Venom Constituents of Rattlesnake Venoms

Subjects: Toxicology | Biochemistry & Molecular Biology

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Venom components are invaluable in biomedical research owing to their specificity and potency. Many of these components exist in two genera of rattlesnakes, *Crotalus* and *Sistrurus*, with high toxicity and proteolytic activity variation.

Keywords: toxin ; venom composition ; *Crotalus* ; *Sistrurus*

1. Introduction

Biomedical research on venom components is invaluable in developing therapeutic strategies owing to their specificity and potency [1]. Pharmacologically significant venomous snakes are mostly front-fanged and fall within three families, namely, *Atractaspidae*, *Elapidae*, and *Viperidae* [2], with a high amount of variability in the composition of their venoms. Such variation in biochemical composition can occur amongst closely related species and within species [3][4][5][6][7]. For example, geographical variation in the venom of pit vipers and adders has been correlated to their diet [4][5][8][9] or topographical features [10][11]. Venom composition variability can be intra-genus [12] or intraspecific [4]. Intraspecific venom compositions vary in their lethality (LD50), thus resulting in varying symptomatology and confused diagnosis or ineffective antivenom treatments, amongst other medical complications, during medical applications [4]. One example is during the treatment of *C. basiliscus* envenomation, which requires many different antivenoms to neutralize specific toxins in the varying venom compositions of just one species [13]. Deshwal et al. (2021) recently explored the variation in snake venom using meta-analysis to tease apart the relationships between different *Crotalus* and *Sisturus* venom components, which could be helpful in biotechnical and biomedical advancements [14]. The diversity observed in snake venom is often due to the recruitment strategy and duplication of toxin-encoding genes [15][16][17][18][19], followed by functional and structural diversification [1][20][21][22][23][24]. The phenomenon of venom diversification occurring at a high rate is supported by the hypothesis suggesting that venom is used for predation [4][22][25][26][27] and prey digestion [4]. Other studies have indicated that prey specificity is not the only driver of the venom diversification within *Crotalus* and *Sistrurus* [28], which is further supported by the studies demonstrating the differences in the venom composition between species despite having similar prey preferences [28][29][30].

Kocholaty et al. (1971) suggested that *Crotalidae*'s venom has the highest toxicity variation with a high proteolytic activity [31]. Rattlesnakes are within the subfamily *Crotalinae,* consisting of two genera, *Crotalus* and *Sistrurus*, with approximately thirty-six species [32]. Their habitats within the Americas range from southern Alberta, Saskatchewan, and southern British Columbia in Canada to central Argentina in a myriad of habitat types: from the Sonoran Desert of northwestern Mexico to alpine and cloud forests in central and southern Mexico [32]. This high variability in habitat type, altitude, associated diet types, and extensive geographical range allows rattlesnakes to have a high variability in their venom composition [14].

The high variability in range and distribution, venom composition, and activity provides ample opportunities to explore the venom components and their properties from biomedical and academic perspectives. However, numerous published works on rattlesnake venoms' variability can sometimes hinder the understanding of critical relationships between different venom components [14].

2. Venom Constituents of Rattlesnake Venoms

2.1. Venom Component Activities and Targets

2.1.1. Disintegrins

It has been demonstrated that the DIS toxins derive from the protein family called A Disintegrin and Metaprotease (ADAM) in snake venoms [33][34]. Such ancestral relationships between cellular ADAMs containing the DIS-like domains and the DIS toxins have been studied in the literature [35][36]. DIS are small, non-enzymatic proteins [34][37][38] with members being

classified into five groups of various sizes and numbers of disulfide bonds: (1) short, (2) medium, (3) long, (4) DIS-like domains in P-III SVMP, and (5) dimeric [33][35][39][40]. The short DIS has four disulfide bonds and around 49–51 residues [34][35]. At the same time, medium-sized DIS have approximately 70 residues and six disulfide bonds [40][41], such as mojastin 1 and 2 [39]. The third group is 100 amino acids long, with eight disulfide bridges [33][34]. As mentioned previously, many P-III SVMPs, like CamVMPII or jarhaggin, contain a DIS-like domain, around 100 amino acids in length, with eight disulfide bonds [37][42][43][44]. Apart from the first four groups, the fifth classification contains both homodimeric and heterodimeric DIS [45][46]. Many of them can bind to cell receptors on many cell types called integrins, which allow extracellular adhesions that implicate cell–cell and cell-matrix interactions [40] needed for cell proliferation, migration, and survival [38], and effectively inhibit the activity of integrins [33][34][41]. Many single-chain DIS have the active tripeptide residues of RGD, such as atroxatin and mojasin [33][34][39][47]. Exceptions in tripeptide residues can be seen with KGD, MVD, KTS, ECD, VGD, MGD, or WGD motifs in other DIS [33][38][40]. The conserved aspartate residue is proposed to be a specific binding site to the β subunits of integrins. At the same time, the other two amino acids handle the binding affinity to the α subunit [48]. This tripeptide is at the top of the loop protruding from the protein core [33], which the integrins would recognize as their ligands [33][40]. For example, the integrins αIIbβ3 recognized both RGD- and KGD-containing DIS [33], and a particular motif like RGD can bind to not just one integrin target [44][47]. However, such recognition differs amongst DIS containing the same tripeptide residues [33]. It has been observed that the dimeric type of DIS exhibits the highest level of diversity in their tripeptide motifs [33]. The inhibition of integrins from these toxins may help the distribution of other venom compounds throughout the tissues [48]. Other reported activities of DIS are anti-angiogenesis [49], the inhibition of platelet aggregation induced by several factors like thrombin, ADP, and collagen [38][39][40], and the inhibition of cancer cell migration and colonization [38][44][50][51][52][53][54][55].

2.1.2. Cysteine-Rich Secretory Proteins

Cysteine-rich secretory proteins (CRiSPs) are a protein superfamily that has gained attention as a potential biopharmaceutical agent. CRiSPs are widely distributed in both genera of rattlesnakes [45][48][56][57][58][59] and have been crystalized and studied from snake venoms [60]. Members are single-chain peptides that contain approximately 230 amino acid residues [49][60], weigh 20–30 kDa [61][62], and contain a consistent pattern of 16 cysteines that are participating in internal disulfide linkages; hence the family name [60][61]. Modeled structures of CRiSPs revealed the two domains of the peptide: an N-terminal globular domain [49] and a C-terminal cysteine-rich domain, which contains 10 of the 16 cysteines [61][63], and a Zinc^{2+} binding motif [49][63]. However, reports of binding to Cd^{2+} have been noted [64]. The family possesses a wide array of biological interactions with ion channels with no precise main functions [63]: blocking ryanodine receptors [65], L-type calcium channels and/or potassium channels [61], and cyclic nucleotide-gated channels [63][66][67]. This binding can inhibit smooth muscle [68], which is its basal activity [36]. These interactions with different channels have been studied but require additional investigation [65][66]. Other intriguing activities from CRiSPs are anti-angiogenic activities [49], antiprotozoal activities (against *Trypanosomes* and *Leishmania*) [61], involvement within the inflammatory processes [65], and the inhibition of human umbilical vascular endothelial cell proliferation [57]. They appear to be non-toxic for mice and insects [49][61]. However, CRiSPs from *Philodryas patagoniensis* can produce mild myotoxicity when injected into gastrocnemius muscle without edema formation, inhibition of platelet aggregation, or hemorrhage [62]. The low toxicity to mammals and insects and the antiprotozoal activities could make CRiSPs a model for developing new pharmaceutical products [61].

2.1.3. C-Type Lectins

Lectins are non-enzyme and non-immune proteins that can bind to carbohydrates. Snake venom is abundant in calcium-dependent (C-type) lectins, which can be grouped into two populations: the true C-type lectins (CTL) and the C-type lectin-like (snaclecs) [69][70]. Lectins are similar in glycan-binding specificities, determined by the proteins' carbohydrate recognition domain (CRD) [71][72]. Such CTL domain has a Q-P-D tripeptides motif to determine the galactose specificities and is mediated by Ca^{2+} [73]. Snaclecs are often heterodimeric with CRD-like domains that cannot interact specifically with sugars [69][70]. In contrast, the true CTLs are homodimeric proteins with two identical disulfide-like subunits (around 15kD each), fully functional CRDs that bind carbohydrates and induce hemagglutination via surface glycoconjugates on erythrocytes [74][75]. Most CTLs' members are galactoside-binding proteins that bind to the terminal galactoside residues using calcium [76]. Although only one rattlesnake lectin from *C. atrox* has been crystalized [77], many species within the *Crotalus* genus have been shown to obtain transcripts [46][78][79][80] and express CTLs [2][79][81]. Many lectins isolated from snake venoms share a high identity degree of 82–97% on the amino acid level, indicating a similar primary structure [76]. Secondary structures of CTLs seem to possess multiple β sheets and a couple of α helices [76][77]. Aside from the hemagglutination effects of CTL, some snake venom lectins can also induce mitogenesis of different cell types [82][83], while others cannot [84]. Similarly, some CTLs can induce platelet aggregation by a proposed mechanism of glycan recognitions on platelet surfaces that induces aggregation by CTLs [83][85], while others cannot [76][85]. Such variety in

biological effects may be due to the carbohydrate specificity of lectins and surface receptors [82]. Other notable activities of CTLs are pro-inflammatory activity (Lomonte et al., 1990), renal toxicity [76], and some propitious activities such as antibacterial [86][87] and anti-tumoral activities in various cell lines [88][89][90], thus providing new avenues for researching this minor venom component.

2.1.4. Bradykinin-Potentiate Peptides

Most toxins with low abundance in snake venom are vasoactive peptides [91], such as bradykinin-potentiate peptides (BPP). BPPs are described as pyroglutamyl proline-rich oligopeptides, 5–14 residues in length (~1 kDa), and with a conserved C-terminus rich in prolines [92][93][94][95]. As the name implies, BPPs can potentiate the bradykinin actions on various organs [96][97]. Such effects lead to hypotensive reactions in many organisms [94][97][98][99] due to the inhibition of bradykinin degradation [94][96][98]. The inhibition may also be accompanied by hyperpermeability of the blood vessels and loss of consciousness because of the potent hypotension [36]. Along with this inhibition, BPPs can also inhibit the conversion of angiotensin I to angiotensin II, the active form of angiotensin, creating a crucial pathway to develop angiotensin-converting enzyme (ACE) inhibitors due to the anti-hypertensive activities these effects induce [94][100][101]. The product called captopril, an ACE inhibitor for treating hypertension and heart failure, is a prime example of a commercialized drug based on snake venoms [93][100]. Recent reports have indicated that there is still much to learn from BPPs, such as the ability to distinguish between the N or C-terminal catalytic domains of ACE [102][103], or the discovery of ACE-independent mechanisms to reduce blood pressure [98][102]. Additionally, attempts to study the biogenesis of BPP have been conducted, and a report of a precursor polypeptide containing multiple sequences for BPP in tandem with a sequence of C-type natriuretic peptide (CNP) at the C-terminal was noted [92][93][104]. However, the processing mechanisms for this precursor are still elusive [92]. Thus, renewed interest in this vasoactive peptide has regained momentum with new BPPs isolated from many different snake species [94], many of which are from rattlesnakes [48][58][92][93][94][105].

2.1.5. C-Type Natriuretic Peptides

Many precursors of BPPs contain another vasoactive peptide, the C-type natriuretic peptide family [92][93][104]. CNP is a member of the mammalian natriuretic peptide (NP) family that contains other subgroups (ANP and BNP), which have the C-terminus extension, and all are usually expressed in various tissues and organs of mammals [106][107][108][109]. CNPs are 22-amino-acid peptides with similar structures to ANP/BNP but differ from them genetically [110]. CNPs usually have an essential conserved ring core by forming an S-S linkage that contains 17 amino acid residues [108]. Snake venom CNPs can be around 30–39 amino acid residues in length, with a small molecular weight of around three kDa [111][112]. Members often bind to the guanylyl cyclase/natriuretic peptide transmembrane receptors (GC/NPR), which have three types (A, B, and C). NPR-C, which acts as a clearance receptor, has a high affinity to all NPs, while NPR-A has a high affinity to ANP/BNP, and NPR-B has a high affinity to CNP [106][107][108][109][113]. Upon binding to NPR-A/B, these peptides convert GTP into cGMP and release it as a second messenger for subsequent downstream pathways to enact its effects [110][113][114]. NPs influence motility in the gastrointestinal system. Specifically, NPs cause the relaxation of the esophagus, stomach, gallbladder, and colon [109][115]. Additionally, NPs produce potent hypotension in their prey during envenomation, contributing to a rapid loss of consciousness [115]. Although lacking the diuretic and natriuretic effects of ANP and BNP due to the absence of a C-terminus extension, CNP seems to have additional advantages [107] due to its less hypotensive effects [110][116], potent anti-proliferative activities, and collagen-suppressing properties [110][113]. Furthermore, CNP also benefits from its signaling receptors not being downregulated in the failing heart [107]. Thus, like BPPs, CNP has been extensively investigated as a therapeutic candidate for cardiovascular diseases [106][113][114][115]. DNP is the most studied NP, isolated from green mamba (*Dendroaspis angusticeps*) venom [106][108][110][117]. The result is a chimeric designer called CD-NP, a fusion of DNP from snake venom and human CNP [107][116], inheriting many novel and beneficial features from DNP and CNP [107][110][113][116]. With many notable activities, CD-NP, under the name Cenderitide, passed the phase I clinical trial [113]. Research avenues for CNPs are still open due to recent discoveries and successful isolations of new, unique CNPs from *Crotalus* [111][118][119] and other snake species [56][106][114][120].

2.1.6. Nerve Growth Factors

Nerve growth factor (NGF) is among the least abundant toxins in snake venom [121][122][123][124][125][126]. Nevertheless, snake venom is considered a rich active source of this peptide [127][128]. Thus, snake venom provides much-needed accessibility to NGF compared to other growth factors and potentially lowers costs [127]. NGF is a peptide neutrophin (NT), important in maintaining nerve cells and repairing damaged cells [127][129][130]. Therefore, its existence within the venom arsenal is initially perplexing [130][131]. However, the family can also produce/enhance anaphylaxis [126][132] and induce mast cell degranulation [132], plasma extravasation, and histamine release [133], consequently leading to vascular permeability and tissue vulnerability, which aids toxin absorption and diffusion [131][133]. Additionally, this neuropeptide has

a variety of non-toxic, ancillary biological activities: wound healing [130][134][135], effect on cartilage metabolism and chondrogenic differentiation [127], inhibition of metalloprotease-mediated degradation [130][131], involvement in inflammatory sites [130][135], and chemotherapy-induced neuropathy [136]. The protein can be isolated as a high molecular weight complex called 7S with 130 kDa in molecular weight composed of 3 subunits: α, β, and γ [125][137]. However, the β subunit (2.5S NGF) is the sole player in the neurotrophic activity of NGF [137]. It has two receptors. First is the tropomyosin kinase receptor A (TrkA), with high and specific affinity [124][138], triggers the MAPK, ERK, and PI3K/AKT cytosolic/endosomal pathways [127][138], leading to proliferation arrest and the induction of differentiation in neuronal cells [137]. Second is the p75 pan-NT receptor (p75NTR) [124][127], with similar affinity but not specificity, is linked to cell apoptosis and growth arrest via the MAPK c-Jun N-terminal kinase pathways [139]. Interestingly, both NGF receptors have been observed to be expressed in tumors in the nervous system and are especially prevalent in breast cancer [128]. NGF can promote or suppress tumor growth depending on tumor types [124][133], with prominent examples of NGFs from cobra venom inhibiting the growth of Ehrlich's adenocarcinoma in vivo [124][128][140], but proliferative activity on breast cancer cell line MCF-7 [133][140][141]. Lately, NGF has been found to have a link to human diseases, including Alzheimer's disease [130][142]. Subsequent NGF therapy for this neurodegenerative illness in phase I clinical trials has been reported [121][130], as well as other neurological disorders (Parkinson's disease, peripheral neuropathy, etc.) [121][136]. Although most research on NGF reported here is based on Cobra species, there are studies on NGF from many snake species [143], including both rattlesnake genera *Crotalus* [125][143][144] and *Sistrurus* [56][58][145], that have indicated potential routes of isolating this active neuropeptide that may resolve the conflicting results of purifying NGF from snake venoms [125].

2.1.7. Kunitz-Type Serine Protease Inhibitors

Kutniz-type inhibitors are a group of serine protease inhibitors that are often found in Elapidae and Viperidae snakes. It is believed that they play a role in interfering with the blood coagulation cascade, thus affecting the prey's homeostasis [146]. Seven of the *Crotalus* species have been reported to have this component in their venom composition. These inhibitors have around 60 residues and bear structural similarities with aprotinin [147]. They are reported to interact with serine protease via an exposed loop in a canonical confirmation, with the P1 residue acting as the primary site [148]. The P1 site also determines the specificity and reactivity of KUN towards its serine proteases [149]. Although KUN members are not highly conserved in their amino acid sequences [148], their overall structural scaffold is conserved, with subtle variations in the binding regions that aid in the functional diversity of KUN [149]. Currently, the members in the KUN family are divided into two major subgroups: non-neurotoxin (i.e., trypsin and chymotrypsin inhibitors) and neurotoxin (potassium and calcium blockers) [148]. Zupunski et al. (2003) report that Viperidae snakes, which include the *Crotalus* and *Sistrurus* genera, only contain the non-neurotoxic KUN members [148].

Due to its ability to bind to serine proteases, KUN offers promising pharmaceutical applications. Textilinin-1, isolated from *Pseudonaja textilis*, is shown to be very specific against plasmin and is one of the examples of promising biomedical applications. Specifically, it is shown to be a very effective and specific anti-bleeding agent with fewer side effects when compared to other agents like Trasylol [150]. Another KUN called tenerplasminin-1, isolated from *Micrurus tener tener*, is said to be a potent antifibrinolytic agent [151]. Such agents can be crucial in treating hyper-fibrinolysis events and excessive bleedings during medical intervention caused by heat strokes, hypotension, dengue infection, etc. [151]. KUN is often listed as a minor component of snake venoms but may prove to be a promising therapeutic agent in various biomedical settings.

2.1.8. Waprin

St Pierre et al. (2008) posited that the KUN and WAP families may have been derived from a common ancestral gene with subsequent duplication and diversification events [152]. Transcripts containing both KUN-WAP have been identified in *Sistrurus catenatus*, further corroborating the relationship between these two components [153]. WAP is first isolated from *Naja nigricollis*, called nawaprin, and structurally resembles whey acidic protein [154]. WAP members are around 50 residues in length with four conserved disulfide bridges [152]. Like KUN, WAP is sorted as a minor component which three *Crotalus* genera and one *Sistrurus* genus are reported to possess. Unlike KUN, which has been studied relatively well, WAP's venom function is poorly characterized and understood [152]. One previous work reported some selective dose-dependent antimicrobial activities of omwaprin, a WAP isolated from *Oxyuranus microlepidotus*, through membrane disruption mechanisms [155]. On the other hand, previously reported nawaprin does not show any antibacterial activity nor does it act as protease inhibitor, a role that whey acidic proteins usually fill [154][156]. Limited information about WAP's physiological functions and potential biomedical applications may warrant further research.

2.1.9. Snake Venom Metalloproteases

The prominent presence of SVMP within rattlesnakes' venoms has been discussed intensively through the Type I/Type II venom profiling dichotomy in *Crotalus* [157][158] and within two species of the genus *Sistrurus* [15]. Not only is SVMP abundant within rattlesnake venoms, with around 11% to over 65% of total venom protein [15][42], but it is also an important protein family present in the general Viperidae snake venoms [42], thus often being referred to informally as one of the major toxins within the world of venoms along with phospholipases and neurotoxins [17]. A high abundance of this protein family is thought to perform generic killing and digestive functions that are not prey-specific [42]. The presence of SVMP across different snake species significantly contributes to several pathological effects on blood coagulation [158] and fibrinogenolysis [16][158], leading to severe bleeding, local and systematic hemorrhage [159], and tissue damage after minutes of injection [158][160]. In numerous venomous snakebites, prothrombin activation [42], apoptotic responses [161], factor X-activating inflammation [42], and necrosis [159] may also occur. The precise mechanism of these effects is still elusive despite recent attempts to unravel the pathological effects of this protein family [160]. However, clues about such processes are given through various studies [162][163]. Briefly, hemorrhagic SVMPs seem to target the basement membrane and surrounding endothelial extracellular matrix, weaken the capillary walls, and reduce the width of endothelial cells, ultimately forming gaps amongst the weakened walls for erythrocytes to flow through [160][164]. Additionally, some endothelial cells are shown to be swollen and forming large blebs [42]. Consequently, several other basement membrane proteins, such as laminin, nidogen, and type IV collagen, seem to be reduced [42]. The SVMP family is classified within the M12 reprolysin family of metalloproteinase and further divides into three groups: P-I, P-II, and P-III [160][164]. P-I SVMPs comprise only one zinc-binding metalloproteinase domain with the lowest molecular weights (20–30 kDa) among SVMP subgroups [42]. A P-II SVMP contains an additional DIS-like domain, which is often released after proteolytic action, along with the zinc-binding metalloproteinase domain, making the protein bigger in the 30–60 kDa range and being thought to diverge from the P-III class [42][164]. Lastly, a P-III SVMP usually has a molecular weight of around 60–100 kDa, containing both the abovementioned domains and an extra cysteine-rich domain [42]. Some subclass members of P-III SVMP may also be linked to C-type lectin-like subunits and belong to the obsolete P-IV class of SVMP [42][160]. It has been reported that the P-III class tends to have higher hemorrhagic activities than P-I due to their size and resistance to α2-M (alpha-2-Marcoglobulin enhances prothrombin activation and thrombin activation) compared to P-I [42][159]. Additionally, the non-catalytic ancillary domains of P-III, namely, the DIS-like and cysteine-rich domains, may play important roles in P-II's original hemorrhagic and additional non-hemorrhagic biological activities [159][164]. The crystal structures of nine P-I SVMPs have been elucidated along with their activities [165][166]. A prominent example is adamalysin II from *Crotalus adamanteus* [167], composed of a single chain of SVMP that needs Zn^{2+} and Ca^{2+} as cofactors for biological activities. However, not all SVMPs need Ca^{2+} to operate [42][167]. Similarly, around seven P-III SVMP crystal structures were found [165]. The crystal structure provided for VAP2B, a *Crotalus atrox* P-III SVMP, did reveal a dynamic, modular architecture of the three domains within P-II SVMP with important intrinsic flexibility for fine-tuning substrate recognition and post-translational regulation [168]. This finding seems to correlate with recent studies on the differences between hemorrhagic and non-hemorrhagic SVMP due to backbone flexibility in specific surface regions of the protein [169]. Despite lacking hemorrhagic activities, these SVMPs can still induce vascular permeability, inflammatory cell migration, and pain [164]. Drawing from such insights, many unexplored aspects of SVMPs would need to be further explored despite the large body of existing literature [42][164].

2.1.10. Snake Venom Serine Proteases

Along with SVMP, SVSP is also considered one of the dominant toxin families within snake venoms [2][164] and has been observed to be present in almost all vipers [2]. SVSP is categorized in the S1 family of serine proteases [164], or the trypsin-like family [46], weighing 26–67 kDa [164]. Its members have evolved from kallikrein-like serine proteases [46][170] with significant gene duplications about the venom production, producing many isoforms [46][171][172]. SVSP was shown to be quite pharmacologically versatile, with a wide array of effects through subtle structural changes (Segura et al., 2017), with some expressing multiple activities [153]. In contrast to SVMP, which usually induces hemorrhages through capillary vessel rupture, SVSP alters the hemostatic systems of the victim [173], induces edema [170], hyperalgesia [164], blood coagulation perturbations [46], fibrinolysis [173][174], and platelet aggregation [170], and alters the kallikrein–kinin systems [80][173], by acting primarily on plasma proteins such as fibrinogen [170] to produce lethal consequences for the victims [164]. Characterized SVSPs are single-chain glycoproteins [153][170], although exceptions such as heterodimeric SVSPs have been found in *Agkistrodon. b. brevicaudus* [171]. Members usually have three substrate-binding sites and a catalytic triad with 12 conserved cysteine residues for six disulfide bridges [170][174]. Additional cysteine residues are usually found in SVSP, along with three N-glycosylation sites, which have been thought to contribute to enzyme stability and selectivity [174]. Due to its resemblance to trypsin, chymotrypsin, and thrombin, the aforementioned catalytic triad (composed of His57, Asp102, and Ser 195) can catalyze the peptide bond cleavage in which histidine is a proton donor/acceptor and serine acts as a nucleophile [170]. SVSP can be inhibited by various synthetic and natural products, such as phenylmethylsulfonyl fluoride (PMSF) [175]. This family of proteins is generally multifunctional, with many different substrates that warrant further investigation [170].

2.1.11. Phospholipases A_2

One of the most diverse classes of esterase is the phospholipase A_2 (PLA_2) enzymes, which prefer cleaving glycerophospholipids [176][177][178]. In a study completed in 2022 by Rodrigues et al., the PLA_2 enzyme family was found to be the most abundant family within the entire *C. durissus* venom composition [179]. While PLA_2 concentration was similar within all *Crotalus* subspecies, there were still differences between the subspecies, with *C. d. durissus* having the highest concentration compared to *C. d. cumanensis*, *C. d. ruruima*, and *C. d. terrificus* [179]. This class of proteins is divided into six families and further classified from I to XVI with capital letters [180]. Rattlesnakes' secretory PLA2 (sPLA2) toxin can be listed in group IIA within the family of $sPLA_2$, while those of cobras and kraits are listed in group IA [178][180]. These proteins are small and stable, with many disulfide bonds that tend to bind to Ca^{2+}, and are highly similar in structures and sequences [176][177][178]. Additionally, within the known PLA_2 secreted in snakes' venoms, members are generally categorized into two groups: (1) catalytically active (D49 variant) and (2) catalytically inactive homologs (K49 variant) [177]. The D49 variants retain the conserved aspartic acid residue at position 49 at the catalytic center, essential for Ca^{2+} binding [177][178]. In contrast, the inactive variants, K49 PLA_2, seem to replace the aspartic acid residue with lysine, thus losing the ability to cleave phospholipids but still having intriguingly crucial activities [177][181], and appear to be a suitable target for pharmacological discoveries [181]. Another form of PLA_2 is a heterodimeric complex named crotoxin (CRTX) from a non-enzymatic polypeptide called crotapotin (CA) and a basic PLA_2 (CB) [182][183][184][185]. Apart from the usual roles in lipid metabolism and membrane modeling, the PLA_2 family of snake toxins displays a diverse array of biological and toxicological functions, including cytotoxicity [176][177], edema forming [176][186], anticoagulant [186][187], antibacterial [181][185], anti-tumoral [185], myotoxicity [176][177], and neurotoxicity activities [183][184][186][188][189]. The PLA_2 family essentially plays both roles of phospholipases and neurotoxins within the venom of rattlesnakes. PLA_2 achieves significant and specific neurotoxic effects on the presynaptic action (β-neurotoxic) [79], inhibiting the release of acetylcholine desensitizing the nicotinic receptors, leading to paralysis [184]. Many of these effects, such as antibacterial and antitumor effects for serum therapy and cancer treatment, are vital to study [177].

2.1.12. Myotoxins

The family of myotoxins in rattlesnakes induces the same paralysis effect as PLA_2 but with a different method [190][191]. This family is believed to derive from a common antimicrobial peptide ancestor called β-defensin in snakes, platypuses, and lizards [192][193]. Within these snakes, this toxin family seems to be exclusively expressed in rattlesnake species, around 11 species of *Crotalus* and *Sistrurus catenatus* [194], with no sign of its expression in other species of *Viperidae*. Myotoxins comprise peptides with around 42 amino acids and six cysteine residues for three disulfide bonds, making up a tight B-sheet core [190][195][196]. Thus, they are generally low in molecular weight and are often essential peptides rich in lysine and amphipathic [191][192][197]. Although they are less abundant than other previously mentioned major toxins, they are regarded as a major toxic component for many rattlesnake species [192][198], accounting for up to 20% of total toxins [199][200], with occasional listing as a minor family [201]. Toxic effects are present in the early and late stages of venom exposure. Myotoxins can disrupt cardiac proteins and cause cascades to destroy cardiac cells, leading to structural damage to the heart. Vascular leakage, swollen muscle fibers, edema, myocytolytic necrosis, and high trophin levels (an indication of heart stress) are all noted in lab rat studies found when exposed to venom from *C. durissus terrificus* [202].

Interestingly, this family has various biological activities like membrane penetration [191][203], nuclear localization [204], anti-tumoral activity, anti-fungal and antimicrobial activity [173][192][198], and irreversible membrane depolarization [191]. Thus, mycotoxin often induces the paralysis and extension of the hind paws by acting on Na^+ and K^+ channels [191][192][197] and inducing skeletal muscle necrosis [190][191][205]. In contrast to the neurotoxic PLA_2, myotoxin's mechanism is considered non-enzymatic and acts extremely rapidly to limit prey escape through hind paw paralysis and death through diaphragm paralysis [190]. Myotoxin was observed to localize in the sarcoplasmic reticulum and bind to its two components, one of which is Ca^{2+}-ATPase, calcium pump, and the other may be a modulator of this calcium pump, which leads to the inhibition of calcium influx into SR and may have partly explained the paralysis effect [205]. Unsurprisingly, myotoxin's structure shares essentially no similarity with PLA_2 [190][200].

Additionally, the structure of myotoxin crotamine with an αββ fold and three disulfide bonds [206] is characteristic of membrane-active peptides and defines its ability to penetrate proliferating active human and murine stem cells [203][206]. Combining this activity with a stable structure scaffold, crotamine is portrayed as a versatile agent that can penetrate cells and cross the blood–brain barrier, with many other myotoxins' features like anti-cancer activities [146][207]. Previous work has shown that crotamine is a potential tumor inhibitor to melanomas in mouse models [207], and a template for a synthetic analogue to deliver anticancer compounds in mammalian cells selectively [208].

Furthermore, previous studies have shown that members of this toxin family, specifically crotamine, are expressed differently amongst individuals and populations of *Crotalus durissus* [199][209][210]. Although the family has been discovered

for over 50 years, many of its activities have been discovered in recent decades, with several more potential therapeutic applications [211].

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