Toxin Reviews

http://informahealthcare.com/txr
ISSN: 1556-9543 (print), 1556-9551 (electronic)

Toxin Rev, Early Online: 1–17
 DOI: 10.3109/15569543.2014.941

informa
healthcare

REVIEW ARTICLE

Rear-fanged snake venoms: an untapped source of novel compounds and potential drug leads

Anthony J. Saviola[1], María E. Peichoto[2], and Stephen P. Mackessy[1]

[1]School of Biological Sciences, University of Northern Colorado, Greeley, CO, USA and [2]Instituto Nacional de Medicina Tropical, Ministerio de Salud de la Nación, Puerto Iguazú, Misiones, Argentina

Abstract

Animal venoms represent a diverse source of potentially valuable therapeutic compounds due to the high specificity and the potent biological activity of many toxins. Snake venom toxins, particularly disintegrins and proteases from viper venoms, have yielded therapeutics with anti-cancer and hemostatic dysfunction activities. However, venoms from rear-fanged ''colubrid'' snakes have rarely been analyzed from the perspective of potential lead compound development. Here, we discuss recent progress in the analysis of these venoms, focusing on several studies of specific venom components as well as transcriptomic and proteomic surveys. Currently available –omic technologies largely circumvent the problematic low venom yields of most rear-fanged snakes, and because their basic biology is often very different from the well-studied front-fanged snakes, there is great potential for novel compound discovery in their venoms.

Keywords

Colubrid, cysteine-rich secretory protein, evolution, metalloprotease, protein, serine protease, structure/function, three-finger toxin, taxon-specific toxicity

History

Received 3 April 2014
Revised 14 June 2014
Accepted 2 July 2014
Published online 24 July 2014

Introduction

Like all venomous snakes, the venoms of rear-fanged ''colubrid'' snakes contain a variety of proteins and peptides that exhibit potent biological functions (Fry et al., 2003a, 2008; Hill & Mackessy, 2000; Mackessy, 2002; Weldon & Mackessy, 2010). However, to date, the amount of published work investigating rear-fanged snake venoms remain relatively low compared to the extensive literature examining the composition and biochemical complexity of venoms from front-fanged elapid and viperid snakes. The reason for this is two-fold. Although at least five genera (*Dispholidus*, *Philodryas*, *Rhabdophis*, *Tachymenis* and *Thelotornis*) contain species responsible for serious (including fatal) human envenomations (Weinstein et al., 2011), rear-fanged colubrids are often considered as non-threatening to humans, and accordingly, research into venom composition and complexity has been relatively under-studied. Second, due to the low-pressure venom delivery system and difficulties associated with venom extractions (see below), low amounts of starting materials are often considered as a significant constraint to colubrid venom research (Mackessy, 2002). However, advancements in laboratory techniques, as well as venom extraction methods, have resulted in an increased understanding of rear-fanged snake venoms, and these ''weak'' venoms may demonstrate a great deal of biological complexity. Venom characteristics similar to those of front-fanged snakes have been documented for several species [refer Mackessy (2002, 2010a,b) for reviews], but due to the tremendous taxonomic diversity of the ''rear-fanged snakes'', encompassing several families, subfamilies and hundreds of species, a variety of different ''venom compositional strategies'' are observed, leading to a high diversity of venom proteomes. Further, rear-fanged colubrids represent very different evolutionary lineages from elapids and vipers (Pyron et al., 2013; Vidal, 2002), providing the potential for discovery of novel proteins and protein families that may represent excellent lead compounds for drug design or development. Rear-fanged snakes are exceptionally diverse, and representative species are found on all continents except Antarctica (Figures 1–4). Expanded research on rear-fanged snake venoms will also provide a better understanding of the broader evolutionary trends among venomous snakes, as well as significant insights into potential therapeutic agents that may be derived from compounds isolated from rear-fanged snake venoms.

Address for correspondence: Stephen P. Mackessy, School of Biological Sciences, University of Northern Colorado, 501-20th Street, CB92, Greeley, CO 80396-0017, USA. Tel: +1 970-351-2429. Fax: +1 970-351-2335. E-mail: stephen.mackessy@unco.edu

The Duvernoy's venom gland

At least one-third of the 2300+ species of non-front-fanged advanced snakes (''colubrids'') produce a specialized venom (Mackessy, 2002; Pyron et al., 2013; Vidal et al., 2007). The Duvernoy's gland of rear-fanged snakes (Figure 5A) is homologous to the venom glands of the front-fanged elapid and viperid snakes (Kochva, 1965; Savitzky, 1980). Unlike the venom glands of front-fanged snakes, which are typically large with a basal lumen capable of storing significant quantities of secreted venom (e.g. Mackessy, 1991), the

Figure 1. Representative African rear-fanged snakes. (A) *Rhamphiophis oxyrhynchus* (Rufous Beaked Snake – Tanzania). (B) *Lioheterodon madagascariensis* (Madagascan Hognosed Snake). (C) *Psammophis sibilans* (Striped Sand Snake – North Africa). (D) *Thelotornis kirtlandii* (Twig Snake – Uganda). Photographs copyright S.P. Mackessy.

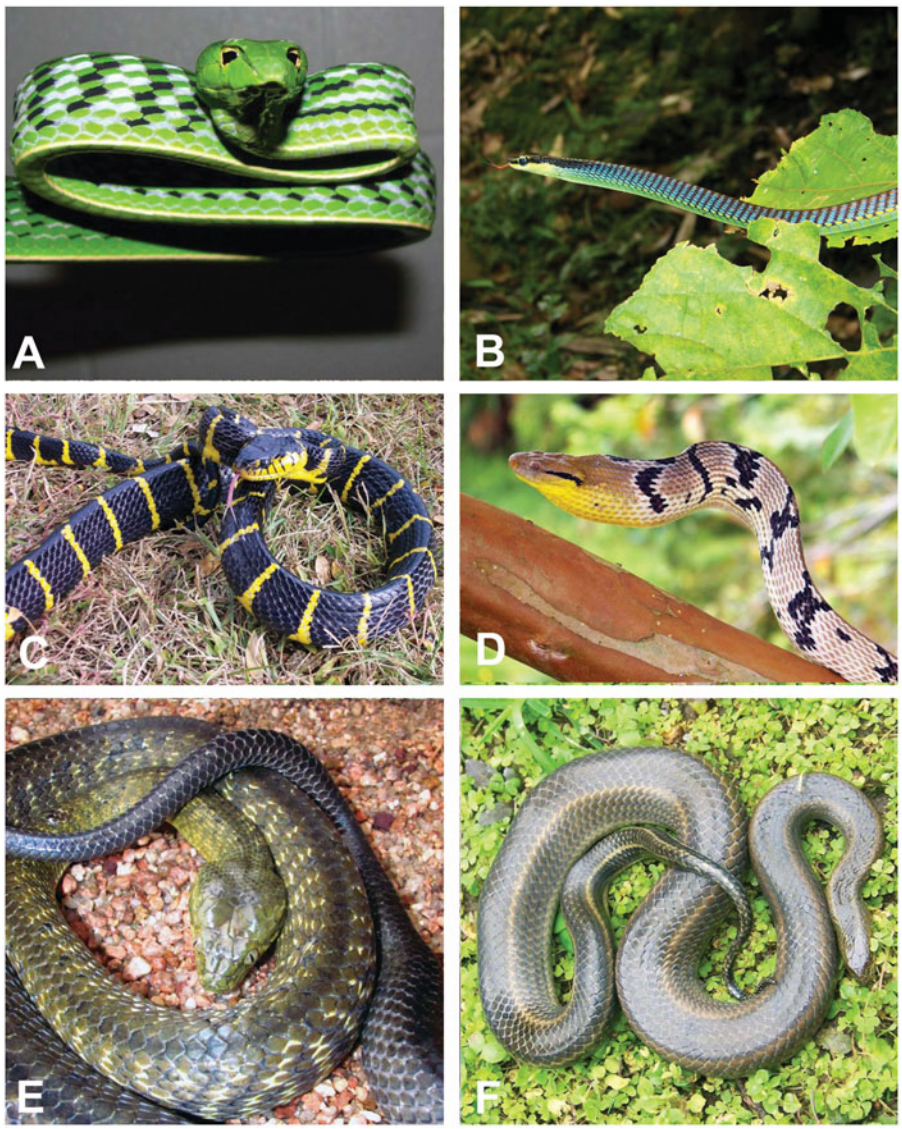

Figure 2. Representative Asian rear-fanged snakes. (A) *Ahaetulla prasina* (Asian Vine Snake – Sumatra). (B) *Dendrelaphis formosus* (Elegant Bronzeback Snake – Malaysia). (C) *Boiga dendrophila* (Mangrove Catsnake – Java). (D) *Boiga cynodon* (Dog-toothed Catsnake – Malaysia). (E) *Boiga irregularis* (Brown Treesnake – Guam). (F) *Enhydris plumbea* (Rice Paddy Snake – Malaysia). Photographs copyright S.P. Mackessy.

Figure 3. Representative North American rear-fanged snakes. (A) *Heterodon nasicus* (Western Hognosed Snake – Colorado, USA). (B) *Trimorphodon biscutatus lambda* (Sonoran Lyre Snake – Arizona, USA). (C) *Thamnophis elegans vagrans* (Wandering Garter Snake – Colorado, USA). (D) *Diadophis punctatus* (Ringnecked Snake – USA). (E). *Gyalopion canum* (Desert Hooknosed Snake – Arizona, USA). (F) *Tantilla nigriceps* (Plains Blackheaded Snake – southwest USA). Photographs copyright S. P. Mackessy.

Duvernoy's venom gland lacks a large basal lumen. Most rear-fanged venomous colubrids also lack hollow fangs, and instead, posterior maxillary teeth, often enlarged and/or grooved (Figure 5B; Mackessy, 2010a; Young & Kardong, 1996), participate in venom introduction into tissues (Kardong & Lavin-Murcio, 1993; Vonk et al., 2008). In general, the Duvernoy's gland is considered a low-pressure system (Kardong & Lavin-Murcio, 1993), but a recent analysis has suggested that grooved enlarged rear maxillary teeth (as seen in many *Boiga* species; Figure 5B) can deliver venom effectively and fairly rapidly into wounds (Young et al., 2011). Although it has been suggested that the term Duvernoy's gland should be abandoned, with the term venom gland used for any toxin-secreting buccal glands (Fry et al., 2003a), rear-fanged snakes possess distinctly different venom delivery systems, and we recommend using the term Duvernoy's venom gland. This retains the historical component of the name (named after G. L. Duvernoy; Taub, 1966) and acknowledges the distinct biomechanical features of the envenomation systems of ''colubrids'', but recognizes the clear embryonic, evolutionary and biochemical homology with front-fanged snake venom glands.

Very few rear-fanged snake venoms are commercially available, in part because of low yields, and the biological and pharmacological activities of these venoms are still poorly known (*cf.* Mackessy, 2002). Larger species, such as *Boiga irregularis*, produce moderate yields which are amenable to standard chromatographic fractionation and analyses; single yields of up to 18.5 mg (90% protein content) have been recorded (Mackessy et al., 2006). Smaller species, such as *Alsophis portoricensis*, yielded proportionally lower amounts, with a dry venom mass averaging 5.9 mg and approximately 89% protein content (Weldon & Mackessy, 2010); even usable amounts of venom can be obtained from the smallest species, such as *Tantilla nigriceps* (body mass ~5 g; Hill & Mackessy, 2000). We routinely use ketamine anesthesia followed by injection with pilocarpine to produce significantly increased yields (Ching et al., 2012; Hill & Mackessy 1997; Mackessy et al., 2006). Other anesthetics, such as Zoletile 100 mg, Tiletamine 50 mg and Zolazepam 50 mg at a dose of 3 mg/kg (Fry et al., 2003a,b) have also been employed. These methods, especially the use of pilocarpine, increase venom yields, are safe for the snake and facilitate handling. Venoms can then be repeatedly sampled from the same snake over time, which may be necessary to obtain sufficient amounts from small species.

When compared to venoms of front-fanged snakes, the diversity of proteins in the venom proteome of rear-fanged snakes is generally less complex (Ching et al., 2006, 2012; Mackessy, 2002; Weldon & Mackessy, 2010). However, as demonstrated by 1D SDS-PAGE (Figure 6A) and MALDI-TOF mass spectrometry (Figure 6B), significant variation can

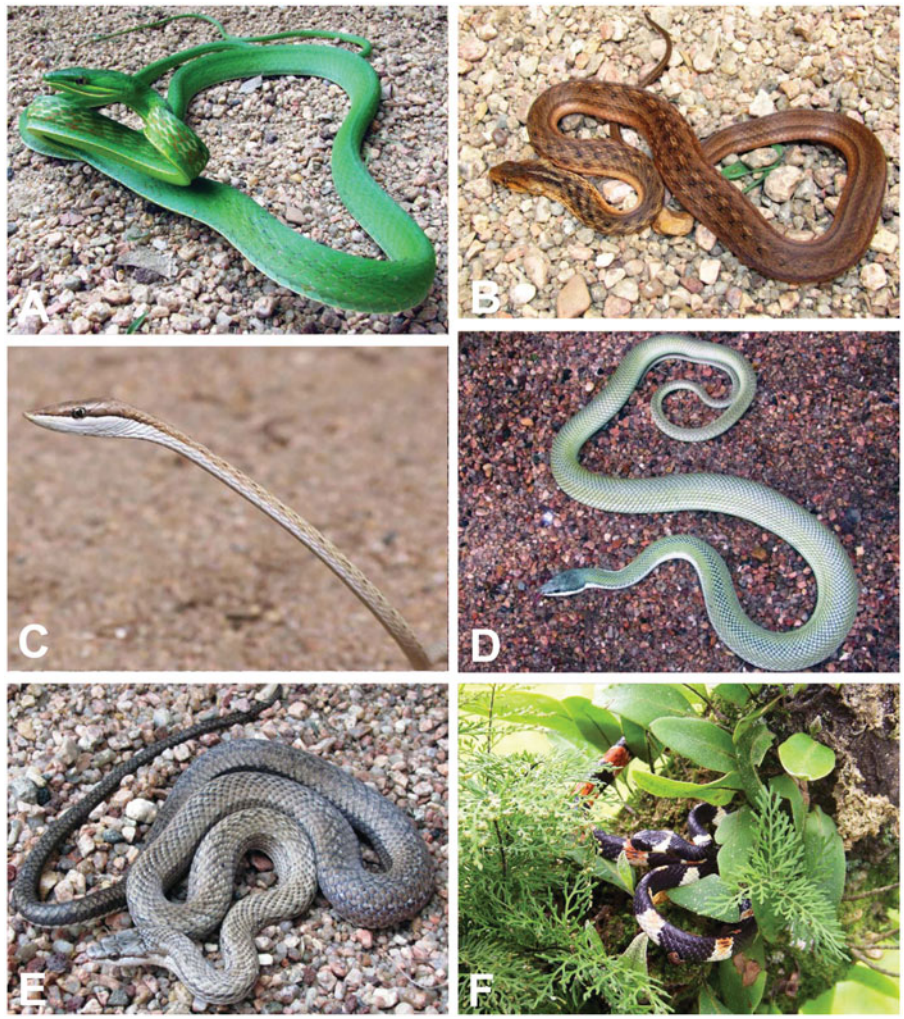

Figure 4. Representative Central and South American Rear-fanged Snakes. (A) *Oxybelus fulgidus* (Green Vine Snake – Suriname). (B). *Thamnodynastes strigatus* (Coastal House Snake – Suriname). (C) *Oxybelis aeneus* (Brown Vine Snake – Honduras). (D) *Philodryas baroni* (Argentine Racer – Argentina). (E) *Alsophis portoricensis* (Puerto Rican Racer – Caribbean). (F) *Sibon anthracops* (Ringed Snail-eating Snake – Costa Rica). Photographs copyright from S. P. Mackessy.

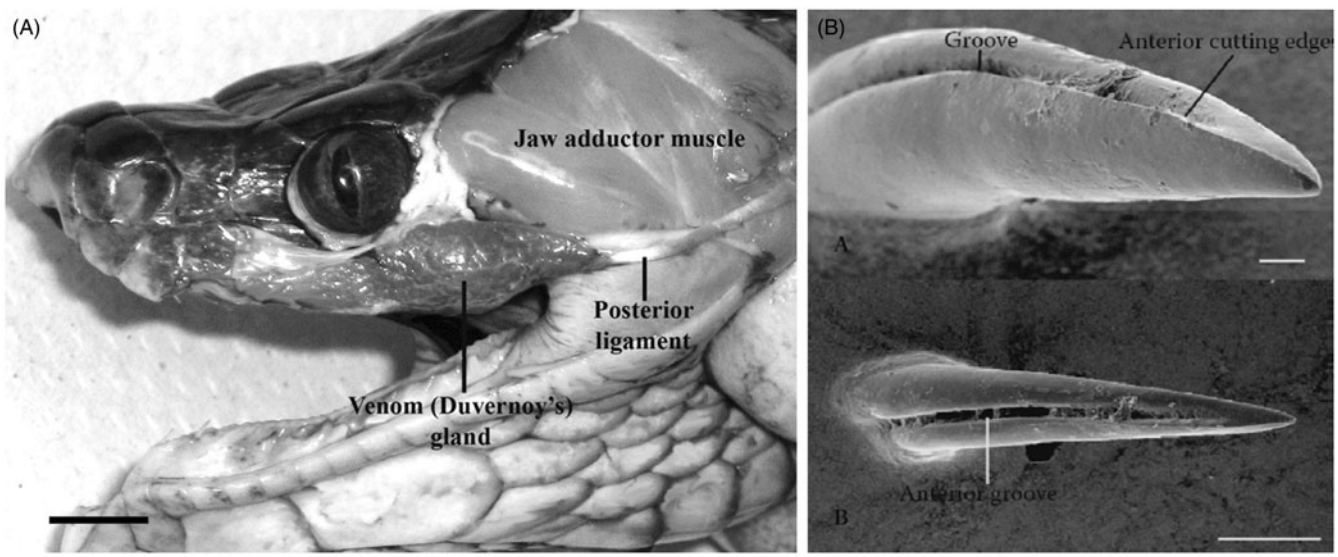

Figure 5. (A) The Duvernoy's venom gland of a rear-fanged snake, the Brown Treesnake *Boiga irregularis* (family Colubridae), common in parts of Indonesia and northeastern Australia and introduced to Guam. Note that the gland lies in the same relative position as that in front-fanged snakes, but unlike viperids, the gland is not surrounded by adductor muscle. Venom delivery occurs via pressure against the skin generated by adductor muscles, and the gland is pulled taught by the posterior ligament. Scale bar = 1 cm. (B) Deeply grooved rear maxillary fangs of the closely related Mangrove Catsnake (*Boiga dendrophila*). Scale bars – top: 100 µm; bottom: 500 µm. Reproduced from Mackessy (2010a).

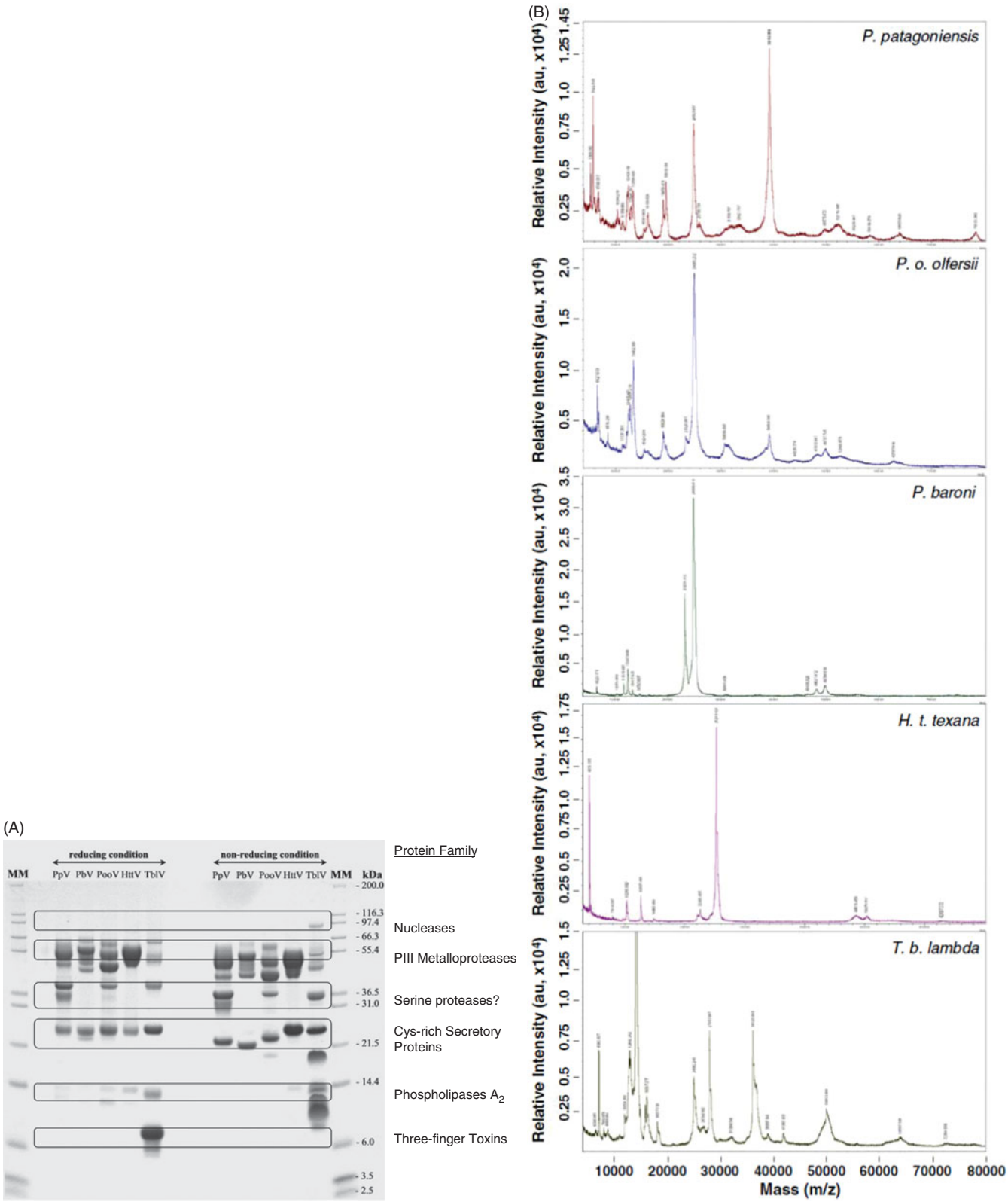

Figure 6. (A) One-dimensional SDS-PAGE of non-reduced and reduced rear-fanged snake venoms of the Americas. Protein families with masses typical of bands seen are listed on the right. PpV, *Philodryas patagoniensis*; PbV, *Philodryas baroni*; PooV, *Philodryas olfersii olfersii*; HttV, *Hypsiglena torquata torquata*; Tbl, *Trimorphodon biscutatus lambda*. (B) Mass spectrograms (MALDI-TOF) of the same venoms. Reproduced from Peichoto et al. (2012).

be exhibited between species. At least eight different protein families are represented in rear-fanged snake venoms (Table 1), and most venoms contain metalloproteases and cysteine-rich secretory proteins (CRiSPs) as dominant venom components. Similar to front-fanged snakes, particularly viperid venoms, rear-fanged snake venoms often contain P-III metalloproteases as well as higher mass enzymes. In addition, venoms of some species possess three-finger toxin proteins structurally similar to those found in elapid venoms. Yet, in both cases, the abundance

Table 1. Some common components of colubrid snake venoms and general characteristics.

Component name	Approximate mass (kDa)	Function	Biological activity	Representative species with detectable activity
Enzymes				
Phosphodiesterase	94–140	Hydrolysis of nucleic acids and nucleotides	Depletion of cyclic, di- and tri-nucleotides; hypotension/shock?	*Amphiesma stolata*, *Diadophis punctatus*, *Heterodon nasicus kennerlyi*, *H. n. nasicus* and *Thamnophis elegans vagrans* (low activity)[1]
Acetylcholinesterase	55–60	Hydrolysis of acetylcholine	Depletion of neurotransmitter; tetanic paralysis (?)	*Boiga dendrophila*, *B. blandingi*, *B. irregularis*[1–3]
Snake venom metalloproteinases	47–58	Hydrolysis of many structural proteins, including basal lamina components, Hydrolysis of fibrinogen (α & β subunits) and cleaving fibrin	Hemorrhage, myonecrosis, prey predigestion	*A. stolata*, *Alsophis portoricensis*, *Boiga cyanea*, *B. dendrophila*, *B. irregularis*, *D. p. regalis*, *H. n. nasicus*, *H. gigas*, *Hypsiglena torquata texana*, *Trimorphodon biscutatus lambda*, *Philodryas patagoniensis*, *P. baroni*, *P. olfersii olfersii*, *Salvadora grahamiae grahamiae*, *Tantilla nigriceps*, *T. e. vagrans*[1,3–6,9]
Alsophinase	56			
Patagonfibrase (pI 5.8)	53.2			
PoFibC1 (pI 6.2)	47			
PoFibC2 (pI 6.2)	47			
PoFibC3 (pI 8.5)	45			
PoFibH (pI 4.6)	58			
Matrix metalloproteinases	21–38	Remodeling of the extracellular matrix/ modulation of bioactive molecules	Hemorrhage, tissue degradation, prey predigestion (?)	*Rhabdophis tigrinus tigrinus*, *Thamnodynastes strigatus*[7,8]
Serine proteases	28–36	Hydrolysis of fibrinogen (α and β subunits)	Hemostasis disruption (?)	*T. b. lambda*, *P. o. olfersii*, *P. patagoniensis*, *P. baroni*[6,9]
PoFibS (pI 4.5)	36			
Phospholipase A_2 enzymes	13–15	Ca^{2+}-dependent hydrolysis of 2-acyl groups in 3-sn-phosphoglycerides	Myotoxicity, myonecrosis, lipid membrane damage	*Boiga dendrophila*, *D. punctatus regalis*, *Dispholidus typus*, *Leptodeira annulata*, *Malpolon monspessulanus*, *Philodryas nattereri*, *P. o. olfersii*, *P. patagoniensis*, *Psammophis mossambicus* (very low activity), *Rhabdophis subminiata*, *Telescopus dhara*, *Thelotornis capensis* (very low activity), *T. strigatus*, *Tomodon dorsatus*, *T. b. lambda*[1,2,10–18]
Trimorphin	13.9			
Non-enzymatic proteins/peptides				
Cysteine-rich secretory proteins (CRiSPs/helveprins)	21–30	Possibly block cNTP-gated channels	Induced hypothermia; prey immobilization (?)	*Philodryas baroni*, *P. o. olfersii*, *P. patagoniensis*, *H. t. texana*, *T. b. lambda*, *D. typus*, *Liophis poecilogyrus*, *Hydrodynastes gigas*, *R. t. tigrinus*[1,6,19–22]
Patagonin	24.8			
Tigrin	30			
Dimeric three-finger toxins	17	Potent inhibitor of neuromuscular transmission; shows taxon-specific effects	Rapid immobilization of prey, paralysis, death	*Boiga irregularis*[23]
Irditoxin	17			
Three-finger toxins	6–10	Potent inhibitors of neuromuscular transmission; may show taxon-specific effects	Rapid immobilization of prey, paralysis, death	*Boiga cynodon*, *B. d. dendrophilia*, *B. d. gemmicincta*, *B. drapiezii*, *B. irregularis*, *B. nigriceps*, *Telescopus dhara*, *T. biscutatus*, *Rhamphiophis oxyrhynchus*, *Oxybelis fulgidus*[23–28]
α-neurotoxins				
α-colubritoxin	8.49			
Boigatoxin-A	8.7			
Denmotoxin	8.5			
Rufoxin	10.66			
Fulgimotoxin	8.1			

Mass in kilodaltons (kDa). Note that this list is not all-inclusive and that masses, functions and activities do not apply to all compounds isolated from all colubrid venoms. Specific venoms may not contain all components. (?) – indicates hypothetical function and/or activity. Isoelectric points (pI) are provided when reported. Largely based on Mackessy (2010a). [1]Hill & Mackessy (2000); [2]Broaders & Ryan (1997); [3]Mackessy et al. (2006); [4]Weldon & Mackessy (2012); [5]Peichoto et al. (2007); [6]Peichoto et al. (2012); [7]Komori et al. (2006); [8]Ching et al. (2012); [9]Assakura et al. (1994); [10]Huang & Mackessy (2004); [11]Christensen (1968); [12]Mebs (1968); [13]Durkin et al. (1981); [14]Ferlan et al. (1983); [15]Rosenberg et al. (1985); [16]DuBourdieu et al. (1987); [17]Lumsden et al. (2004); [18]Zelanis et al. (2010); [19]Yamazaki et al. (2002); [20]Peichoto et al. (2009); [21]Mackessy (2002); [22]Fry et al. (2006); [23]Pawlak et al. (2009); [24]Lumsden et al. (2004); [25]Fry et al. (2003b); [26]Lumsden et al. (2005); [27]Pawlak et al. (2006); [28]Heyborne & Mackessy (2013).

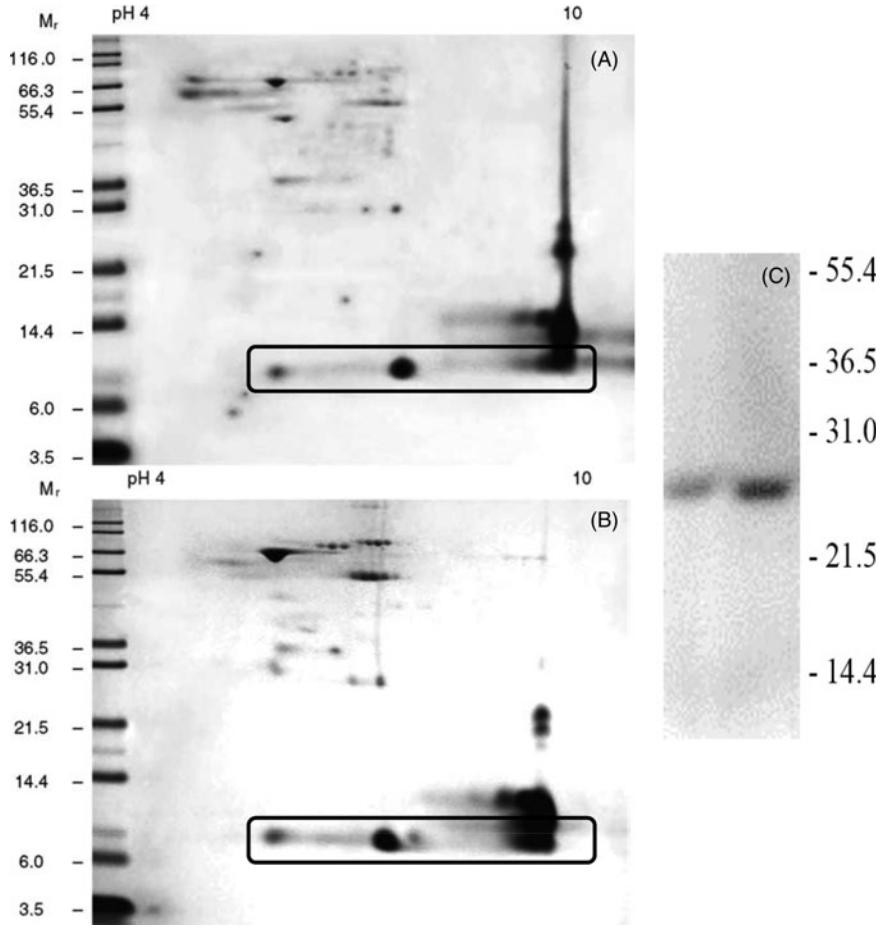

Figure 7. (A, B) Two-dimensional SDS-PAGE of Brown Treesnake (*Boiga irregularis*) venoms. Approximately 40 protein spots are visible (Coomassie blue), less than half the number typically observed with rattlesnake venoms. Three-finger toxins, abundant in this venom, are boxed; both acidic and basic toxins are observed in both neonate (A) and adult (B) snake venoms. (C) Western blot demonstration of a 25 kDa cysteine-rich secretory protein (CRiSP) in neonate (left) and adult snake venoms. Reproduced from Mackessy et al. (2006).

and diversity of these compounds in rear-fanged snake venoms are generally lower. This lower diversity of proteins is also seen following two dimensional SDS-PAGE (Figures 7A and B); gels of both adult and neonate Brown Treesnake (*Boiga irregularis*) venoms showed approximately 40 protein spots, whereas typical rattlesnake venoms often display 100+ proteins (including isoforms) classified into numerous protein families. Western blotting of the same venoms with polyclonal antibodies for tigrin (Yamazaki et al., 2002) revealed a single CRiSP band (Figure 7C).

Toxins to drugs: colubrid venoms in drug discovery

The development of possible therapeutics from toxins is becoming increasingly emphasized in venom research, and numerous compounds found in snake venoms have been utilized as a source for protein drugs and additional novel drug leads (Fox & Serrano, 2007; Mukherjee et al., 2011; Parkes et al., 2013; Takacs & Nathan, 2014). Through the introduction of ''conscripted'' homologs of homeostatic regulators, venom components disrupt important physiological processes. The observation that snake venom genes have orthologs among normal vertebrate genes, rather than the evolution *de novo* of toxic components, provided the logical connection to the development of toxins as drugs. When appropriately investigated and evaluated, toxins have vast potential for applications in numerous fields of biomedical research and may provide the molecular scaffold for developing potential peptide drugs.

Several novel therapeutics marketed for human use have been successfully designed from animal poisons and venoms, with several more currently in clinical trials. The first successful venom-based drug, captopril, which is currently on the market as an anti-hypertensive drug, was designed from the structure of a bradykinin-potentiating peptide from the venom of *Bothrops jararaca*. Since the development of captopril in 1975, numerous compounds have been developed from the often highly conserved and stable molecular scaffolds of venom proteins. Tirofiban (aggrastat), an anti-platelet drug, and integrilin (eptifibatide), used to treat acute coronary ischemic disease, were both designed based on the structure of two viperid venom disintegrins, echistatin (Gan et al., 1988), and barbourin (Scarborough et al., 1993), respectively. Venoms from rear-fanged snakes also have the potential to contain compounds that could be used as pharmacological investigational tools and provide significant leads in drug design or development.

Drugs targeting coagulopathies

Snake venoms contain a vast array of pro- and anti-coagulants that exhibit potent interactions with the hemostatic system, leading researchers to examine these compounds for potential therapeutic use. Anti-coagulants in snake venoms include enzymatic proteins such as metalloproteinases, serine proteinases and phospholipase A_2 enzymes. Further, some non-enzymatic proteins such as C-type lectins and three-finger toxins have also demonstrated anti-coagulant functions

(Kini, 2006; Sajevic et al., 2011). Serine proteinases are abundant enzymes in numerous snake venoms, and certain classes of these enzymes, the thrombin-like enzymes (TLEs), demonstrate functional similarities to thrombin (Mackessy, 2010b). TLEs often target the plasma glycoprotein fibrinogen that is normally cleaved specifically by thrombin, releasing fibrinopeptides A and B and leading to fibrin polymerization and stabilization by factor XIIIa and the formation of insoluble clots (Tanaka et al., 2009). However, following viperid envenomations, a common sequelae is hypofibrinogenemia, and the loss of this critical clotting factor leads to uncontrolled and often severe hemorrhage and bleeding. A TLE isolated from the venom of *Calloselasma* (*Agkistrodon*) *rhodostoma*, ancrod (Au et al., 1993), showed promise for treating acute ischemic stroke by cleaving fibrinogen A(α), depleting competent fibrinogen and further inhibiting activation of factor XIII or any other coagulation components (Sherman, 2002). Initial results with ancrod (drug name viprinex) were promising; however, in late 2008, this compound failed phase III clinical trials, and is no longer being developed as a drug for human use in strokes. Further, the company developing viprinex (Neurobiological Technologies) filed for bankruptcy and ceased to exist shortly thereafter, a potent reminder of the labile nature of drug development.

Serine proteinase activity has been detected in several rear-fanged venoms (Assakura et al., 1994; Ching et al., 2006, 2012; Hill & Mackessy, 2000; Weldon & Mackessy, 2010), but activity-based assays, in addition to transcriptomic data, indicate that these enzymes are in relatively low abundance (<1% in *Thamnodynastes strigatus*, ~2.6% in *Philodryas olfersii*; Ching et al., 2006, 2012). Venoms from *P. o. olfersii*, *Trimorphodon biscutatus lambda*, *P. patagoniensis*, *P. baroni* and *A. portoricencis* all exhibited low serine proteinase activity toward thrombin-like paranitroaniline-derived peptide substrates. The most potent serine proteinase activity has been seen in *P. olfersii* venom (Peichoto et al., 2012), likely due to the serine proteinase PoFib S (Assakura et al., 1994). This 36 kDa, acidic (pI 4.5) proteinase hydrolyzed both the A(α) and B(β)-chains of fibrinogen and also exhibits fibrinolytic and moderate hemorrhagic activities. It further hydrolyzed only the Arg-Gly(22–23) peptide bond in oxidized insulin B-chain. Ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline, both metalloproteinase inhibitors, did not prevent PoFib S activity, but dithiothreitol (DTT) significantly inhibited the activity of this enzyme. Serine proteinase assays of rear-fanged snake venoms show higher activity towards paranitroaniline-coupled peptide substrates for thrombin-like serine proteases (TosylGlyProArg-pNA), whereas serine proteinase activity in rattlesnake venoms exhibits different substrate preferences, with the highest activity typically towards BzPheValArg-pNA substrate (Mackessy, 1993, 2008; Peichoto et al., 2012; Weldon & Mackessy, 2010). Prothrombin activators have been reported from *Dispholidus typus* venom long ago, but these remain poorly characterized (Hiestand & Hiestand, 1979; Mackessy 2002). This prothrombin activator may be the same protein as that detected in an early proteomic analysis of *Dispholidus* venom, which identified a 65 kD metalloprotease in the venom (Kamiguti et al., 2000). Many other venoms from rear-fanged snakes do not appear to exhibit thrombin-like properties (Hill & Mackessy, 2000).

Fibrin(ogen)olytic metalloproteinases may also be attractive candidates for potential thrombolytic drugs. Under normal physiological conditions, the serine proteinase plasmin is responsible for the degradation of fibrin clots, and therefore the disruption of clot formation may be a useful therapeutic option for thromboses and other clotting disorders. To date, the most promising fibrin(ogen)olytic drug from snake venom was derived from the 23 kDa zinc metalloproteinase, fibrolase. Isolated from *Agkistrodon contortrix contortrix* venom (Bajwa et al., 1982; Guan et al., 1991), fibrolase rapidly cleaves the A(α)-chain and at a slower rate the B (β)-chain of fibrinogen, and further exhibits similar activity towards fibrin, by rapidly cleaving the α-chain followed by a slower cleaving of the β-chain. Amgen developed alfimeprase, a recombinant form of fibrolase by truncating the first two amino acid residues and replacing the third residue, arginine, with a serine. Phase I and II clinical trials were promising; however, in 2008, alfimeprase did not meet expectations in Phase III trials, and the development of the compound was discontinued (Markland & Swenson, 2010).

Transcriptomic and proteomic analyses indicate that snake venom metalloproteinases (SVMPs) are one of the most abundant enzymes found in rear-fanged venoms (Ching et al., 2006, 2012; Peichoto et al., 2012; Weldon & Mackessy, 2010). Further, many purified SVMPs have direct fibrin (ogen)olytic activity, by cleaving either the A(α) or B(β)-chains of fibrinogen and cleaving fibrin (Peichoto et al., 2012). It also appears that most fibrin(ogen)olytic SVMPs hydrolyze the A(α) chain at cleavage sites different from the serine protease plasmin (Lu et al., 2005a). Although significant hemorrhagic activity is shown by most SVMPs, at least three (PoFibC1, C2, and C3) from *P. olfersii* venom exhibit fibrin(ogen)olytic proteolysis without hemorrhagic activity in mice at doses of 6 μg protein (Assakura et al., 1994). After 5 min incubation, PofibC1, a 47 kDa acidic (pI 6.2) SVMP, exhibited partial hydrolysis of A(α)-chain of fibrinogen, whereas proteolysis of fibrinogen by PofibC2 (47 kDa, pI 6.2) and a fourth hemorrhagic metalloproteinase PoFibH (58 kDa; pI 4.6) resulted in rapid degradation of the A(α)-chain after 30 s. These SVMPs showed no effect towards the B(β)-chain after 5 min incubation. Also from *P. olfersii* venom, the basic metalloproteinase PofibC3 (45 kDa; pI 8.5) rapidly degraded A(α)-chain within 30 s, and within 3 min it produced partial degradation of the B(β)-chain. In addition, all five enzymes isolated from *P. ofersii* venom (PoFibC1, C2, C3, H and the serine proteinase PoFib S mentioned above) digest the α-polymer and α-chains of fibrin, while the β and gamma chains are not affected (Assakura et al., 1994).

Similar to PoFibH, patagonfibrase, a 53.2 kDa (pI 5.8) SVMP from *Philodryas patagoniensis* venom, degraded the A (α)-chain of fibrinogen, classifying it as a α-fibrinogenase, and clots were not produced when patagonfibrase was incubated with plasma or fibrinogen. This enzyme appears to degrade fibrinogen, yet does not appear to produce fibrinopeptides A or B, hydrolytic steps which are necessary for fibrin polymerization. Patagonfibrase also caused prolonged clotting of human citrated plasma when incubated

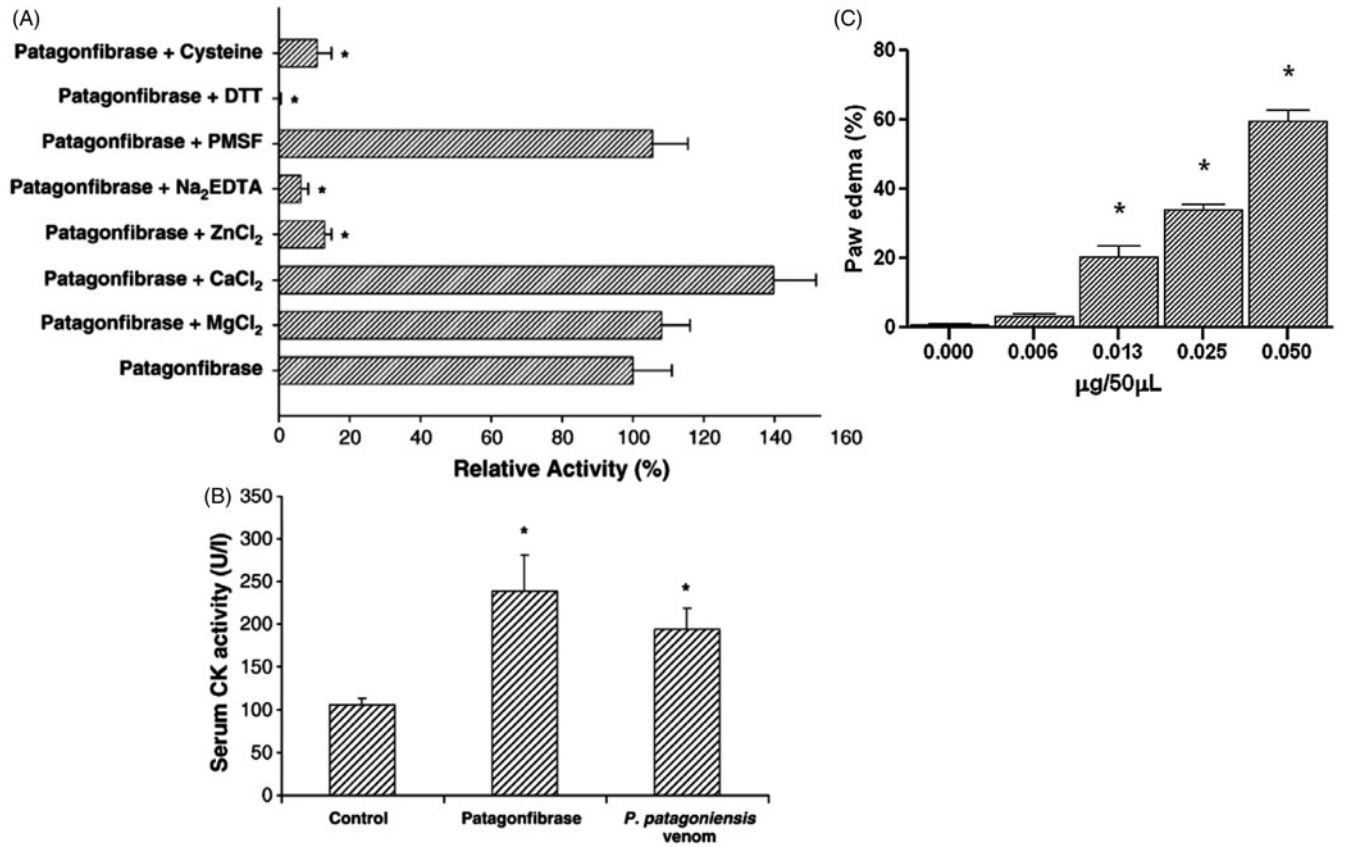

Figure 8. Activity of patagonfibrase, a PIII metalloprotease isolated from the venom of the dipsadid *Philodryas patagoniensis*. (A) Effects of metal ions and inhibitors on protease activity – Ca^{2+} stimulated activity, while Zn^{2+}, EDTA, DTT and cysteine were strongly inhibitory. (B) Patagonfibrase increased mouse serum CK levels by >2-fold; this activity was presumed to result from necrotic effects on skeletal muscle. (C) Intensity of mouse paws edema induced by different doses of patagonfibrase. A and B reproduced from Peichoto et al. (2007) and C from Peichoto et al. (2011b). *, significantly different from controls ($p < 0.05$).

with thrombin. The caseinolytic activity of patagonfibrase drastically increased in the presence of Ca^{2+}, whereas Zn^{2+}, cysteine, dithiothreitol and Na_2EDTA inhibited almost all activity (Figure 8A) (Peichoto et al., 2007). This SVMP caused hemorrhagic myonecrosis and edema when injected into mouse gastrocnemius muscle (Figure 8B) and hind paws (Figure 8C), respectively (Peichoto et al., 2007, 2011b).

Due to significant SVMP concentration, crude venom from *Alsophis portoricencis* (family Dipsadidae) showed immediate hydrolysis of the α subunit of fibrinogen, with slight degradation of the β subunit only after 60 min incubation (Weldon & Mackessy, 2010). Alsophinase, a basic, monomeric 56 kDa P-III SVMP purified from *A. portoricencis* venom was quite sensitive to the metal ion chelator 1,10-phenanthroline (Figure 9A), and it produced rapid cleavage of the α subunit of fibrinogen when incubated at a concentration of 1.5 µg/100 µl, indicating that this SVMP is an α-fibrinogenase. It showed 65% *N*-terminal sequence identity with patagonfibrase, and similar to other SVMPs (including fibrolase), it cleaved the Ala14–Leu15 bond of oxidized insulin B chain (Figure 9B; Weldon & Mackessy, 2012). However, the Tyr16–Leu17 bond was cleaved at a much lower rate, and unlike viperid SVMPs, no other cleavage fragments were observed, even following 24 h digestion. These results suggest that the specificity of

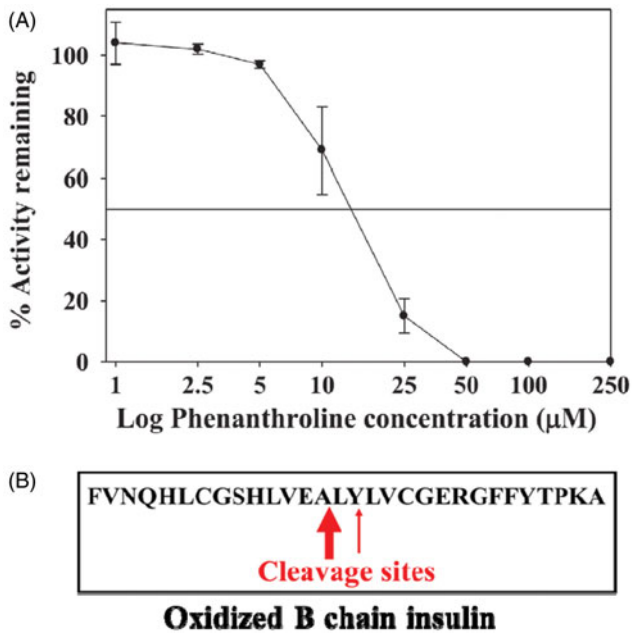

Figure 9. Activity of alsophinase, a PIII metalloprotease purified from the venom of the dipsadid *Alsophis portoricensis*. (A) Protease activity was strongly inhibited by the metal chelator 1,10-phenanthroline. (B) Alsophinase shows strong cleavage preference for the carboxyl side peptide bond of A14 (thick arrow) of the oxidized B chain of bovine insulin; the peptide bond of Y16 (thin arrow) is cleaved at a much lower frequency, and no other degradation products were observed. Reproduced from Weldon and Mackessy (2012).

alsophinase may be greater than observed for many other P-III SVMPs.

Because metalloproteinases are one of the most abundant enzymes in the transcriptome and proteome of rear-fanged snakes, further research may provide additional leads to possible drug development and therapeutic use of these enzymes. It should be noted that P-I or P-II classes of SVMPS precursors have not been identified in transcriptomes of *T. strigatus* or *P. olfersii* or in proteomes of any rear-fanged snake venom studied to date. However, snake venom matrix metalloproteinases (svMMPs) have recently been identified in the transcriptome and proteome of *T. strigatus* (Ching et al., 2012), and an apparent 38 kDa matrix-metalloproteinase was isolated from *Rhabdophis tigrinus tigrinus* (Komori et al., 2006), indicating that novel venom proteins are present in rear-fanged snake venoms, and these may demonstrate unique biological activities.

Additional snake venom proteins demonstrate potent interactions with components of the hemostatic system. C-type lectins target a wide array of coagulation factors, platelet receptors and other proteins critical to hemostasis (Lu et al., 2005a). Bothrojaracin, a C-type lectin-like protein from *Bothrops jararaca* venom, has been shown to bind to and exhibit potent inhibitory activity on thrombin (Monteiro et al., 2001). Botrocetin, also from *B. jararaca* venom, is used as an established reagent for examining von Willebrand factor (vWF)/platelet adhesion and is further used for detection of abnormalities leading to von Willebrand's disease, as well as glycoprotein Ib-vWF-related disorders such as Bernard–Soulier syndrome (Lu et al., 2005b). In rear-fanged snake venoms, full length sequences of C-type lectins have been reported from the venom gland transcriptome of *P. olfersii* (Ching et al., 2006) and more recently two isoforms of C-type lectin-like proteins have been identified in the proteome of *T. strigatus* (Ching et al., 2012), most likely corresponding to the alpha and beta subunits of this heterodimeric protein. True C-type lectins have also been found in the transcriptome and proteome of the rear-fanged snake *Cerberus rhynchops* (family Homalopsidae), as well as several new proteins, named ryncolins, speculated to have platelet aggregating or complement activating activities (OmPraba et al., 2010). However, no biological activities of any of these proteins have yet been examined. It is quite probable that other rear-fanged snake venoms also contain C-type lectin and C-type lectin-related proteins.

Venom disintegrins, which inhibit platelet aggregation and have led to the development of two current therapies (see above), are not found in rear-fanged snake venoms. However, incubation of the P-III SVMP patagonfibrase without Ca^{2+} resulted in an autolytic hydrolysis and release of the intact disintegrin-like and cysteine-rich domains (Peichoto et al., 2010). This has also been documented in *Philodryas olfersii*, with residue sequence from a 32 kDa protein matching only the disintegrin-like/cysteine-rich region (Ching et al., 2006), as well as with alsophinase from *A. portoricencis* venom (Weldon & Mackessy, 2012). Due to differences in disulfide bond structure near the integrin-binding site, the activity of these P-III disintegrin-like domains (if any) are likely drastically different from those of true disintegrins and disintegrin-containing proteins (Fox & Serrano, 2005).

Cysteine-rich secretory proteins and other toxins

Cyclic nucleotide-gated (CNG) channels are involved in signal transduction of sensory epithelium of visual and olfactory neurons (Zimmerman, 1995). In response to stimulus-induced changes within intracellular levels of cyclic nucleotides, CNG channels generate electrical signals in response to light or odor (Matulef & Zaggota, 2003; Zimmerman, 1995). CNG channels are also found in non-sensory tissue such as brain, kidney and endocrine tissues (Kaupp & Seifert, 2002); however, their physiological roles still remain relatively unknown (Biel et al., 1996; Distler et al., 1994). The isolation of pseudechetoxin, a 24 kDa CNG blocker and later identified as a cysteine-rich secretory protein (CRiSP), has led to advances in the understanding of CNG channels, and has stimulated research into a relatively new class of venom proteins – the venom CRiSPs (Brown et al., 1999). CRiSPs are 20–30 kDa, highly conserved monomeric proteins, broadly distributed in the venoms of many front and rear-fanged snakes. Although most CRiSP have unknown functions, the broad distribution of this protein among venoms suggests a significant biological role in venom (Mackessy, 2002). A recent proteome study involving *Philodryas baroni*, *P. o. olfersii*, *P. patagoniensis*, *Hypsiglena torquata texana* and *T. b. lambda* venoms indicated that CRiSPs are a major protein present in all five venoms (Peichoto et al., 2012; Figures 2 and 3). Sequence data have been reported for several CRiSPs from venoms of *Dispholidus typus*, *Liophis poecilogyrus*, *Philodryas olfersii*, *Trimorphodon biscutatus* (Fry et al., 2006), *Hydrodynastes gigas*, *Hypsiglena torquata* (Hill & Mackessy, 2000; Mackessy, 2002), *Rhabdophis tigrinus tigrinus* (Yamazaki et al., 2002) and *P. patagoniensis* (Peichoto et al., 2009).

Like all CRiSPs, those from rear-fanged venoms exhibit 16 highly conserved cysteine residues forming eight disulfide bonds (Heyborne & Mackessy, 2010), yet many of these proteins appear to have very different pharmacological activities. Some CRiSPs from front-fanged snake venoms are non-enzymatic proteins exhibiting specific blocking activities towards cyclic nucleotide-gated channels (Brown et al., 1999; Yamazaki et al., 2002), high conductance calcium-activated potassium channels (Wang et al., 2005) and L-type Ca^{2+}channels (Yamazaki et al., 2002). Patagonin, a 24.8 kDa CRiSP isolated from the venom of *P. patagoniensis*, showed unique necrotic activity toward murine gastrocnemius muscle when injected intramuscularly at doses of 43 and 87 μg, possibly by binding to the ion channels (Peichoto et al., 2009). However, at 20 μg, patagonin did not induce edema or hemorrhage, and it had no effect on the aggregation of human platelets or platelet-rich plasma (at concentrations as high as 100 nM). Patagonin did neither induce platelet aggregation directly nor inhibit platelet aggregation induced by ADP, collagen, convulxin, thrombin, ristocetin or the divalent cation ionophore A23187. This protein failed to inhibit collagen-induced platelet adhesion and showed no proteolytic activity toward azocoll, azocasein or fibrinogen.

Rear-fanged snake venom CRiSPs exhibited distinctive behavior on endothelium denuded rat thoracic aortic rings when compared to CRiSPs isolated from elapid and viperid venoms, as neither patagonin nor tigrin showed activity toward smooth muscle contractility (Peichoto et al., 2009). In normal Krebs-bicarbonate solution, patagonin (2 μM) did neither affect the basal tension of the denuded thoracic aortic rings, nor affect contractions of rat aortic smooth muscle induced by 60 mM K^+. Tigrin, a 30 kDa CRiSP from *Rhabdophis tigrinus tigrinus*, showed no effect on high K^+- or caffeine-induced contraction of helical strips of endothelium-free rat-tail arterial smooth muscle (Yamazaki et al., 2002). Other venom CRiSPs, such as albumin from *Agkistrodon blomhoffi* venom, may also exhibit activity towards L-type Ca^{2+}-channels (Yamazaki et al., 2002), which play a role in several important physiological processes. L-type Ca^{2+} channel blockers have received significant biomedical attention as they have been used to treat hypertension (Rich et al., 1992) and cardiac arrhythmias (Bodi et al., 2005). Since CRiSPs appear to be a common protein in the venoms of many rear-fanged snakes, further isolation and characterization of these proteins will not only increase our understanding of CRiSP-receptor interactions, but may also provide useful insights into novel therapeutics for targeting specific receptors such as CNG channels.

Additional compounds isolated from rear-fanged venoms may hold promise for development into useful anti-hypertension therapeutics. Precursors of hypotensive and vasodilator agents such as natriuretic peptides (NPs) have been identified in front-fanged snake venoms (Higuchi et al., 1999; Schweitz et al., 1992). The C-type natriuretic peptides (CNP), which act as biological messengers and hypotensive/vasodilator agents, have been identified in a several organisms, and their ability to control blood vessel tone has received significant attention (Barr et al., 1996; Lumsden et al., 2010). Analyses of *P. ofersii* venom gland identified relatively high abundance (~6.6% of expressed sequence tags) of CNP precursors that exhibit N-terminal sequences similar to elapid venom NPs, with C-terminal sequence similar to viperid venom CNP precursors (Ching et al., 2006). The identification of CNPs in a rear-fanged snake venom suggests the potential for discovery of other CNPs in rear-fanged venoms; additionally, it helps clarify the evolutionary links between rear-fanged colubrid venom CNPs and the NPs of elapid and viperid snake venoms. As NPs isolated from snake venoms exhibit significant differences in structure and function compared to mammalian NPs, they may represent possible therapeutic options for treatment of hypertension.

Paralytic toxins

Three-finger toxins (3FTxs), including the well-characterized α-neurotoxins, are 60–79 amino acid non-enzymatic proteins common in the proteome of elapid venoms (Kini & Doley, 2010; Hegde et al., 2010; Nirthanan & Gwee, 2004). However, it is now apparent that these compounds are often abundant in the venoms of many rear-fanged snakes (Figures 10 and 11; Fry et al., 2003a, 2008; Heyborne & Mackessy, 2013; Lumsden et al., 2005; Mackessy, 2002; Pawlak et al., 2006, 2009; Weinstein et al., 1993). The often subtle differences in non-structural residues of 3FTxs allow different members of this protein family to recognize, with a high degree of specificity, an array of targets such as nicotinic and muscarinic acetylcholine receptors (mAChRs; Kini & Doley, 2010), the integrin $\alpha_{IIb}\beta_3$, L-type Ca^{2+} channels (Kini, 2002), coagulation factor VIIa (Banerjee et al., 2005) and β1/β2-adrenergic receptors (Rajagopalan et al., 2007). The wide array of pharmacological activities on a conserved molecular fold has made these proteins important models for structure–function studies and has provided significant insights into protein–receptor interactions, with high potential for novel drug design. The mAChRs M1 and M4 have been

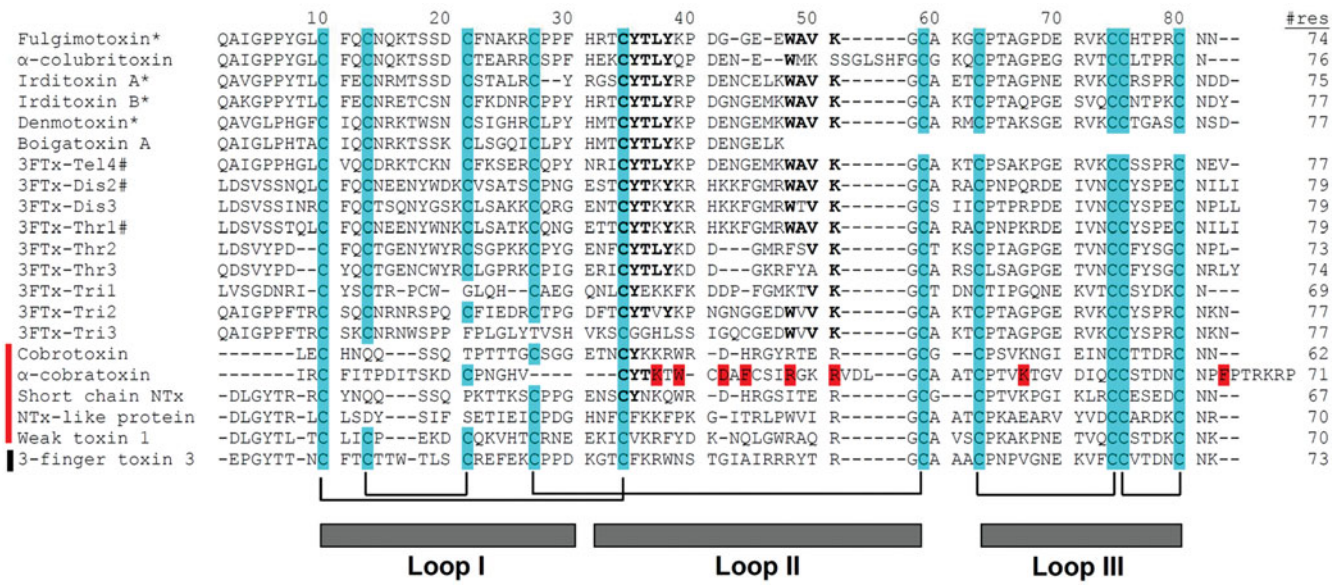

Figure 10. Sequence alignment of three-finger toxins (3FTxs) from several rear-fanged snake venoms and from five elapid venoms (red bar) and one viperid venom (black bar). The bolded residues in loop II of rear-fanged snake toxins (asterisk) with taxon-specific effects toward lizards and birds are absent from most rear-fanged snake 3FTxs and all elapid and viperid 3FTxs. Loop II residues of α-cobratoxin in red boxes are known to be involved in receptor binding. Reproduced from Heyborne and Mackessy (2013).

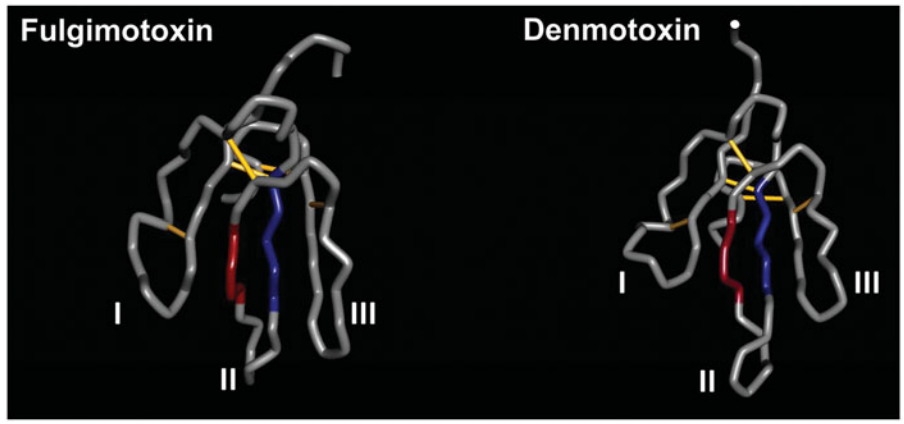

Figure 11. Backbone structural models of taxon-specific three-finger toxins from a neotropical colubrid (fulgimotoxin: *Oxybelis fulgidus*) and an Asian colubrid (denmotoxin: *Boiga dendrophila*). Note that both show three-dimensional structures very similar to elapid three-finger toxins. The red- (WAVK) and blue-colored (CYTLY) regions of loop II are hypothesized to be involved in the taxon-specific effects of these toxins (Heyborne & Mackessy, 2013). The five disulfides which stabilize the canonical scaffold are shown in yellow.

important pharmaceutical targets for conditions such as Alzheimer's and Parkinson's diseases, respectively; however, finding compounds with specific selectivity for mAChR subtypes has been challenging (Bradley, 2000). The production of chimeric 3FTxs with novel pharmacological activity toward muscarinic receptors has shown occasionally to exceed binding affinities expressed by the natural toxins (Fruchart-Gaillard et al., 2012), and so the possibility exists that the 3FTx scaffold could serve as a mutable template for drug discovery efforts.

Neurotoxic effects of colubrid venoms towards neuromuscular junctions have been documented in numerous species, and this activity appears to follow phylogenetic patterns (Lumsden et al., 2004). At 10 μg/ml, venoms from *Boiga cynodon*, *B. d. dendrophilia*, *B. d. gemmicincta*, *B. drapiezii*, *B. irregularis*, *B. nigriceps*, *Telescopus dhara* and *Trimorphodon biscutatus* induced time-dependent inhibition of indirect twitches of chick isolated biventer cervicis nerve muscle. At the same concentrations, all of these venoms (except *Trimorphodon biscutatus*) inhibited contractile responses to acetylcholine (1 mM) and carbachol (20 μM) but not potassium chloride (40 mM; Lumsden et al., 2004). In the same study, venom of *Psammophis mossambicus* showed time-dependent inhibition of indirect twitches; however, this activity was reversed after 30 min incubation. *Ahaetulla prasina*, *Enhydris chinensis* and *Lioheterodon madagascariensis* venoms (10 μg/ml) all lacked inhibitory effects on indirect twitches as well as on contractile responses.

α-Colubritoxin, purified from *C. radiatus* venom is an 8.49 kDa, 79 amino acid 3FTx that exhibits reversible antagonism at the nicotinic acetylcholine receptor (Fry et al., 2003b). This reversibility differs from α-bungarotoxin from the elapid *Bungarus multicinctus*, which is significantly limited as an investigational pharmaceutical tool due to its irreversibility. Boigatoxin-A, from *Boiga dendrophilia* venom, an 8.7 kDa 3FTx, also exhibited weak reversible post-synaptic blockage as indicated by inhibition of indirect twitches to acetylcholine (1 mM) and carbachol (20 μM), in addition to producing a reversible inhibition of electrically stimulated twitches of the prostatic segment of the rat vas deferens preventing the release of neurotransmitters (Lumsden et al., 2005). Isolated from the venom of *Rhamphiophis oxyrhynchus*, rufoxin, a 10.66 kDa neurotoxin, showed time-dependent inhibition of indirect twitches of chick-biventer cervicis nerve-muscle preparation, with partial recovery of twitch height after 60 min washing. Rufoxin also significantly inhibited contractions to nicotinic receptor agonist such as acetylcholine and carbachol, but not potassium chloride. Interestingly, this neurotoxin lacks N-terminus sequence homology with other rear-fanged neurotoxins such as α-colubritoxin, boigatoxin-A and denmotoxin (Lumsden et al., 2007).

Taxon-specific effects of crude venom suggested that for rear-fanged snake venoms, the inbred mouse model was likely insufficient to evaluate biologically relevant pharmacological effects (Mackessy et al., 2006). Denmotoxin, another 3FTx from *B. dendrophilia* venom, contains 77 amino acid residues, has a mass of 8.5 kDa, shares less than 30% sequence homology with elapid 3FTXs and has approximately 50% homology with α-colubritoxin. It exhibits potent and irreversible neuromuscular blockade of chick biventer cervicis nerve muscle. Denmotoxin showed approximately 100-fold weaker and reversible inhibition of indirectly stimulated twitches in mouse hemidiaphragm nerve-muscle preparations and was unable to produce complete blockage (Pawlak et al., 2006). These results demonstrate that denmotoxin is able to discriminate between the peripheral nicotinic acetylcholine receptors from two distinct prey types (birds versus mammals), and toxin specificity correlates with the feeding ecology of these snakes (Pawlak et al., 2006). Similarly, irditoxin, a covalently linked heterodimeric 3FTx from *B. irregularis* venom, induced taxon-specific lethality via respiratory paralysis in both chicks (LD_{50} = 0.22 μg/g) and lizards (LD_{50} = 0.55 μg/g), indicating a peripheral post-synaptic neurotoxic effect. However, irditoxin was non-toxic to mammalian prey (*Mus musculus*) at doses as high as 25 μg/g. Irditoxin also showed potent post-synaptic neuromuscular inhibition of avian skeletal muscle, and this effect was three orders of magnitude lower on mammalian motor endplate preparations (Pawlak et al., 2009). Another rear-fanged 3FTx with taxon specificity was recently purified from the venom of the Green Vinesnake (*Oxybelis fulgidus*), and its structural features were analyzed for clues concerning the observed specific toxicity toward lizards (Heyborne & Mackessy, 2013). Comparative analyses of 3FTxs from many colubrid and elapid snakes indicated that only those toxins with known taxon-specific effects contained two canonical sequences in loop two: CYTLY and WAVK (Figure 10). This same region of loop II has been shown to be critical to

acetylcholine receptor-binding of α-cobratoxin, strongly suggesting that this region of the toxin is important to rear-fanged 3FTx binding as well. Molecular modeling of fulgimotoxin and comparison with the X-ray crystal-based structure of denmotoxin further showed that these sequences occur in the same place within a highly spatially conserved region of loop II (Figure 11). Further, modifications of 3FTx loops can drastically influence binding affinity to specific receptors. For example, synthesis of a chimeric 3FTx with an additional loop on the central finger increased toxin binding to neuronal α7 AchR by 20-fold when compared to the native toxin (Mourier et al., 2000). In addition, loop grafting of loops 1 and 3 of muscarinic toxin 7 increased affinities towards α_{1A}-adrenoceptor significantly, up to 6000 times greater than that exhibited by the native toxin (Fruchart-Gaillard et al., 2012). It is now becoming clear that 3FTxs are abundant and important components in the venoms of rear-fanged snakes, particularly colubrine colubrids, and with their unique receptor specificities, taxon-specific toxins may have utility in design of compounds for potential therapeutics.

Phospholipase A_2

Phospholipase A_2 (PLA_2) enzymes are esterolytic enzymes that are one of the major pharmacologically active compounds found in reptile venoms (Mackessy, 2010a). They are one of the best-studied venom compounds, and these enzymes induce varying pharmacological effects which disrupt normal physiological processes (Kini, 1997); a single venom may have several different isozymes with distinct activities, some of which are independent of any enzyme hydrolytic activity. Significant research has characterized many PLA_2 enzymes from the venoms of elapids and viperids (refer Doley et al., 2010 for a review), yet most rear-fanged snake venoms were once thought to lack PLA_2 activity (Weinstein & Kardong, 1994). However, further research suggests that these enzymes are broadly (if not commonly) distributed among these venoms. In fact, PLA_2 activity has been detected in venoms of *Boiga dendrophila*, *Diadophis punctatus regalis*, *D. typus*, *Leptodeira annulata*, *Malpolon monspessulanus*, *Philodryas nattereri*, *P. olfersii*, *P. patagoniensis*, *Psammophis mossambicus* (very low activity), *Rhabdophis subminiata*, *Telescopus dhara, Thelotornis capensis* (very low activity), *Thamnodynastes strigatus*, *Tomodon dorsatus* and *Trimorphodon biscutatus lambda* (Broaders & Ryan, 1997; Christensen, 1968; DuBourdieu et al., 1987; Durkin et al., 1981; Ferlan et al., 1983; Hill & Mackessy, 2000; Huang & Mackessy, 2004; Lumsden et al., 2004; Mebs, 1968; Rosenberg et al., 1985; Zelanis et al., 2010). In a transcriptomic analysis of *P. olfersii* venom gland, transcripts coding for PLA_2 were not observed (Ching et al., 2012), and at present, non-enzymatic PLA_2 toxins have not been detected in rear-fanged snake venoms.

The first PLA_2 characterized and partially sequenced from a rear-fanged venom was trimorphin, a monomeric 13.9 kDa PLA_2 (Figure 12A) from *T. biscutatus lambda* (Huang & Mackessy, 2004). This PLA_2 shared a high degree of sequence homology with the group IA PLA_2s, particularly the Asp-49 enzymes characterized from several hydrophiine elapid venoms, and had optimal activity towards 4-nitro-3 -(octanoyloxy) benzoic acid substrate at pH of 7.5 (Figure 12B; Huang & Mackessy, 2004). More recently, this enzyme was shown to have potential application as an anti-leishmanial drug, and *in vitro* assays showed potent cytotoxicity ($IC_{50} = 0.25\,\mu M$; Figure 12C) toward log-phase promastigote stage *Leishmania major* (Peichoto et al., 2011a). As *T. b. lambda* venom does not show cytotoxicity toward an immortal, mammalian cell line (MCF-7 breast cancer; Bradshaw et al., *in review*), it appears that effects of trimorphin may be specific for *Leishmania* parasites.

Cytotoxic effects of rear-fanged snake venoms

Many snake venoms are generally cytotoxic, often due to the presence of high levels of PLA_2. However, a screen of several different colubrid venoms indicated that they generally lacked cytotoxicity toward human melanoma (A375) cell lines (Figure 13). However, this lack of cytotoxicity does not mean these venoms should be disregarded for further examination of potential anti-neoplastic therapeutics. Compounds in these venoms may inhibit or slow cell proliferation when incubated with cells for relatively longer periods of time, or they may impede pathways critical for metastasis without exhibiting any cytoxicity towards cell lines, as is observed with disintegrins such as vicrostatin (Minea et al., 2010).

Anti-parasitic effects

Five venoms from rear-fanged snake species were recently evaluated for potential anti-leishmanial activity. Several species of the protistan parasites in the genus *Leishmania* cause disfiguring cutaneous diseases in humans, and several others cause debilitating (and sometimes fatal) visceral leishmaniasis (Desjeux, 1992; Rath et al., 2003). Exposure to relatively high levels of rear-fanged snake venoms resulted in cytotoxicity toward cultured promastigote stages of *L. major*, but venom of only one species tested, *T. b. lambda*, showed significant cytotoxicity at lower doses (Peichoto et al., 2011a). It was also shown that the PLA_2, trimorphin (mentioned above), previously isolated from this venom (Huang & Mackessy, 2004) was responsible for potent cytotoxic effects, as demonstrated by the effects of the purified toxin (Figure 8C).

Future directions in rear-fanged snake venom research

Investigations of venom systems allow for a detailed understanding of the biological roles of venom compounds (Saviola et al., 2013) and evolutionary relationships among venomous snakes (Lomonte et al., 2014), and it provides information that may be utilized for novel drug discovery (Takacs & Nathan, 2014; Vonk et al., 2011). As trophic adaptations, venoms can be expected to vary significantly based on diet, as different prey types are differentially sensitive to specific toxins. The broad array of prey preferences seen among rear-fanged snakes (Greene, 1997) suggested that venom composition may vary drastically compared to elapids or viperids, and we are beginning to see that this prediction holds true, as exemplified by several novel venom protein families found in rear-fanged snake venoms (Ching et al., 2012;

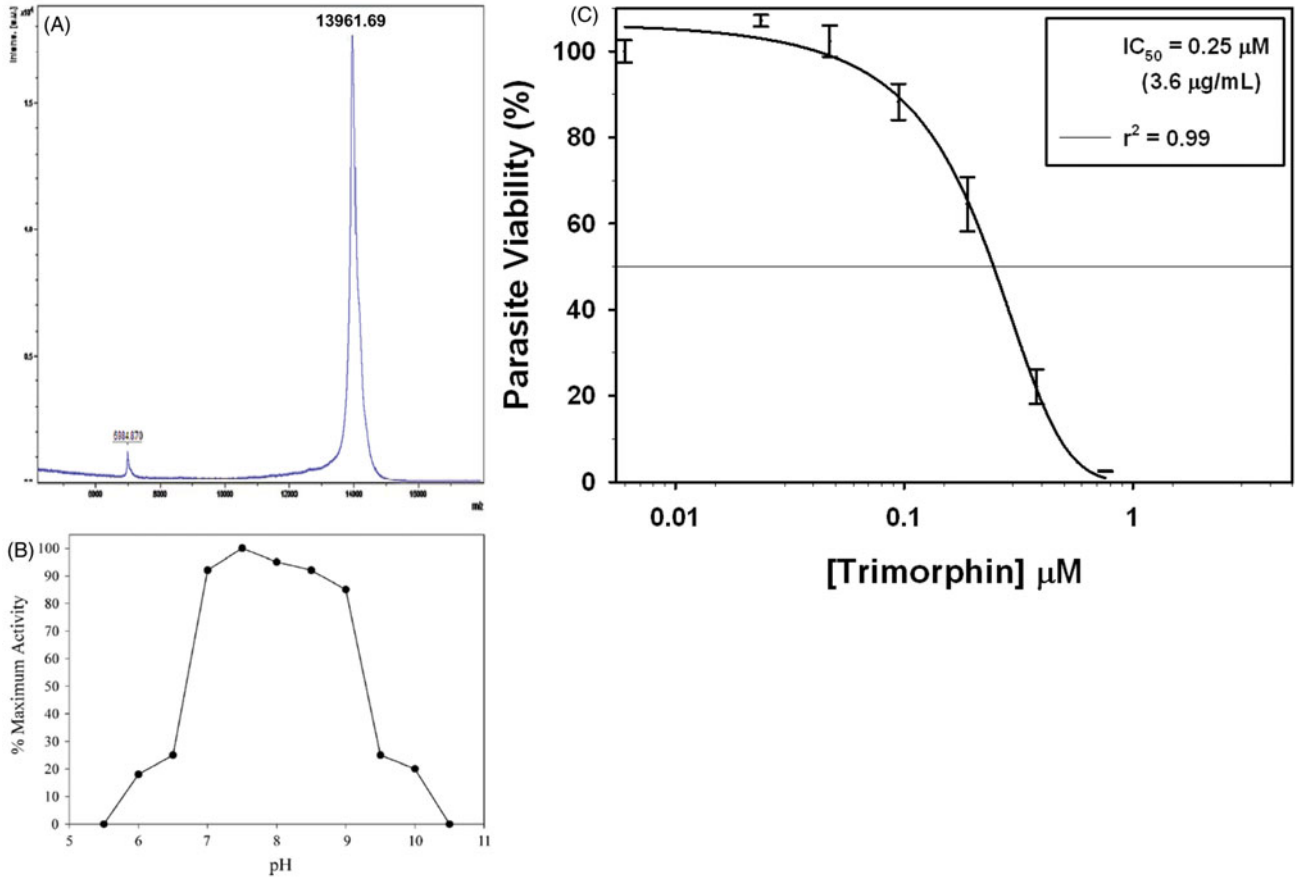

Figure 12. (A) MALDI-TOF MS analysis of trimorphin, a PLA_2 isolated from the venom of the Sonoran Lyre Snake (*Trimorphodon biscutatus lambda*). (B) Trimorphin shows a broad pH optimum toward synthetic substrates. (C) Potent *in vitro* anti-leishmanial effects of trimorphin toward log-phase promastigote stage of *L. major.* A and C reprinted from Peichoto et al. (2011a) and B from Huang and Mackessy (2004).

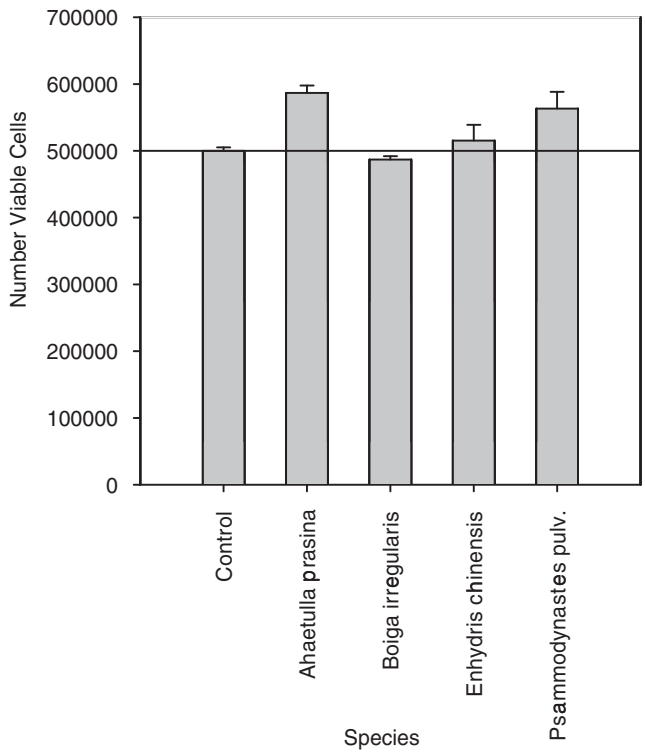

Figure 13. The 24 h cytotoxicity assays of selected rear-fanged snake venoms toward human A375 melanoma cancer cells. Note that the venoms show no cytotoxicity. *Psammodynastes pulv.*, *Psammodynastes pulverulentus*. Reproduced from Bradshaw et al. (in review).

OmPraba et al., 2010). Although rear-fanged snake venoms still remain relatively understudied, within the last decade, research investigating these venoms has significantly increased, providing a better understanding of the composition, functions and biological roles of venoms generally. These venoms are proving to be rich sources of compounds with potent biological activities, and they represent a vast and largely untapped source of toxin diversity which is likely to contain further novel compounds and new pharmacological activities. Proper evaluations of the biological activities of venom compounds are essential to further our understanding of and therapeutic benefit from these components. As venomic approaches (Calvete, 2013) are applied to rear-fanged snake venoms, identification and characterization of proteins and peptides will accelerate, and discovery of unique biochemical and pharmacological properties may also lead to the development of novel protein drugs. Further transcriptomic and proteomic analyses of these venoms, coupled with functional assays of venom proteins, will also help clarify our understanding of evolutionary trends among all venomous snakes, as well as identify species that may be of medical importance with regards to human envenomations.

Acknowledgements

Our work with venoms from rear-fanged snakes has benefited immensely from collaborations and discussions with numerous colleagues, including J. J. Calvete, T. Castoe,

J. M. Gutiérrez, R. M. Kini, B. Lomonte, L. Sanz, M. Sasa and many students at UNC.

Declaration of interest

Financial support for this work was provided in part by a BioScience Discovery grant from the Colorado Office of Economic Development and International Trade (SPM) and by Agencia Nacional de Promoción Científica y Tecnológica, PICT-2010-1908 (MEP).

References

Assakura MT, Reichl AP, Mandelbaum FR. (1994). Isolation and characterization of five fibrin(ogen)olytic enzymes from the venom of *Philodryas olfersii* (green snake). Toxicon 32:819–31.

Au LC, Lin SB, Chou JS, et al. (1993). Molecular cloning and sequence analysis of the cDNA for ancrod, a thrombin-like enzyme from the venom of *Calloselasma rhodostoma*. Biochem J 294:387–90.

Bajwa SS, Kirakossian H, Reddy KN, et al. (1982). Thrombin-like and fibrinolytic enzymes in the venoms from the Gaboon viper (*Bitis gabonica*), Eastern cottonmouth moccasin (*Agkistrodon p. piscivorus*) and Southern copperhead (*Agkistrodon c. contortrix*) snakes. Toxicon 20:427–32.

Banerjee Y, Mizuguchi J, Iwanaga S, et al. (2005). Hemextin AB complex-a unique anticogulant protein complex from *Hemachatus haemachatus* (African Ringhals cobra) venom that inhibits clot initiation and factor VIIa activity. J Biol Chem 280:42601–11.

Barr CS, Rhondes P, Struthers AD. (1996). C-type natriuretic peptide. Peptides 17:1243–51.

Biel M, Zong X, Ludwig A, et al. (1996). Molecular cloning and expression of the modulatory subunit of the cyclic nucleotide-gated cation channel. J Biol Chem 271:6349–55.

Bodi I, Mikala G, Koch SE, et al. (2005). The L-type calcium channel in the heart: the beat goes on. J Clin Invest 115:3306–17.

Bradley KN. (2000). Muscarinic toxins from the green mamba. Pharmacol Ther 85:87–109.

Broaders M, Ryan MF. (1997). Enzymatic properties of the Duvernoy's secretion of Blanding's tree snake (*Boiga blandingi*) and of the mangrove snake (*Boiga dendrophila*). Toxicon 35:1143–8.

Brown RL, Haley TL, West KA, et al. (1999). Pseudechetoxin: a peptide blocker of cyclic nucleotide-gated ion channels. Proc Natl Acad Sci USA 96:754–75.

Calvete JJ. (2013). Snake venomics: from the inventory of toxins to biology. Toxicon 75:44–62.

Ching AT, Rocha MM, Paes Leme AF, et al. (2006). Some aspects of the venom proteome of the Colubridae snake *Philodryas olfersii* revealed from a Duvernoy's (venom) gland transcriptome. FEBS Lett 580: 4417–22.

Ching AT, Paes Leme AF, Zalanis A, et al. (2012). Venomics profiling *of Thamnodynastes strigatus* unveils matrix metalloproteinases and other novel proteins recruited to the toxin arsenal of rear-fanged snakes. J Proteome Res 11:1152–62.

Christensen PA. (1968). The venoms of Central and South African snakes. In: Bucherl W, Buckley EE, Deulofeu V, eds. Venomous animals and their venoms. New York: Academic Press, 437–61.

Desjeux P. (1992). Human leishmaniases: epidemiology and public health aspects. World Health Stat Q 45:267–75.

Distler M, Biel M, Flockerzi V, et al. (1994). Expression of cyclic nucleotide-gated cation channels in non-sensory tissues and cells. Neuropharmacology 33:1275–82.

Doley R, Zhou X, Kini RM. (2010). Snake venom phospholipase A_2 enzymes. In: Mackessy SP, ed. Handbook of venoms and toxins of reptiles. Boca Raton: CRC Press/Taylor & Francis Group, 173–205.

DuBourdieu DJ, Kawaguchi H, Shier WT. (1987). Molecular weight variations in the diversity of phospholipase A_2 forms in reptile venoms. Toxicon 25:333–43.

Durkin JP, Pickwell GV, Trotter JT, et al. (1981). Phospholipase A2 electrophoretic variants in reptile venoms. Toxicon 19:535–46.

Ferlan I, Ferlan A, King T, Russell FE. (1983). Preliminary studies on the venom of the colubrid snake *Rhabdophis subminatus* (red-necked keelback). Toxicon 21:570–4.

Fox JW, Serrano SM. (2005). Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. Toxicon 45:969–85.

Fox JW, Serrano SM. (2007). Approaching the golden age of natural product pharmaceuticals from venom libraries: an overview of toxins and toxin-derivatives currently involved in therapeutic or diagnostic applications. Curr Pharm Des 13:2927–34.

Fruchart-Gaillard C, Mourier G, Blanchet G, et al. (2012). Engineering of three-finger fold toxins creates ligands with original pharmacological profiles for muscarinic and adrenergic receptors. PLoS One 7: e39166.

Fry BG, Wüster W, Ryan Ramjan SF, et al. (2003a). Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications. Rapid Commun Mass Spectrom 17:2047–62.

Fry BG, Lumsden NG, Wüster W, et al. (2003b). Isolation of a neurotoxin (a-colubritoxin) from a 'nonvenomous' colubrid: evidence for early origin of venom in snakes. J Mol Evol 57:446–52.

Fry BG, Vidal N, Norman JA, et al. (2006). Early evolution of the venom system in lizards and snakes. Nature 439:584–8.

Fry BG, Scheib H, van der Weerd L, et al. (2008). Evolution of an arsenal structural and functional diversification of the venom system in the advanced snakes (Caenophidia). Mol Cell Proteomics 7:215–46.

Gan ZR, Gould RJ, Jacobs JW, et al. (1988). Echistatin. A potent platelet aggregation inhibitor from the venom of the viper, *Echis carinatus*. J Biol Chem 263:19827–32.

Greene HW. (1997). Snakes: the evolution of mystery in nature. Berkeley, CA: University of California Press.

Guan AL, Retzios AD, Henderson GN, et al. (1991). Purification and characterization of a fibrinolytic enzyme from venom of the southern copperhead snake *Agkistrodon contortrix contortrix*. Arch Biochem Biophys 289:197–207.

Hegde RP, Rajagopalan N, Doley R, Kini RM. (2010). Snake venom three finger toxins. In: Mackessy SP, ed. Handbook of venoms and toxins of reptiles. Boca Raton: CRC Press/Taylor & Francis Group, 287–301.

Heyborne WH, Mackessy SP. (2010). Cysteine-rich secretory proteins in reptile venoms. In: Mackessy SP, ed. Handbook of venoms and toxins of reptiles. Boca Raton: CRC Press/Taylor & Francis Group, 325–36.

Heyborne WH, Mackessy SP. (2013). Identification and characterization of a taxon-specific three-finger toxin from the venom of the Green Vinesnake *Oxybelis fulgidus*; family Colubridae). Biochimie 95: 1923–32.

Hiestand PC, Hiestand RR. (1979). *Dispholidus typus* (boomslang) snake venom: purification and properties of the coagulant principle. Toxicon 17:489–98.

Huang P, Mackessy SP. (2004). Biochemical characterization of phospholipase A2 (trimorphin) from the venom of the Sonoran Lyre Snake *Trimorphodon biscutatus lambda* (family Colubridae). Toxicon 44:27–36.

Higuchi S, Murayama N, Saguchi K, et al. (1999). Bradykinin-potentiating peptides and C-type natriuretic peptides from snake venom. Immunopharmacology 44:129–35.

Hill RE, Mackessy SP. (1997). Venom yields from several species of colubrid snakes and differential effects of ketamine. Toxicon 35: 671–8.

Hill RE, Mackessy SP. (2000). Characterization of venom (Duvernoy's secretion) from twelve species of colubrid snakes and partial sequence of four venom proteins. Toxicon 38:1663–87.

Kardong KV, Lavin-Murcio PA. (1993). Venom delivery of snakes as high-pressure and low-pressure systems. Copeia 644–50.

Kamiguti AS, Theakston RDG, Sherman N, Fox JW. (2000). Mass spectrophotometric evidence for P-III/P-IV metalloproteinases in the venom of the boomslang (*Dispholidus typus*). Toxicon 38:1613–20.

Kaupp UB, Seifert R. (2002). Cyclic nucleotide-gated ion channels. Physiol Rev 82:769–824.

Kini RM. (1997). Phospholipase A_2: a complex multifunctional protein puzzle. In: Kini RM, ed. Venom phospholipase A_2 enzymes: structure, function and mechanism. New York: Wiley, 1–28.

Kini RM. (2002). Molecular molds with multiple missions: functional sites in three-finger toxins. Clin Exp Pharmacol Physiol 29:815–22.

Kini RM. (2006). Anticoagulant proteins from snake venoms: structure, function and mechanism. Biochem J 397:377–87.

Kini RM, Doley R. (2010). Structure, function and evolution of three-finger toxins: mini proteins with multiple targets. Toxicon 56:855–67.

Kochva E. (1965). The development of the venom gland in the opisthoglyph snake *Telescopus fallax* with remarks on *Thamnophis sirtalis* (Colubridae, Reptilia). Copeia 1965:147–54.

Komori K, Konishi M, Maruta Y, et al. (2006). Characterization of a novel metalloproteinase in Duvernoy's gland of *Rhabdophis tigrinus tigrinus*. J Toxicol Sci 31:157–68.

Lomonte B, Tsai WC, Ureña-Diaz JM, et al. (2014). Venomics of New World pit vipers: Genus-wide comparisons of venom proteomes across *Agkistrodon*. J Proteomics 96:103–16.

Lu Q, Clemetson JM, Clemetson KJ. (2005a). Snake venoms and hemostasis. J Thromb Haemost 3:1791–9.

Lu Q, Navdaev A, Clemetson JM, et al. (2005b). Snake venom C-type lectins interacting with platelet receptors. Structure–function relationships and effects on haemostasis. Toxicon 45:1089–98.

Lumsden NG, Fry BG, Kini RM, et al. (2004). In vitro neuromuscular activity of 'colubrid' venoms: clinical and evolutionary implications. Toxicon 43:819–27.

Lumsden NG, Fry BG, Ventura S, et al. (2005). Pharmacological characterisation of a neurotoxin from the venom of *Boiga dendrophila* (Mangrove catsnake). Toxicon 45:329–34.

Lumsden NG, Banerjee Y, Kini RM, et al. (2007). Isolation and characterization of rufoxin, a novel protein exhibiting neurotoxicity from venom of the psammophiine, *Rhamphiophis oxyrhynchus* (Rufous beaked snake). Neuropharmacology 52:1065–70.

Lumsden NG, Khambata RS, Hobbs AJ. (2010). C-type natriuretic peptide (CNP): cardiovascular roles and potential as a therapeutic target. Curr Pharm Des 16:4080–8.

Mackessy SP. (1991). Morphology and ultrastructure of the venom glands of the northern Pacific rattlesnake *Crotalus viridis oreganus*. J Morphol 208:109–28.

Mackessy SP. (1993). Kallikrein-like and thrombin-like proteases from the venom of juvenile northern Pacific rattlesnakes (*Crotalus viridis oreganus*). J Nat Toxins 2:223–39.

Mackessy SP. (2002). Biochemistry and pharmacology of colubrid snake venoms. J Toxicol Toxin Rev 2:43–83.

Mackessy SP. (2008). Venom composition in rattlesnakes: trends and biological significance. In: Hayes WK, Cardwell MD, Beaman KR, Bush SP, eds. The biology of rattlesnakes. Loma Linda: Loma Linda University Press, 495–510.

Mackessy SP. (2010a). The field of reptile toxinology: snakes, lizards and their venoms. In: Mackessy SP, ed. Handbook of venoms and toxins of reptiles. Boca Raton: CRC Press/Taylor & Francis Group, 3–23.

Mackessy SP. (2010b). Thrombin-like enzymes in snake venoms. In: Kini RM, Clemetson KJ, Markland FS, McLane MA, Morita T, eds. Toxins and hemostasis: from bench to bedside. Berlin: Springer-Verlag, 519–57.

Mackessy SP, Sixberry NM, Heyborne WH, Fritts T. (2006). Venom of the Brown Treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. Toxicon 47:537–48.

Markland FS, Swenson S. (2010). Fibrolase: trials and tribulations. Toxins 2:793–808.

Matulef K, Zagotta WN. (2003). Cyclic nucleotide-gated ion channels. Annu Rev Cell Dev Biol 19:23–44.

Mebs D. (1968). Analysis of *Leptodeira annulata* venom. Herpetologica 1968:338–9.

Minea RO, Helchowski CM, Zidovetzki SJ, et al. (2010). Vicrostatin – an anti-invasive multi-integrin targeting chimeric disintegrin with tumor anti-angiogenic and pro-apoptotic activities. PLoS One 5:e10929.

Monteiro RQ, Bock PE, Bianconi ML, et al. (2001). Characterization of bothrojaracin interaction with human prothrombin. Protein Sci 10: 1897–904.

Mourier G, Servent D, Zinn-Justin S, et al. (2000). Chemical engineering of a three-fingered toxin with anti-α7 neuronal acetylcholine receptor activity. Protein Eng 13:217–25.

Mukherjee AK, Saikia D, Thakur R. (2011). Medical and diagnostic applications of snake venom proteomes. J Proteins Proteomics 2: 31–40.

Nirthanan S, Gwee MCE. (2004). Three-finger a-neurotoxins and the nicotinic acetylcholine receptor, forty years on. J Pharm Sci 94: 1–17.

OmPraba G, Chapeaurouge A, Doley R, et al. (2010). Identification of a novel family of snake venom proteins Veficolins from *Cerberus rynchops* using a venom gland transcriptomics and proteomics approach. J Proteome Res 9:1882–93.

Parkes DG, Mace KF, Trautmann ME. (2013). Discovery and development of exenatide: the first antidiabetic agent to leverage the multiple benefits of the incretin hormone, GLP-1. Expert Opin Drug Disc 8: 219–44.

Pawlak J, Mackessy SP, Fry BG, et al. (2006). Denmotoxin, a three-finger toxin from the colubrid snake *Boiga dendrophila* (mangrove catsnake) with bird-specific activity. J Biol Chem 281:29030–41.

Pawlak J, Mackessy SP, Sixberry NM, et al. (2009). Irditoxin, a novel covalently linked heterodimeric three finger toxin with high taxon-specific neurotoxicity. FASEB J 23:534–45.

Peichoto ME, Teibler P, Mackessy SP, et al. (2007). Purification and characterization of patagonfibrase, a metalloproteinase showing α-fibrinogenolytic and hemorrhagic activities, from *Philodryas patagoniensis* snake venom. Biochim Biophys Acta 1770:810–19.

Peichoto ME, Mackessy SP, Teibler P, et al. (2009). Purification and characterization of a cysteine-rich secretory protein from *Philodryas patagoniensis* snake venom. Comp Biochem Physiol C Toxicol Pharmacol 150:79–84.

Peichoto ME, Leme AFP, Pauletti BA, et al. (2010). Autolysis at the disintegrin domain of patagonfibrase, a metalloproteinase from *Philodryas patagoniensis* (Patagonia Green Racer; Dipsadidae) venom. BBA-Proteins Proteom 1804:1937–42.

Peichoto ME, Tavares FL, DeKrey G, et al. (2011a). A comparative study of the effects of venoms from five rear-fanged snake species on the growth of *Leishmania major*: identification of a protein with inhibitory activity against the parasite. Toxicon 58:28–34.

Peichoto ME, Zychar BC, Tavares FL, et al. (2011b). Inflammatory effects of patagonfibrase, a metalloproteinase from Philodryas patagoniensis (Patagonia Green Racer; Dipsadidae) venom. Exp Biol Med 236:1166–72.

Peichoto ME, Tavares FL, Santoro ML, et al. (2012). Venom proteomes of South and North American opisthoglyphous (Colubridae and Dipsadidae) snake species: a preliminary approach to understanding their biological roles. Comp Biochem Physiol D Genom Proteom 7: 361–9.

Pyron RA, Burbrink FT, Wiens JJ. (2013). A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol Biol 13:93–145.

Rajagopalan N, Pung YF, Zhu YZ, et al. (2007). β-Cardiotoxin: a new three-finger toxin from *Ophiophagus hannah* (king cobra) venom with β-blocker activity. FASEB J 21:3685–95.

Rath S, Trivelin LA, Imbrunito TR, et al. (2003). Antimoniais empregados no tratamento da leishmaniose: estado da arte. Quim Nova 26:550–5.

Rich S, Kaufmann E, Levy PS. (1992). The effect of high doses of calcium-channel blockers on survival in primary pulmonary hypertension. N Engl J Med 327:76–81.

Rosenberg HI, Bdolah A, Kochva E. (1985). Lethal factors and enzymes in the secretion from Duvernoy's gland of three colubrid snakes. J Exp Zool 233:5–14.

Sajevic T, Leonardi A, Križaj I. (2011). Haemostatically active proteins in snake venoms. Toxicon 57:627–45.

Saviola AJ, Chiszar D, Busch C, et al. (2013). Molecular basis for prey relocation in viperid snakes. BMC Biol 11:20.

Savitzky AH. (1980). The role of venom delivery strategies in snake evolution. Evolution 34:1194–204.

Scarborough RM, Rose JW, Naughton MA, et al. (1993). Characterization of the integrin specificities of disintegrins isolated from American pit viper venoms. J Biol Chem 268:1058–65.

Schweitz H, Vigne P, Moinier D, et al. (1992). A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). J Biol Chem 267: 13928–32.

Sherman DG. (2002). Ancrod. Curr Med Res Opin 18:s48–52.

Takacs Z, Nathan S. (2014). Animal venoms in medicine. In: Wexler P, ed. Encyclopedia of toxicology, 3rd ed. London: Elsevier, 252–9.

Tanaka KA, Key NS, Levy JH. (2009). Blood coagulation: hemostasis and thrombin regulation. Anesth Analg 108:1433–46.

Taub AM. (1966). Ophidian cephalic glands. J Morphol 118:529–41.

Vidal N. (2002). Colubroid systematics: evidence for an early appearance of the venom apparatus followed by extensive evolutionary tinkering. J Toxicol Toxin Rev 2:21–41.

Vidal N, Delmas AS, David P, et al. (2007). The phylogeny and classification of caenophidian snakes inferred from seven nuclear protein-coding genes. C R Biol 330:182–7.

Vonk FJ, Admiraal JF, Jackson K, et al. (2008). Evolutionary origin and development of snake fangs. Nature 454:630–3.

Vonk FJ, Jackson K, Doley R, et al. (2011). Snake venom: from fieldwork to the clinic. Bioessays 33:269–79.

Wang J, Shen B, Guo M, et al. (2005). Blocking effect and crystal structure of natrin toxin, a cysteine-rich secretory protein from *Naja atra* venom that targets the BK_{Ca} channel. Biochemistry 44:10145–52.

Weldon CL, Mackessy SP. (2010). Biological and proteomic analysis of venom from the Puerto Rican Racer (*Alsophis portoricensis*: Dipsadidae). Toxicon 55:558–69.

Weldon CL, Mackessy SP. (2012). Alsophinase, a new P-III metalloproteinase with α-fibrinogenolytic and hemorrhagic activity from the venom of the rear-fanged Puerto Rican Racer *Alsophis portoricensis* (Serpentes: Dipsadidae). Biochimie 94:1189–98.

Weinstein SA, Stiles BG, McCoid MJ, et al. (1993). Variation of lethal potencies and acetylcholine receptor binding activity of Duvernoy's secretions from the brown tree snake, *Boiga irregularis* Merrem. J Nat Toxins 2:187–98.

Weinstein SA, Kardong KV. (1994). Properties of Duvernoy's secretions from opisthoglyphous and aglyphous colubrid snakes. Toxicon 32: 1161–85.

Weinstein SA, Warrell DA, White J, et al. (2011). Venomous bites from non-venomous snakes: a critical analysis of risk and management of ''Colubrid'' snake bites. London: Elsevier, 364.

Yamazaki Y, Koike H, Sugiyama Y, et al. (2002). Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. Eur J Biochem 269:2708–15.

Young BA, Herzog F, Friedel P, et al. (2011). Tears of venom: hydrodynamics of reptilian envenomation. Phys Rev Lett 106:198103.

Young BA, Kardong KV. (1996). Dentitional surface features in snakes (Reptilia: Serpentes). Amphibia-Reptilia 17:261–76.

Zelanis A, Teixera de Rocha MM, Domingues Furtado MF. (2010). Preliminary biochemical characterization of the venoms of five Colubridae species from Brazil. Toxicon 55:666–9.

Zimmerman AL. (1995). Cyclic nucleotide gated channels. Curr Opin Neurobiol 5:296–303.