).

Determination of sequence and synthesis of the hemolytic polypeptide

N-gel digestion and MALDI TOF analysis of RPTE showed several partial peptide masses which, by post source decay analysis and database searching, were attributed to one protein of 5.5 kDa molecular weight. Protein database search confirmed

that the complete sequence corresponded to Ae1 toxin (AAB46904.1) and Delta-actitoxin-Aeq2a (Q9NJQ2) isolated from *A. equina* (Fig. 1E).

Cytolytic activity of synthesized neurotoxin Ae1

Figure 2A showed cell membranes lysis and the presence of erythrocyte ghosts after treatment of RBCs with the synthetic Ae1 peptide.

The synthetic Ae1, starting with final concentration of 125 μ g, was assayed for its hemolytic activity by a spectrophotometric quantization. Lysis was recorded and after 1 h of incubation in polyethylene tubes a value of 58 % hemolysis was evaluated and the level of cytotoxicity decreased in a dose dependent manner (Figs 2B and C). As shown in Figure 2B, a visible hemoglobin release is present in tube that contained the synthetic hemolysin. It was almost transparent in the control. The time course of the reaction indicated a lysis after 10 min, but the stable value was obtained after 1 h (data not shown). The statistical evaluation was performed with one-way analysis of variance (ANOVA), and different groups were compared by using Tukey's t-test. Standard deviations were calculated on three experiments. $p < 0.05$ was considered statistically significant.

Discussion

In sessile Anthozoa, cytolytic polypeptides have been classified at least in four groups. They were classified based on their molecular weight in a) 5 - 8 kDa peptides with antihistamine activity, b) ~20 kDa pore-forming proteins with activity of lysis versus cell membrane inhibited by sphingomyelin, c) ~30-40 kDa cytolysins with or without phospholipase A2-activity, and d) a putative group of proteins represented at present only by a 80 kDa (Stabili *et al.*, 2015). Two most known molecules isolated from the sea anemone *Actinia equina* are isoforms of Equinatoxin II (EqII).

Toxins can act on common sites or on different subtypes of specific channels for Na^+ showing toxic and biologically significant differences (Bosmans and Tytgat, 2007). In this work, a neuropeptide from acid (10 % acetic acid) extract of *A. equina* tentacles has been isolated by HPLC and the lytic capability against mammalian erythrocytes has been demonstrated.

The tentacles crude extract exhibited many bands in SDS page and resulted mainly enriched in the component of 20 kDa correspondent to cytolysin Equinatoxin II (EqII). Instead, a new hemolysin of about 6 kDa was isolated by filtration and size exclusion and reverse phase HPLC. Following mass spectrometry, the low molecular weight hemolysin was characterized, sequenced and identified as a Na^+ channels neurotoxin (Ae1) (Lin *et al.*, 1996).

The neurotoxic lethal activity to crab Na^+ channel toxin (Ae1) was distinguished from cellular lytic activity suggesting that "in gel filtration on Sephadex G50 of crude extract toxin(s) with lethal activity to crab was distinguished from hemolysis reasonable assumed to be equitoxins the former appears at around fraction 26 and the latter fraction 21" (Lin *et al.*, 1996).

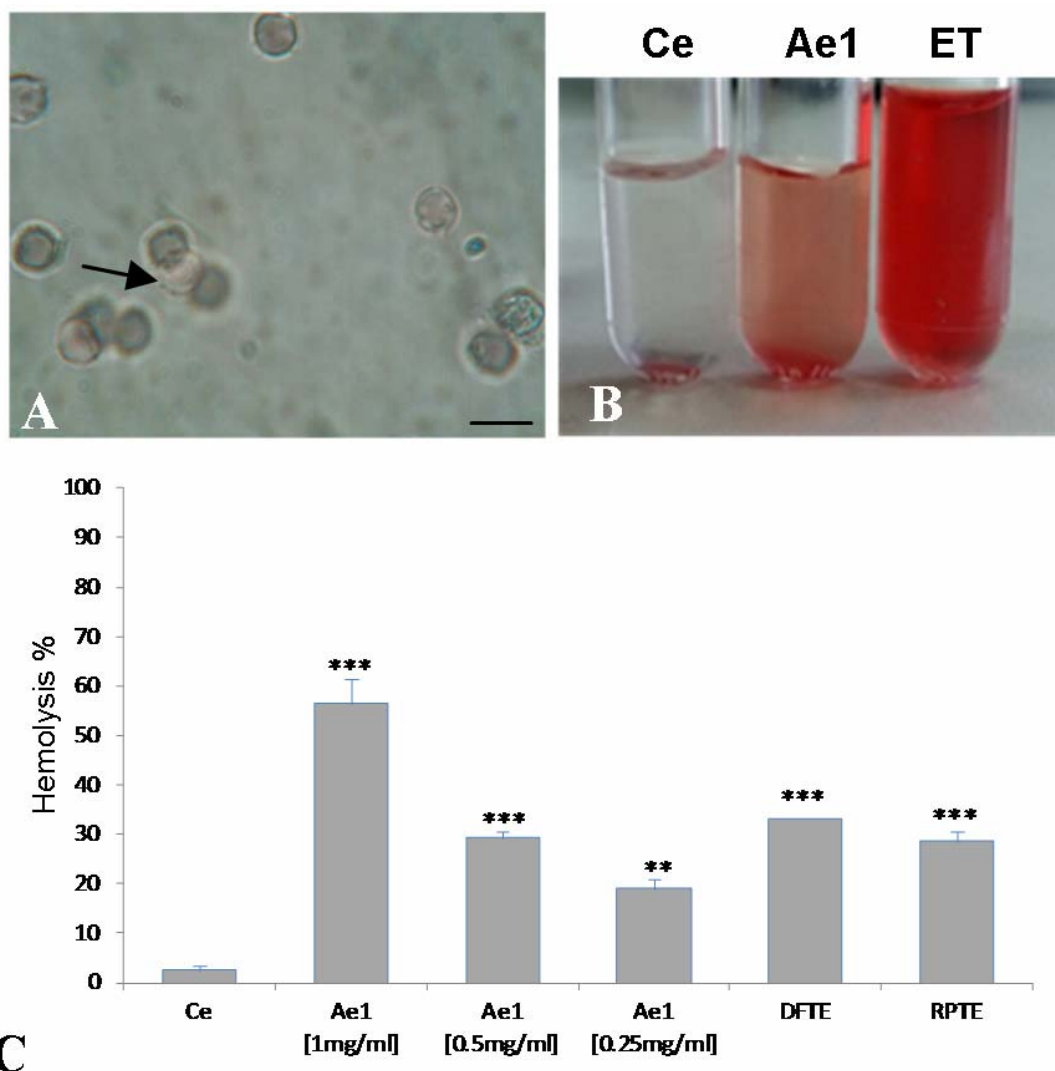


Fig. 2 A) Sheep erythrocytes (RBCs) observed at the light microscope after treatment with synthetic peptide Ae1 (125 µg/ml). Arrows indicate the ghosts of erythrocytes. (Bar: 10 µm); B) Release of hemoglobin after 30 min of incubation with synthetic peptide. Control (Ce), total lysis in distilled water (ET) and Lysis after treatment with synthetic peptide Ae1 (125 µg/ml); C) Hemolytic activity evaluation of synthetic Ae1 from *A. equina* toward RBCs in TBS at different concentrations. Ce: control of erythrocytes suspended in TBS; The values were at least the mean of three experiments, each performed in duplicate. ** $p < 0.01$ and *** $p < 0.001$ respect to the control.

In this study a hemolysin with low molecular weight from acid tissue tentacle extracts of sea anemone *Actinia equina* was isolated by HPLC starting with a <10 kDa fraction in which the equinotoxin (20 kDa) was clearly separated from Ae1 containing fraction.

In order to verify the hemolytic activity of this identified neurotoxin by sequencing, a synthetic peptide was also produced. The results of quantification assay for hemoglobin release by red blood cells lysis indicated that, the synthetic peptide AE1 shows also hemolytic activity as well as the purified hemolysin.

Since voltage-gated Na⁺ channels are responsible for the conduction of electrical impulses

in most excitable tissues in the majority of animals, they have become important targets for the toxins of venomous animals, from sea anemones to mollusks, scorpions, spiders and even fishes (Wanke *et al.*, 2009).

The discovery of cytolytic activity in addition to inhibitory effect could support the hypothesis of evolutionary trend to combine two venomous functions in one compound: ion channel inhibitor and membranolytic activity. Another example of neurotoxic/cytotoxic proteins resides in the components isolated from the sea anemones *Stichodactyla mertensii* and *S. haddoni* (Veeruraj *et al.*, 2008) in which hemolytic activity was estimated toward several types of erythrocytes.

Moreover, molecules with variable functions are found in animals including vertebrate could also take advantage of the multi-functionality of some of their bioactive molecules (Ovchinnikova *et al.*, 2006; Peigneur *et al.*, 2011; Trapani *et al.*, 2014).

The unique feature of a multiple role within one molecule is intriguing. The production of toxins is an energy demanding task for an organism and the dual effects contained within one molecule can be seen as an efficient and economic manner to save energy with a maximal pharmacological output (Trapani *et al.*, 2016). Neurotoxin-like protein with cytotoxic activity against different human cancer cell lines was purified from the venom of the snake *Naja haje* (El Hakim *et al.*, 2008). Its mechanistic role in the cell death was explored too. CsTx-1, the main neurotoxic acting peptide in the venom of the spider *Cupiennius salei* possess membranolytic activities caused by its C-terminal tail (Nentwig *et al.*, 2012). This peptide exhibits neurotoxin function because it inhibits L-type Ca²⁺ channels and acts as a lytic peptide toward prokaryotic and eukaryotic cell membranes.

Evolution has ensured sessile animals as sea anemones to develop a cysteine-rich peptides capable of affecting different extracellular sites of channel proteins. The venom in fact is abundant of low molecular mass neurotoxins with differences in structure, site of action and target preference.

The mechanisms enable specimens to subdue a broader range of prey even if some of them do not express specific ion channels that are targeted by neurotoxins. At the same time this mechanism could prevent the development of resistance to a single venom compound. Although the cytolytic mechanism of action is unknown, the hypothesis of combination of two venomous functions in one compound is certainly an evolutionary fascinating strategy.

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