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Review

Small peptides, big world: biotechnological potential in neglected bioactive peptides from arthropod venoms¶

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Abstract: Until recently, a toxinologist's tasks involved the search for highly toxic or lethal toxins in animal venoms that could explain the harmful effects in clinically observed symptoms. Most of these toxins were put on evidence using a function to structure approach, in which a biological phenomena observation usually guided the isolation and characterization of the causative molecule. Paving this way, many toxins were promptly purified because of their readily observed effect. Nevertheless, small molecules with micro-effects that are not easily visualized can be relatively neglected or poorly studied. This situation has changed now with the advent of the sensitivity, resolution and accuracy of techniques such as mass spectrometry and proteomic approaches used in toxinology. Taking advantage of these methodologies, small peptides with 'newly exploited' biological activities such as vasoactive, hormone-like, antimicrobial and others have been recently given much more attention, enlarging the known repertoire of bioactive molecules found in animal venoms. This article aims to review current knowledge on small biologically active peptides (<3 kDa) found in arthropod venoms and discuss their potentialities as new drug candidates or therapeutic lead compounds.

Keywords: bioactive peptides; arthropod venom; Bradykinin potentiating peptides; antimicrobial peptides; hormone-like peptides

INTRODUCTION

Animal venoms have been broadly recognized as one of the main sources of biologically active molecules. In fact, animal venoms are a successful evolutionary outcome that has evolved differently along Metazoa Phylum in both predatory and defense senses. To illustrate this extraordinary repertoire, Theakston and Kamiguti [1] have listed more than 2500 animal toxins and other natural products that showed any biological activity. This number tends to increase enormously, since it has been demonstrated that the number of molecules can easily reach 50–300 in each venom, although many of them are still unknown to date [2–9].

Such a richness can be useful to biotechnology in many ways, with the prospection of new drug candidates or new chemical entities – that can be used as therapeutic lead compounds – being the most promising. A rough classification of toxins can be made on the basis of their chemical nature (proteins, glycoproteins, peptides, alkaloids, polyamines, biogenic amines and others), their pharmacological or biological effects (neurotoxins, myotoxins, vasoactive peptides, hemolytic, cytolytic, necrotic, hemorrhagic, anti-inflammatory, antitumoral, analgesic, antimicrobial and others), their molecular level effects (ionic channel ligands, agonists or antagonists of ionotropic or metabotropic receptors, enzymes, enzymatic inhibitors, others) and, finally, on the basis of their sub-molecular binding sites (such as α- and β-toxins from scorpion venom that bind sites 3 and 4 respectively, from sodium channels).

It is interesting to note that among the myriad of molecules that can be found in animal venoms, many of them have their molecular level actions related to receptors and enzymes, which constitute the two main classes of targets for drug action [10]. It is also noteworthy that many of the worldwide top-selling pharmaceutical products are natural products or synthetic and semisynthetic analogs of natural products [11], and yet, proteinaceous molecules and their scaffold templates are poorly exploited.

In this review, attention has been paid to small peptides (up to 3 kDa) from arthropod venom sources that act as hormone-like, vasoactive or antimicrobial substances and, for that reason, can be envisaged both as potential drugs and as lead compounds by the pharmaceutical industry.

AN OVERVIEW ON NEGLECTED PEPTIDES

Small peptides such as hormones, neuropeptides, cytokines and enzyme inhibitors play a key role in many

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physiological and regulatory processes that maintain the steady-state of the organism but, surprisingly, have been put aside from many proteomic projects [12]. However, the interest in these small structures is increasing as the access to microscale analytical technologies, such as mass spectrometry, and to peptide synthesis is becoming affordable.

Frequently, small peptides are represented in very low concentrations in the venom of arthropods and it is a tedious and cumbersome task to gather enough material to be analyzed by conventional analytical techniques. Therefore, technical difficulties in addition to the poorly visualized toxicological effects, inherent to many small bioactive peptides, were responsible for toxinologists neglecting some of these molecules for many years.

With modern analytical platforms, very small quantities of raw material are needed to profile a given venom, with the possibility to sequence small peptides by using a combination of liquid chromatography coupled with mass spectrometry (LC/MS) and liquid chromatography coupled with tandem mass spectrometry(LC/MS/MS), for example. Chemical synthesis of these peptides enables further characterization of their biological activities in a subsequent step. Taking advantage of these techniques, many unfolded or poorly reticulated peptides have been found in arthropod venoms and have attracted attention because of their potential use in biotechnology both for the biological roles they perform and as they can be easily synthesized and engineered.

Despite the fact that the main efforts to prospect new peptide scaffolds and functions in arthropod venoms have focused on ion-channel targeting proteins, a great potential of small peptides (<3 kDa) can be perceived in proteomic studies of these venoms [2,5,6,8].

VENOM PEPTIDOMICS AND STRUCTURE TO FUNCTION APPROACHES

Although there is no clear definition about the ranging size of a peptide, terming small proteins up to 10 kDa as peptides is commonly acceptable. In the scope of this review, peptidyl molecules up to 3 kDa are defined as small peptides. It is interesting to note that within this molecular mass range, peptides can be easily subjected to MS/MS and/or Edman's automated sequencing, which facilitates the primary structure identification and posterior synthesis.

Obtaining a molecular mass list (venom mass fingerprinting), which functions as a static image of the multicomponent venom, has become a good practice in toxinology (Figure 1). Direct analysis using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS or coupling LC/MS, in both off-line or on-line modes, have been used to photograph whole venoms [2,5,6,9,13–15]. Such photos produce valuable information on structural families that are present in the studied venom.

In some cases, the first passage by a MS technique gives enough resolution to perform MS/MS *de novo* sequencing without the necessity of LC fractionation. Alternatively, venom can be subjected to LC techniques prior to MS analysis and MS/MS sequencing to increase resolution and MS signal gaining (Figure 1).

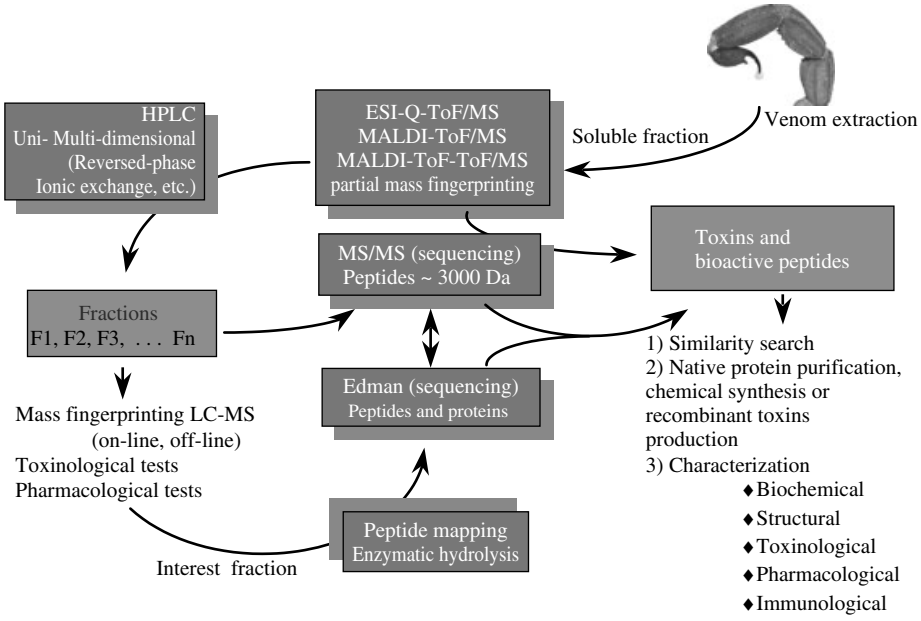

Figure 1 Workflow chart with methodological strategies on the animal venom peptidomic approach for prospecting bioactive molecules.

De novo sequencing by MS/MS has become one of the main analytical techniques to overcome difficulties in both raw material gathering and peptide isolation. By this technique, e.g. Konno and coworkers [16] used only two venom sacs from the solitary wasp *Cyphononyx dorsalis* to sequence two novel peptides (Cd-125 and Cd-146) and the known peptide Thr6-Bradykinin. Sequences were corroborated by Edman's degradation and by solid-phase synthesis. More recently, Mendes and coworkers [17], by using the LC/ESI-MS/MS (Electro-spray ionization mass spectrometry) technique, were able to sequence two novel chemotactic and mastoparan peptides, named *Agelaia-CP* and *Agelaia-MP*, which occurs in a low-level concentration in the venom of the social wasp *Agelaia pallipes pallipes*. Many other authors report the use of MS/MS techniques to sequence low abundance peptides from the whole venoms of arthropods [6,18,19], conus shells [20,21], amphibian skin secretions [22] and snakes [23] followed by chemical synthesis for further biological characterizations.

BRADYKININ POTENTIATING PEPTIDES

Among the bioactive peptides found in animal venoms, the Bradykinin Potentiating Peptides (BPPs) family is by far the one that has received most attention over the past decades. The history of this peptide family began late in the 1960s when it was first observed that *Bothrops jararaca* venom was able to enhance the Bradykinin (BK) hypotensive effect [24]. The BPPs were verified to act as Angiotensin Converting Enzyme (ACE) inhibitors and were used as a novel prototype of antihypertensive drug, leading to the development of captopril [25], the first commercial ACE inhibitor and probably one of the most successful examples of peptide as a lead compound in the pharmaceutical industry.

Since then, many other BPPs were described in different venoms from arthropods, amphibians and snakes, most of them being ACE inhibitors (as reference see [1]). In the case of BPPs isolated from snake venoms, these molecules are recognizable by a common structural pattern (Pyr-$EX_nPXPXIPP$, where Pyr is pyroglutamic acid and X is any amino acid residue), with the *C*-terminus sequence PXIPP crucial for the binding in the ACE catalytic site (for review see [23] and [26]).

Surprisingly, many arachnid venoms have also been reported to contain BPPs, although they do not share the common structural pattern observed for snake venoms, except by the fact that proline residues can be found at the *C*-terminal ending. Ferreira *et al.* [27] have isolated and characterized a molecule from the venom of the Brazilian scorpion *Tityus serrulatus* that was verified to potentiate the effects of BK on the isolated guinea pig ileum preparation and on arterial blood pressure in the anesthetized rats. Also, this peptide inhibited the hydrolysis of both BK and angiotensin I using *in vitro* assays. Interestingly, the described sequence has high homology with the *N*-terminal portion of TsIV, or Tityustoxin, an α-toxin known to inhibit the Na^+ current inactivation, prolonging the action potential [28]. Structure-function studies are in progress in our laboratory using peptide synthesis envisaging a deeper knowledge on this structural convergence, the results will be published later on. Shortly after, Meki *et al.* [29] put on evidence a peptide (peptide K12) found in the venom of the Egyptian scorpion *Buthus occitanus*, which acts as BK potentiator in smooth muscle preparations, by inhibiting ACE. The peptide K12 is 21 residues-long and does not share the main structural features of snake BPPs, except by the presence of two proline residues at the *C*-terminal region, which ends with an additional alanine residue. A family of linear peptides that was able to potentiate the BK hypotensive effect was put on evidence by our group [30]. These peptides named *Tityus serrulatus* Hypotensins (TsHpT) have molecular masses ranging approximately from 1190 to 2700 Da and are somehow related to peptide K12, but with unique structural features. Structural and functional studies on this family of peptides will be published later on. Other arachnid BPPs – with unique structural features – were reported to be found in the venom of the scorpions *Buthus martensii Karsch* [31] and *Leiurus quinquestriatus* [32] and spiders *Scaptocosa raptoria* [33,34] and *Latrodectus tredecimguttatus* [35].

ANTIMICROBIAL PEPTIDES

If, on the one hand, BPPs were the first deeply observed family of small peptides in animal venoms, on the other hand, antimicrobial peptides may be considered as the current excitement of toxinology in the recent years. This is partially an outcome from a gold rush to discover new chemical entities and scaffolds to deal with emerging bacterial resistance [36] and partially because it is becoming widely accepted that peptides are an essential part of the innate immune system, and therefore could be potentially used as antimicrobial therapeutics [37].

Antimicrobial peptides can be classified into three families according to different structural features: (i) α-helical linear peptides, (ii) disulfide-bridged cyclic and open-ended cyclic peptides and (iii) peptides whose primary structures have a high content of some amino acid residues (e.g. proline, glycine or histidine rich). Most of these peptides adopt an amphipathic structure with both cationic and hydrophobic properties that facilitate their interaction with anionic cell walls and membranes of microorganisms. Antimicrobial peptides usually display a broad spectrum of action against gram-positive and gram-negative bacteria, fungi and

protozoa, and some of them have highly hemolytic and insecticidal effects [38,39].

Most of the antimicrobial peptides described so far are constitutive from hemolymph and only a few have been found in venom gland secretion from arthropods. Apart from some K^+-channel blocker toxins, found in scorpion venoms, which are structurally related to some defensins [5,40], all of the so far isolated antimicrobial peptides from arthropod venoms are linear amphipathic peptides [18,41]; for review see Ref. 38.

Four linear peptides (IsCT, IsCT2, IsCTf and IsCt2f) were isolated from the venom of the primitive scorpion *Opisthacanthus madagascariensis* [41,42]. The authors have shown that IsCTf and IsCt2f are, in fact, enzymatic fragments from IsCT, IsCT2. Interestingly, both IsCTf and IsCt2f have shown to loose their cytolytic activity and structure observed for IsCT, IsCT2, which are amidated at *C*-terminus and are able to adopt amphipathic structures in aqueous TFE solution. Other antimicrobial α-helical peptides were described from the venom of the scorpions *Hadrurus aztecus* (hadrurin), *Opistophtalmus carinatus* (opistoporin 1 and 2), *Parabuthus schlechteri* (parabutoporin) and *Pandinus imperator* (pandinin 1 and 2) [43–46].

From Hymenoptera venom, antimicrobial peptides have been described in wasps [19], bees [47] and ants [48]. The amino acid sequences of Anoplin – from the venom of the solitary wasp *Anoplius sumariensis*, and crabrolin, isolated from the venom of *Vespa crabro* – have high similarity with the mastoparan-X from *Vespa xanthoptera* venom and, in addition to the antimicrobial activity, these peptides have also been described as mast cell degranulators [19].

Antimicrobial α-helical peptides have also been described in the venoms of spiders. Antimicrobial peptides have been described from the venom of wolf spiders *Lycosa singoriensis* [18], *L. carolinensis* [39], *L. erythrognatha* [49] and *Oxyopes kitabensis* [50]. Kuhn-Nentwig and coworkers [51] put on evidence a series of linear peptides ranging from 3 to 4 kDa that display high antimicrobial, hemolytic and insecticidal activities.

HORMONE-LIKE PEPTIDES

An emerging and important facet of peptidomics in animal venoms is the crescent discovery of structures related to peptidyl hormones. Despite the fact that these low-represented small peptides do not display, in most cases, any important observed effect from the toxicological point of view, hormone-like peptides seem to have an important role in the disturbance of the steady-state of the inflicted victim. Together with antimicrobial peptides, hormone-like peptides are richly represented in amphibian skins (for reference see [1,22,38]).

An increasing number of BK-like peptides have been found in hymenoptera venoms. Venom from ants, social and solitary wasps have been reported to contain such peptides (for review see [52]). In the case of solitary wasps, it was previously shown that BK-like peptides (megascoliakinin and Thr6-BK) identified in the venom of the scoliid wasps *Megascolia flavifrons* and *Colpa interrupta* presynaptically block nicotinic acetylcholine receptors in the insect central nervous system [53,54]. By using MALDI-TOF MS, Konno and coworkers [55] surveyed the venom of 26 species of solitary wasps to assess the presence of kinins and they were able to positively assign the presence of Thr6-BK in four of them (*Cyphononyx dorsalis, Megacampsomeris prismatica, Campsomeriella annulata annulata* and *Carinoscolia melanosoma fascinata*). Because of this synaptic blockage, these authors postulated that BK-like peptides found in the venom of wasps play a crucial role in paralyzing action for capturing prey. Also, BK-like peptides cause severe pain when injected into vertebrate animals, thus playing an important role in the defense against predators [55].

Recently, our group reported the primary structures of 15 isoforms of Phonetachykinins (PhTkP-I to PnTkP-XV), a tachykinin-related family of peptides found in the venom of the aggressive spider *Phoneutria nigriventer* [6]. This family of peptides was first put on evidence in the early 1990s when a fraction issued from gel filtration chromatography of the whole *P. nigriventer* venom, named *Fraction M*, was verified to be able to induce smooth muscle contraction in guinea pig ileum preparations [56]. Although there was an initial interest to further work on this fraction at the time, attempts to characterize these peptides were largely unfruitful, both because of their low levels in the whole venom and because their *N*-termini were somehow blocked preventing the sequencing by Edman degradation. This puzzle was recently solved by using MS/MS *de novo* sequencing when all isoforms containing a pyroglutamic acid residue at *N*-terminus was verified [6]. Chemically synthesized peptides were constructed to both corroborate the primary structure and to better characterize their pharmacological properties (to be published elsewhere). Tachykinin-related peptides are widely distributed along the phylogenetic scale and have been found in other animal venoms (for reference, see [1]).

PEPTIDES TO DRUGS

Many aspects have to be observed to fulfill all requirements needed for a drug candidate to finish as a drug-store shelf product, including storage and *in vivo* stability, administration device and its suitability via, toxicology, immunological aspects and, of course, pharmacodynamic and pharmaco-kinetic aspects. Although a

deeper discussion of these aspects is not in the scope of this review, attention is drawn to *in vivo* stability and administration which are two of the potential problems of using peptides as drugs.

Peptides found in animal venoms are usually secreted with an aim to reach a variety of targets within the inflicted organism. For that reason, *in vivo* chemical stability of such molecules has been tested by millions of years of evolution in bloodstream and other circulatory systems of invertebrate animals. To achieve such stability, toxins from animal venoms rely on some known posttranslational modifications such as disulfide bridges [57,58], glycosylation [59] and modified amino acid residues such as cyclization of glutamine into pyroglutamic acid [6,23], amidation of *C*-terminal residue [4,6,8,16–19,39,49,57], acetylation [60] and others. Some other primary structure features can also act as a protective shield against enzymatic cleavages. This is promptly observed in proline rich peptides or by duets of aminoacid residues such as adjacent positive-negative charges (Pimenta *et al.*, unpublished data). Amidation of *C*-terminal residue has also been demonstrated to be important in enhancing biological activity [61]. Besides stability, the potency of the effect and specificity of the target may have contributed to the success of such small peptides as toxins. These factors would make it possible for labile peptides to achieve a strong biological effect despite a rather short half-life in the bodily fluids.

Mimicking peptides or chemically modifying the peptide structure can be of crucial importance to increase *in vivo* stability of a proteinaceous drug. In this way, the use of noncanonical amino acid residues has been shown to be an interesting alternative [37,62]. To protect the protein structure, mimicking the effect obtained by glycosylation rendering potential enzymatic sites hidden, e.g. PEG-conjugation, liposomes or micelles microreservoir delivery systems or cyclodextrin-coated proteins were found to be advantageous [63–66].

Proteinaceous drugs need suitable delivery systems in order to optimize absorption and bioavailability. Injections using hypodermical needles have been used for many years by insulin-dependent diabetic patients, while recombinant interferons (Intron®, Roferon®, Avonex®, Rebif® and Betaferon®) have also been administered by injection route in patients suffering from a variety of diseases, from viral infections to cancer and multiple sclerosis [64]. In many cases, injections must be given on a daily basis leading to a high percentage of negligence in treatment. Painless and easier methods for the administration of peptidyl or proteinaceous drugs have been described with relative success: oral, nasal, pulmonary, transdermal patches, microorganisms and plant delivery systems [63,65–71].

CONCLUDING REMARKS

Venoms and toxins from arthropods constitute rich sources of molecules with high a therapeutical and biotechnological potential, since many of them have receptors, membranes and enzymes as primary molecular targets. The use of proteomic approaches and mass spectrometry has given a new dimension to toxinology, increasing the possibilities of new bioactive peptides that were neglected because of their low toxicity or because of the lack of visible biological activity to be discovered and characterized.

As compared to well-established state-of-the-art ion channel-targeting toxins, the structural and biological activity characterizations of small peptides from arthropod venoms are in their early stages. Only recently, attention has been drawn to the biotechnological potential of these molecules. The turning point for this was the increasing capabilities of prospecting and characterizing such small molecules from few amounts of starting raw material, achieved by microscale analytical techniques and the recognition by pharmaceutical industries that animal venoms can be a valuable source of new drug candidates and novel scaffolds for lead compounds. Also, the increasing research and application efforts to improve peptidyl and proteinaceous drug delivery systems are noteworthy.

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