Snake evolution and prospecting of snake venom

Vonk, F.J.

Chapter 2: Snake venom: from fieldwork to the clinic.

Vonk FJ, Jackson K, Doley R, Madaras F, Mirtschin PJ and N Vidal.

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Abstract

Snake venoms are recognized here as a grossly under-explored resource in pharmacological prospecting. Discoveries in snake systematics demonstrate that former taxonomic bias in research has led to the neglect of thousands of species of potential medical use. Recent discoveries reveal an unexpectedly vast degree of variation in venom composition among snakes, from different species down to litter mates. The molecular mechanisms underlying this diversity are only beginning to be understood. However, the enormous potential that this resource represents for pharmacological prospecting is clear. New high-throughput screening systems offer greatly increased speed and efficiency in identifying and extracting therapeutically useful molecules. At the same time a global biodiversity crisis is threatening the very snake populations on which hopes for new venom-derived medications depend. Biomedical researchers, pharmacologists, clinicians, herpetologists and conservation biologists must combine their efforts if the full potential of snake venom derived medications is to be realized.

Introduction

Snakes are represented on earth today by some 3150 species [1] . Of these the vast majority (ca. 2700 species, see Fig. 1) represent a single massive diversification event which occurred after the K-T boundary and extinction of the dinosaurs. This large and relatively recent group is known as Caenophidia or "advanced snakes" and characterized by the possession of a venom-delivery system or components of such a system [2]. Snakes traditionally considered venomous are the 600 or so species with tubular front fangs, muscularized venom glands, and a bite significantly dangerous to humans (Viperidae, Elapidae and Atractaspidinae) - including well-known examples as the cobras, seasnakes, vipers and rattlesnakes. The remaining caenophidians were traditionally classified as "Colubridae", meaning snakes with a venom gland whose venom poses no danger to humans, and who lack the fangs at the front of the mouth for injecting it. The "Colubridae" has been shown to be paraphyletic, and most of its subfamilies have recently been elevated to a familial rank in order to reflect their evolutionary distinctiveness (Fig. 1) [1,3].

"Colubrid" snakes have been largely neglected in venom research, because of the sole fact that bites have not been perceived as of medical importance, except few species as the African boomslang (*Dispholidus typus*) and twigsnakes (*Thelotornis* spp.) and the Asian yamakagashi (*Rhabdophis tigrinus*). However, during the last few years, there has been a trend towards studying the venoms of these harmless venomous snakes to, primarily, increase our understanding of venom evolution. It became evident that these harmless snakes do secrete a strong acting venom with powerful toxins, although in a significantly lower amount and without an efficient injection mechanism [2,4]. This influenced the ongoing quest for venom molecules that may be useful in fighting human disease - a snake need not be dangerous to humans for its venom to still have a profound effect upon the human body. This field of biodiscovery is now rapidly emerging, especially due to the development of modern high-throughput screening assays that allow rapid identification of potential therapeutic agents.

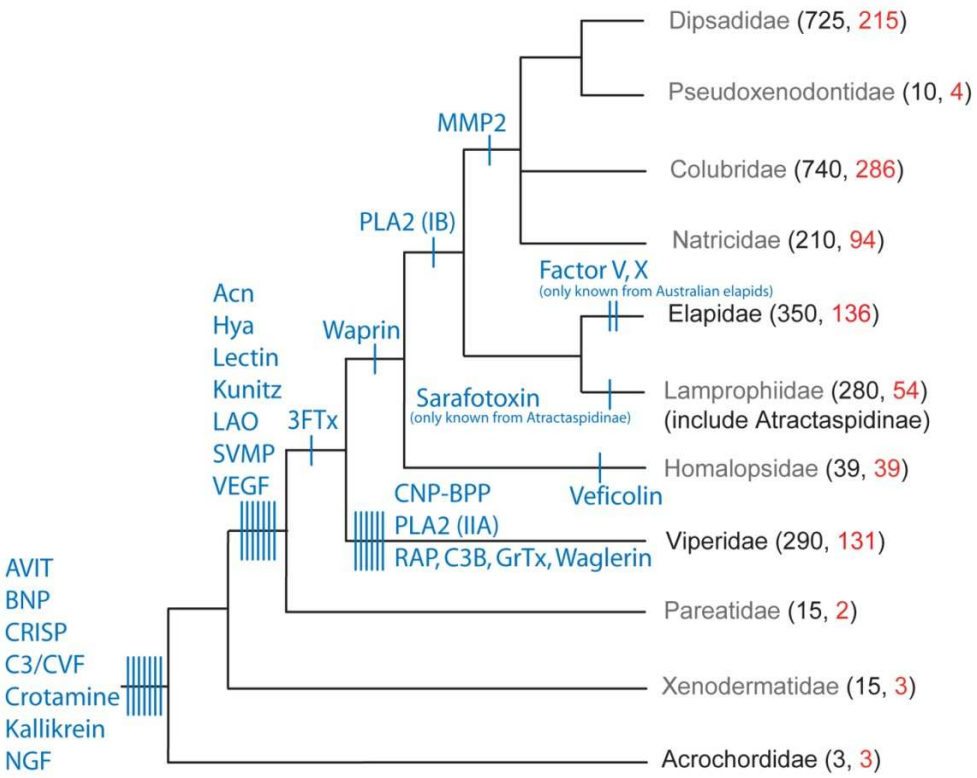

Figure 1 | Phylogenetic tree showing the distribution of 28 snake venom protein families among advanced snakes, with the number of currently known species of each family and the number of red listed species. Families in grey used to make up the old traditional "Colubridae". Phylogeny based on [1,5].

Snake venom contains a mixture of powerful proteins and peptides that have evolved to be targeted to receptors, ion channels or enzymes [6], in addition to some carbohydrates, nucleosides, lipids, and metal ions, whose functions are not all known [7,8]. They interact with a wide variety of mammalian proteins and can disrupt the central and peripheral nervous systems, the blood coagulation cascade, the cardiovascular and neuromuscular systems, and homeostasis in general. These venom proteins act with great precision – different toxins recognize different subtypes of certain receptors with only subtle differences – and are very biologically active. The precision and power with which they work lies right at the centre of why they form such a valuable resource to biochemists, biomedical researchers, evolutionary biologists and others.

Several major human drugs or diagnostics have been developed based on snake venom components. In addition, some fundamental biological processes have been revealed by using toxins as probes to study cells and their receptors. Here we review the state of this field and emphasize that the emerging field of high-throughput screening assays applied to a wide range of unstudied venoms, has the potential to provide a solid basis for the discovery of new lead compounds for new drugs.

Forces driving evolutionary divergence in venom composition: diet, phylogeny, biogeography and ontogeny

Only in relatively recent years has the remarkable variability of venom composition at the genus, species, subspecies, population and even individual levels been fully appreciated, and the underlying ecological forces and mechanisms of mutation at the molecular level started to be identified and understood. Variation in venom composition has far-reaching implications. For the treatment of snakebite, for example, the importance of using pooled venom (mixtures of venom from several individuals of the same species representing different ages and geographical origins) has been emphasized in the production of antivenom to – in some cases - produce a serum that will be maximally effective against a bite from any snake of that species [9]. However, it should be noted that many antivenoms provide excellent – sometimes even better – cross reactions against antigens not even included in the original mixture, a phenomenon which is not yet well understood.

Since different species of prey differ in their physiological reaction to venom molecules, it follows that venom composition is linked to diet and varies from the species level all the way to individuals. In saw-scaled vipers (*Echis*), venom from those species that feed on arthropods was highly toxic to scorpions [10]. By contrast, scorpions were unaffected by venom from those species that feed on mammals [10]. Phylogenetic analysis revealed repeated instances of co-evolution of venom composition with prey preference. Other examples of variation in venom composition, at the individual to the species level, correlated with diet have been found in coral snakes (*Micrurus*) [11], rattlesnakes (*Crotalus* and

Sistrurus) [9,12-14], Malayan pitvipers (*Calloselasma*) [15], lanceheads (*Bothrops*) [16,17], puff adders (*Bitis arietans*) [18], and others.

Specificity of venom to prey type has also been found by studies approaching the question by analyzing the composition of the venom. A single species of coral snake (*Micrurus s. surinamensis*), distinctive within its genus in feeding on fish, was found to have venom containing neurotoxins lethal to fish and not known from the venom of other *Micrurus* species [11]. Significant differences in the protein composition were also found in the venoms of three subspecies of the rattlesnake *Sistrurus catenatus*, attributable to dietary differences between the subspecies [19]. Of the 11 protein families represented in the venom of this species, variation between the subspecies was found in all protein families, but variation was greater in some than in others. The metalloproteinases proved to be the most conserved while the PLA2s were the most divergent. PLA2s are often associated with neurotoxins – i.e the neurotoxin molecules also have PLA2 activity (e.g. notexin, taipoxin, crotoxin, β-Bungarotoxin etc.), so it could be that the neurotoxins vary more to accommodate the different prey types. Some toxins may assist in prey breakdown (digestion) but others – like neurotoxins causing paralysis or haemotoxins causing rapid low blood pressure or circulation – are perhaps more critical, they may determine whether the snake gets the meal in the first place.

In addition to diet, other variables such as phylogeny, biogeography and ontogeny may also be forces driving the evolutionary divergence of venom proteins (these factors may well be correlated with diet in many cases) – and perhaps just even separation time between populations due to genetic drift [20]. Although most studies documenting variation attribute it to a single factor, considering these categories in isolation may result in an incomplete understanding of the true scenario. For example, individuals of the common lancehead (*Bothrops asper*) from two different localities and of different ages displayed significant differences in venom proteome [21]. Variation in symptoms of bite victims from individuals of this species varying in age or geographic origin has been observed though never formally documented. Ontogenetic differences in venom compositions are most probably explained by ontogenetic shifts in diet

[22]. The venom of neonates was found to differ significantly from that of adults, with a trend toward increasing complexity with age [22]. In the proteomes of neonate and adult individuals of the rattlesnake *Crotalus simus* the venom of adults and neonates had only 50% of their proteome in common, with a trend from neurotoxic changing to hemorrhagic properties from neonate to adult [23].

While ontogenetic variation certainly, and biogeographical variation in some cases, may well be explained by variation in prey type, observations of variation in the venom proteome of male versus female neonates from a single litter of the South American viper *Bothrops jararaca* [24] are puzzling and underscore just how much remains to be learned about the evolutionary forces driving variation in venom composition.

For the pharmacological prospector, variation is right at the heart of the potential goldmine of molecules that snake venom represents since a high degree of variation in venom composition increases the number of potentially useful novel molecules. Recognizing the crucial biomedical significance of variation in venom underscores the importance of efforts by those field biologists working with snakes to understand and conserve biological diversity, from the species down to the individual level. This emphasizes the importance of fieldwork in biodiscovery and the collection of venom samples from a wide range of species and specimens of different geographical localities and ages (Fig. 2).

Figure 2 | Due to the enormous diversity in venom composition and the accelerated evolution of different isoforms of each of the toxin types in Fig. 1, fieldwork is essential to obtain samples from a wide range of species and specimens of different geographical localities and ages to exploit the full pharmacological potential of venom. A: a rare species of "colubrid", the green tree snake (*Dipsadoboa viridis*), in the Republic of Congo (Africa), photo by K.J. B: one of the authors (F.J.V.) with a wild king cobra (*Ophiophagus hannah*) – the longest venomous snake in the world - on the island of Java in Indonesia, photo by Smarley. C. The same author obtaining a venom sample from an Australian King brown (*Pseudechis rossignoli*), photo by H-W Herrmann. D. one of the authors (K.J.) removing a watersnake (*Grayia ornata*), mimic of the venomous Water cobra (*Naja annulata*) from a net in the Republic of Congo.

Sources of toxin diversity: multiple splicing, exon insertion, exon switching, post-translational modification and domain switching

Snake toxin genes are the result of gene-duplications of normal body proteins that are subsequently selectively expressed in the venom gland [25], often followed by accelerated point mutations in the protein coding regions [26]. Gene duplication creates redundancy and allows the duplicated gene to escape the pressures of negative selection and acquire new functions through accelerated adaptive molecular evolution [26]. Acetylcholinesterase is the only currently known exception, because both the toxin and the normal enzyme are encoded by the same gene but differentially expressed using alternative splicing [27].

The molecular mechanisms that cause accelerated evolution – a bias towards nucleotide mutations that lead to amino acid changes (nonsynonymous substitutions) compared to those that don't (synonymous substitutions) – are currently not understood. When the first snake genome becomes available, this may shed light upon the molecular mechanisms that operate on the venom genes. Interestingly, nucleotide sequences appear to determine the accelerated rate of point mutations [27]. Specific triplets were found to be more 'stable' with regards to mutations than others, and the stable triplets were found to be higher in abundance in venom introns while the non-stable triplets were found higher in abundance in venom exons. There also appears to be a bias for transversions over transitions in nucleotide substitutions in some toxins [28].

There are several mechanisms by which molecular diversity of venom toxins is generated. First, alternative splicing allows multiple different functional proteins to be created using the same exons (Fig. 3a) [29,30] as it may cause binding to different receptors [31] or change of target altogether. Second, exons may be inserted into existing genes - as in denmotoxin (Fig. 3b), a three-finger toxin in the venom of the "colubrid" mangrove catsnake (*Boiga dendrophila*) with bird-specific activity [32]. Third, part of the intron may be retained in the mRNA due to error in splicing (Fig. 3c). Also, a recent discovered mechanism termed accelerated segment switch in exons to alter targeting (ASSET) may play an important role in

generating the molecular diversity in snake venom molecules [33] (Fig. 3d). During ASSET certain parts of exons are changed through accelerated segment switch and generate a functionally new toxin with a conserved structural fold. Sometimes, synergistic action between two different toxins may enhance their potency [34,35].

Post-translational modifications such as disulphide bridge formation and proteolysis play important roles in structural modification and acquisition of new functional sites. Both covalent and non-covalent interactions between similar and dissimilar proteins can form complexes that may exhibit a much higher pharmacological activity compared to the individual components. Sometimes formation of hetero/homo dimeric or trimeric complexes may lead to recognition of new targets as protein-protein interaction in complexes may expose critical amino acid residues that were otherwise buried in monomers [28].

New interaction sites are also formed in certain proteins to exhibit higher pharmacological potencies through domain swapping. In domain swapping, exchange of identical structural elements takes place between two or more molecules to form dimers or oligomers. For example, with the exchange of domains by α and β subunits of IX/X binding protein, isolated from the venom of the Okinawa habu (*Protobothrops flavoviridis*) [36], the hinge region forms a concave structure between the subunits and provides a new functional site in the heterodimer [28]. Without this swapping the loop would fold back and the ligand binding site would be absent.

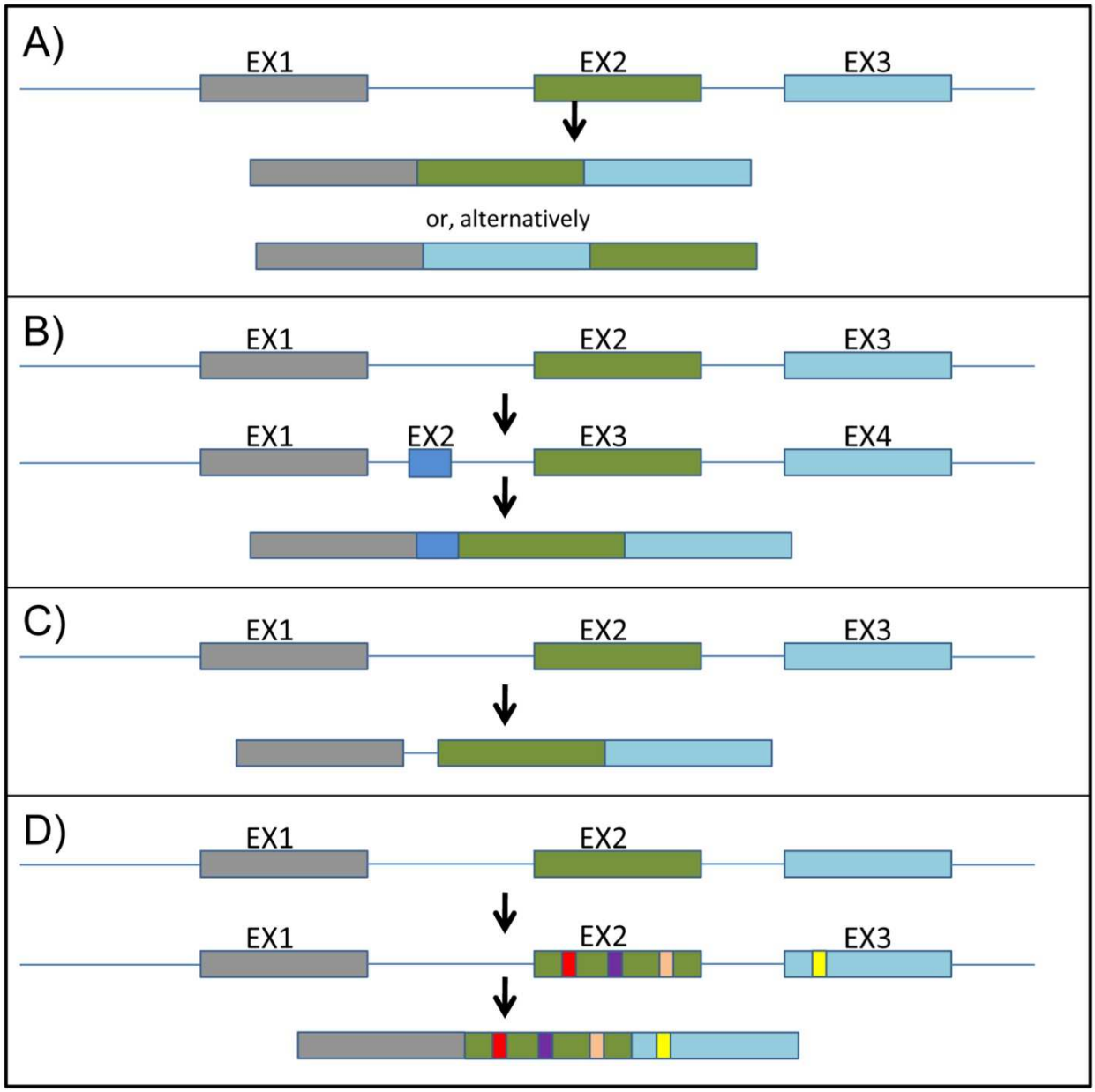

Figure 3 | Some molecular mechanisms by which the diversity of toxin proteins is achieved. A: normal and alternative splicing. B: insertion of exon, as in denmotoxin [32]. C: intron retention due to error in splicing. D: accelerated segment switch in exons to alter targeting (ASSET) sas in a 3FTX from the venom of the rattlesnake *Sistrurus catenatus* [33]. Colored segments (red, purple, pink, yellow) represent exchanges of segments.

Snake venom enzymes and toxins

Of the following enzymes, some have currently been described in all snake venoms, and others from only a limited number of species: PLA2s, metalloproteinases, serine proteases, acetylcholinesterases, LAOs, and hyaluronidases (Fig. 1). Two important and diverse families are the PLA2s and the metalloproteinases. PLA2 enzymes exhibit a wide variety of pharmacological effects in prey/victims and are therefore interesting for the pharmacological prospector [37]. Catalytically inactive Lys49 PLA2 homologs – found in many viperid venoms and being strongly myotoxic – work through a mechanism independent of hydrolysis [38]. Furthermore, although different in their pharmacological and enzymatic activity, the PLA2-like and PLA2 toxins are highly similar in sequence and structure and differ only for the substation of Asp-49 with Lys or Ser [39] - an example of how puzzling the mechanisms of toxicity can be. Some of the neurotoxic PLA2 are either homo/heterodimeric complexes. For example, β–bungarotoxin contains a covalently linked kunitz-type serine protease inhibitor which is involved in the blocking activity [40].

SVMPs are responsible for major local symptoms in snakebite - causing hemorrhage, edema, hypotension, hypovolemia, inflammation and necrosis. They are divided into three groups (P-I to P-III) based on the presence of other domains in the mature protein [41]. PI SVMP's have a prodomain and a single metalloproteinase domain that overall causes less hemorrhagic action then the other types, but still displays a variety of biological activities. The PII SVMP's are composed of a metalloproteinase and disintegrin domain, along with a prodomain.

Non-enzymatic venom proteins include 3FTXs, Kunitz-type serine protease inhibitors, sarafotoxins, CRISP, disintegrins, C-type lectins, waprins, veficolins and vespryns. 3FTXs have three beta-stranded loops resembling three-fingers and are mainly found in the venoms of elapids, some "colubrids" [32,42] and, in low quantity, in some viperids [43,44]. The structure is stabilized by four conserved disulphide bridges [45,46]. However, these structurally related molecules differ greatly in their biological functions [47,48]. Functional characterization of this family of proteins has contributed significantly to our

understanding of the mechanisms of venom toxicity and of normal physiological processes [48]. For example, characterization of α-bungarotoxin, a three-finger neurotoxin found in the venom of the banded krait (*Bungarus multicinctus*), enabled the isolation of the human nAChR [49] and contributed to our understanding of myasthenia gravis [50]. Significant contributions have been made in determining the distribution of specific receptors or ion channels in particular tissues or cells, identification of subtypes of receptors, imaging receptor trafficking [51-53] as well as in the development of therapeutic agents for treatment of adrenomyeloneuropathy and multiple sclerosis [54].

Snake venom kunitz-type serine protease inhibitors like dendrotoxin, calcicludine and the B chain of β-bungarotoxin, act as Ca^{2+} and K^{+} channel blockers respectively [55-57]. Textilinin, a kunitz-type serine protease inhibitor from the venom of the highly dangerous Australian brown snake (*Pseudonaja textilis*) is a reversible plasmin inhibitor and has promising potential for development of anti-bleeding agent [58].

Waprins have only recently been identified in snake venom and show homology to whey acidic proteins [59]. So far their functions are not really understood except for omwaprin, isolated from the Inland taipan (*Oxyuranus microlepidotus*), which possesses selective antimicrobial activities [60], useful for developing potential antibiotics.

CRISP toxins have molecular weights of 20-30 kDa and 16 conserved cysteine residues. Functional characterization of some CRISP has revealed that they are involved in blocking cyclic nucleotide-gated ion channels and block potassium-stimulated smooth muscle contraction [61]. Therefore in addition to their involvement in disrupting the normal physiological functions of victims they can be used for studying ion channel chemistry. A CRISP toxin from the venom of the "colubrid" snake the Patagonia green racer (*Philodryas patagoniensis*) was recently shown to cause damage to the murine gastrocnemius muscle, an action never before shown for any CRISP toxin [62] – showing the potential "colubrid" venoms have to find new toxins.

Snaclecs comprise two subclasses of protein, C-type lectins (CTLs) and C-type lectin-related proteins (CLRPs), found in venoms of most families of advanced snakes. Snake venom CTLs are involved in hemagglutinating and platelet aggregation activities during envenomation [63-66]. The CLRPs are involved in anticoagulant, procoagulant and agonist/antagonist of platelet activation [67,68]. Pharmacological characterization reveals that they either enhance or inhibit the function of coagulation factors which underscores their potential in drug discovery for blood related diseases. Their immaculate specificity also helps in understanding platelet physiology.

Disintegrins are a class of non-enzymatic that bind to integrins expressed on platelets and other vascular endothelial cells as well as some tumor cells [69] – they are an important class of cell surface receptors that are critically involved in cell–cell and cell–matrix interactions and are therefore good candidates to understand the interaction between extracellular matrix and cells [70]. In addition to their role in antiplatelet activity, these molecules are used in the diagnosis of cardiovascular diseases as well as serving as prototypes for therapeutic molecules in treatment of cancer.

The medicinal use of snake venom

A large number of venom proteins affect the haemostatic system [71] and can have procoagulant, anticoagulant, fibrinolytic or platelet active activities. Ancrod (Arvin) from the venom of the Malayan pitviper (*Calloselasma rhodostoma)*, Batroxobin (Reptilase) from the common lancehead (*Bothrops atrox*), Crotalase from the Eastern diamondback rattlesnake (*Crotalus adamanteus*) have all been used as defibrinogenating agents for several clinical conditions including deep vein thrombosis, myocardial infarction, pulmonary embolus, and many others [72]. Venoms with anticoagulant properties are extensively studied for possible medical applications. The drug Aggrastat (tirobifan) was developed from a compound in the venom of the saw-scaled viper (*Echis carinatus*), and is used as an anti-platelet drug (glycoprotein IIb/IIIa inhibitors) [73] and given to those with unstable angina (Fig. 4). Many venoms with procoagulant properties find application in the diagnosis of clotting abnormalities, and most of the clotting pathways can be assayed by some venom component. For example “Reptilase time” (*B. atrox)* assays for thrombin

inhibitors [74], "Ecarin" (*E. carinatus*) and "taipan time" (*O. scutellatus*) assays for phrothrombin, and Russell's viper (*Daboia russelii*) venom assays for factor X and for monitoring anticoagulant therapy [75].

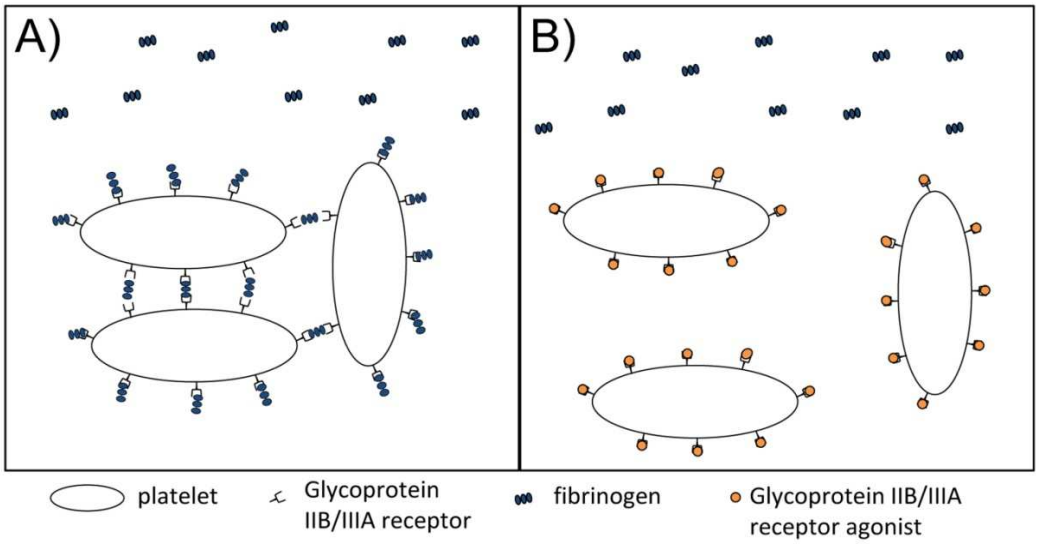

Figure 4 | Mechanisms of action of the anticoagulant Aggrastat developed from a compound in the venom of the Indian saw-scaled viper (*Echis carinatus*). A: aggregated platelets by formation of fibrinogen bridges between the glycoprotein IIb/IIIa receptors. B: glycoprotein IIB / IIIA receptor antagonists like Aggrastat prevent platelet aggregation, and are mainly used in patients with acute coronary syndromes.

A number of snake venoms create a transient condition of depressed blood pressure in envenomed patients. ACE-inhibitors were developed from a bradykinin potentiating enzyme isolated from the venom of the Brazilian pitviper (*Bothrops jararaca*) and approved in 1979 by the FDA [76] to treat high-blood pressure and heart disease (Fig. 5). They work by blocking the switch between angiotensin-I and angiotensin-II, the latter being a vasoconstrictor. These inhibitors are now prescribed worldwide and have saved the lives of millions.

Many venoms have analgesic properties. Hannalgesin, derived from the venom of the king cobra (*Ophiophagus hannah*, see Fig. 2b) is already in clinical trials [77]. Promising toxins have been isolated from the tropical rattlesnake (*Crotalus durissuss terrificus*) and some other related species. Compounds derived from the Asiatic cobra (*Naja kaouthia*) are already in use in alternative medicine. Being more

powerful than morphine, cobra venom was used in the 1930's for treating intractable pain in cancer sufferers.

There is great deal of research currently been done into the anti-cancer properties of venoms. For example malignant brain and spinal-cord tumors (gliomas) are not curable by surgery because they invade the surrounding brain tissue without clear boundaries, making removal impossible. Disintegrins, like contortrostatin from American copperhead (*Agkistrodon contortrix*) venom, prevent cells from sticking together, and inhibit their interaction with surrounding tissue and result in a blockage of cell motility and invasiveness [78].

It has been demonstrated that fibrin(ogen) plays separate and distinctive roles at different stages of tumor growth and dissemination. At the primary site, fibrin deposition around the tumor could form a protective barrier, but also limit tumor progression. On the other hand, fibrin deposits formed by metastatic tumor cells may help disseminating these tumor cells [79]. Anticoagulants and the removal of fibrin could be an effective therapy. One of the earliest reports on the successful use of a venom defibrinogenating enzyme was that of Wood and Hilgard [80].

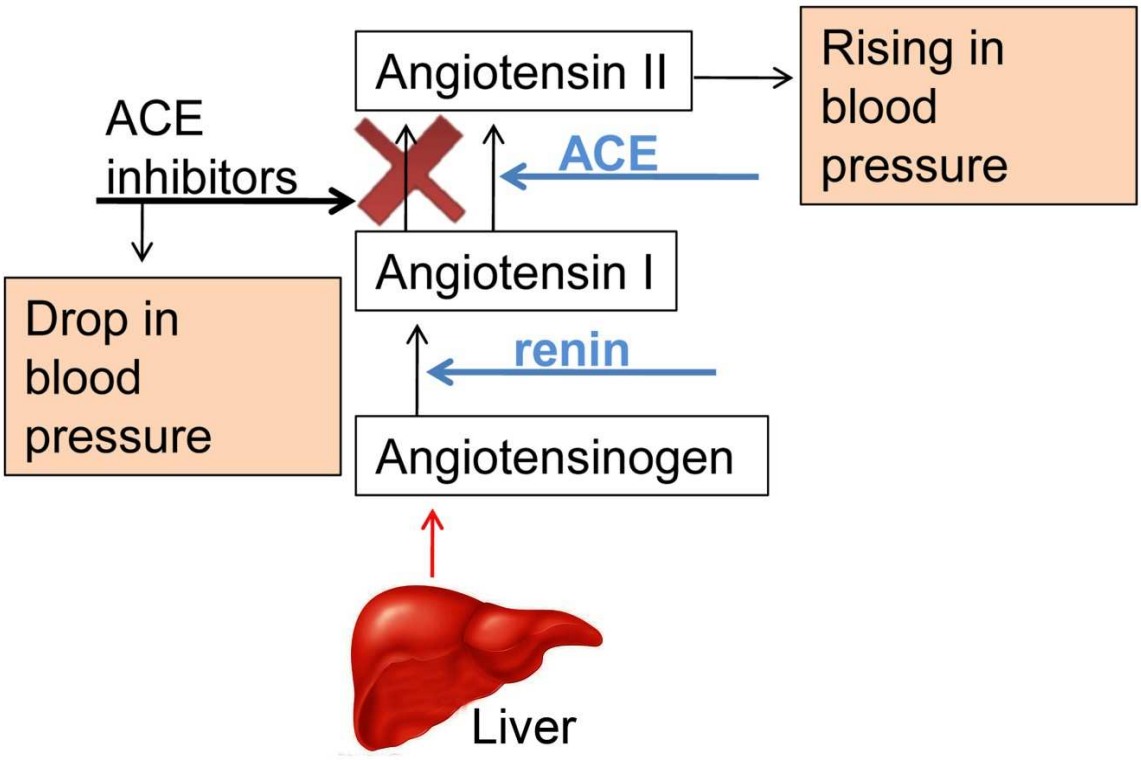

Figure 5 | Mechanism of action of ACE inhibitors developed from a bradykinin potentiating enzyme isolated from the venom of the Brazilian pitviper (Bothrops jararaca) and approved in 1979 by the FDA [75] to treat high-blood pressure and heart disease. Angiotensin I causes vasoconstriction which raises the blood pressure.

Many cobra venoms contain Cobra Venom Factor (CVF), that activates and depletes the mammalian immune-complement system [81]. They are structural and functional analogues of the mammalian serum complement factor C3 [82]. CVF is used as a tool to study various aspects of the complement system and in this respect CVF is a uniquely useful venom component that has also been found useful as an immuno suppressant agent in tissue transplantation and in cancer therapy [83].

Many venoms have antibacterial properties. For example, Stiles *et al* [84] found 2 antibacterial bioactive L-amino acid oxidase components in King brown (*Pseudechis australis*) venom that were 70 and 17.5 times more effective *in-vitro* than tetracycline, a drug of choice for *Aeromonas* infections.

Antibacterial and antiparasitic effects of the venom of the Marajó lancehead (*Bothrops marajoensis*) were shown to be caused by PLA2 and and L-amino acid oxidase toxins [85].

Antiviral activity has been demonstrated in several venoms, although no commercialization of any of these compounds has yet taken place. The venom of the tropical rattlesnake (*Crotalus durissus terrificus*) from Brazil has been shown to be active against the measles virus [86]. The purified PLA2 venom neurotoxin "taipoxin" from the coastal taipan (*Oxyuranus scutellatus*), Nigexine from the African black necked cobra (*Naja nigricolis*) and a basic PLA2 from the Mozambique spitting cobra (*Naja mossambica*) have been shown to have potent anti-viral activities against HIV-1 virus [87].

Myasthenia gravis, a chronic autoimmune disorder affecting 2 out of every 100,000 people, results in progressive skeletal muscle weakness with rapid fatigue and loss of strength. It primarily affects mastication, facial and swallowing muscles and in advanced cases, respiratory muscles. Autoimmune antibodies destroy acetylcholine receptor sites at the neuromuscular junctions preventing nerve impulses from reaching the muscles. A rapid, quantitative and sensitive radioimmunoassay using human acetylcholine receptor, affinity labeled with purified neurotoxin, alpha-bungaratoxin, from the venom of the Taiwan krait (*Bungarus multicinctus*) is used to diagnose the condition [88,89]. Most venoms possessing alpha neurotoxins are potential candidates for this purpose.

High-throughput screening to identify potential new medicines in venoms

A major obstacle in exploring snake venom for new leads into drug discovery is the low amount of venom usually obtained during the process of milking (extracting venom from the venom glands of a live snake, see Fig. 2c). In addition, it is difficult to extract venom from many of the "colubrid" snakes. Because of this, venom research has focused mainly on those snakes that are dangerous to humans, but this doesn't necessarily reflect the potential of their venom for drug discovery. Some of the harmless "colubrid" venom toxins have been shown to be as potent as those of some deadly elapids, only produced

in small amounts [4,90]. For example, the venoms of the Patagonia green racer (*Phildryas patagoniensis*) and Lichtenstein's green racer (*P. olfersii*) have been shown to posses high proteolytic activity, degrading fibrinogen and the vascular wall [91], and strong edematogenic and myotoxic activity [92], respectively. In addition, a completely new family of toxins has recently been found in the venom of the Asian dog-faced water snake (*Cerberus rynchops*). These toxins, called veficolins, may induce platelet aggregation and/or initiate complement activation [93]. Hence, the bias towards deadly snakes in exploring venoms for drug discovery is completely unwarranted.

Alternatively with the use of molecular tools the venom gland transcriptome can be complied. This approach not only allows us to understand the expression profile and the evolution of the venom proteins [94], but also reveals the lowly expressed proteins which will expand our resource for pharmaceutically active molecules.

Many of the medically significant venomous snakes produce hundred to a couple hundred milligrams of dry venom in a single milking [95]. Large species of elapids and viperids may produce from several hundred mg's up to more than a gram of venom in a single milking [95]. The recently described giant spitting cobra (*Naja ashei*) has been shown to produce the immense amount of 3 gm's of weight of dry venom [96], the largest amount ever collected during a single milking. The amount of venom that a snake produces during milking is determined by the species, its geographic origin, its body size and relative head size, and by the time of the year that it is milked, as well as by interactions among these factors, body size being the primary factor [97].

When biomedical researchers are looking for lead therapeutic agents against diseases, they can first look at those venoms of which the snakebite symptoms involve the same pathways as the disease, for example, using venom that causes vasodilatation to look for potential blood pressure regulators. However, snake venom is very complex and contains many different isoforms, some of which may only be present in very low quantities in the venom. For example, nerve growth factor is only present in a concentration

of about 0.1-0.5% [98]. A fraction that stimulates neurite outgrowth was first identified in tumour cell extracts, but venom from cottonmouth vipers (*Agkistrodon piscivorus*), was then found to be up to 6,000 times more potent [99] – a discovery that allowed the authors to study mechanisms which regulate cell and organ growth and for which they were awarded the Nobel prize in 1986. The problem is that venom compounds that are present only in low concentrations don't necessarily contribute to the bite symptomology and may thus be easily overlooked, although they can be of high potential biomedical interest. To exploit the full potential of snake venoms, a wide variety of them need to be fractionated and all fractions tested separately in a high-throughput screen of interest. Mice have typically been used as model organisms, in for example identifying genes involved in pain and anxiety, and in screening for analgesic peptides [100]. However, using mice is ethically controversial and low-throughput, screens also require relatively large amounts of the precious venoms. Zebrafish (*Danio rerio*) are now more and more used as model organisms to screen for new drugs, and this provides a significant window of opportunity for using snake venom (or venom from other venomous animals) to screen. Zebrafish are cheaper to maintain than rodents, their eggs and embryos are transparent so phenotypic changes can be visualized, and they can be easily scaled up into high-throughput assays [101] that require minimal amounts of venom, and automated imaging and analysis systems are available [102,103]. Morpholino knockdowns can be performed by injecting the yolk sac of embryos, allowing live imaging studies, and many disease-related genes identified in humans have orthologs in zebrafish [101]. Although zebrafish will never replace mammalian models completely in the drug development pipeline, they do form a cost-effective bridge between cell-based models on the one hand and rodent whole-organism models on the other [101].

Conservation of snakes

A recent report by conservation biologists [104] documented a disturbing trend - a global decline in non-related snake species over the world during the same time period. Although they only looked at a small number of snakes (17 populations of eight species), we need to keep in mind that any species of snake that goes extinct may have held a new drug in its venom. Species having small home ranges, sedentary

habits and ambush feeding strategies are likely to be the most vulnerable as they rely on sites with specific types of ground cover that are disrupted by anthropogenic activities, and as ambush foraging is associated with a suite of life-history traits that involve low rates of feeding, growth and reproduction [105].

Current research on snake venoms is phylogenetically biased. Of the 1622 snake venom toxin sequences available on Universal Protein Resource database, only 49 are sequenced from snakes that do not possess any danger to humans. But with the high-throughput systems currently being developed this may hopefully soon change.

Very little data actually exist on the true conservation status of snakes worldwide. Out of 2711 extant caenophidian species, 26 are CITES listed, i.e. less than 1%, while 967 are listed on the IUCN Red List of Threatened Species, i.e. 36%. The main causes identified for their threatened status are caused by humans: annual & perennial non-timber crops (272 sp.), logging & wood harvesting (179 sp.), housing & urban areas (143 sp.), livestock farming & ranching (98 sp.), hunting & trapping (64 sp.) and natural system modifications (57 sp.). Although the majority of the species listed have been categorized as Least Concern (60%), this does not necessarily reflect their true status [104], and for another 22% of the species the data are insufficient to categorize them accordingly. In addition, 87% of the species (837) have been added to the Red List since 2007, highlighting the lack of proper attention in the years before.

If biogeography is considered, relatively well protected regions are North America (123 red listed sp.), Meso America (325 sp.), the Philippines (84 sp.) and Europe (29 sp.). On the other hand, several biodiversity hotspots such as South America (113 sp.), Sub-Saharan Africa (82 sp.), and the Caribbean Islands (16 sp.), among others are neglected while these areas may represent the locations with the highest potential for discovering snake species new to science, each representing new potential for advancing the frontiers of clinical medicine.

Conclusions and future perspectives

While the use of snake venom for medicinal purposes dates back to ancient times, the past few decades have seen several new drugs derived from components of snake venom becoming available to patients worldwide. Here, we have reviewed recent advances in areas ranging from evolutionary to molecular biology that taken altogether indicate new possibilities for contributions to clinical medicine and medical research from snake venom. First, advances in snake systematics demonstrate a high degree of phylogenetic bias in previous molecular prospecting in snake venom, increasing the number of species from which medically useful molecules may be obtained. Secondly, discoveries in snake venom composition have revealed an unexpectedly high degree of variation of snake venoms of the same species linked to variables such as diet, geographical distribution, ontogeny and others. These two factors increase the potential pool that snake venom represents for pharmacological prospecting. Finally, new and innovative techniques in high throughput systems offer increased speed and efficiency in identifying and extracting desirable molecules from snake venom. In conclusion, we emphasize the challenges faced by the conservation of snake biodiversity, since it is ultimately on this that hopes for the development of new therapeutic agents from snake venom depends.

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