Utilisation of compounds from venoms in drug discovery

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Running Title

Venoms in drug discovery

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Abstract

Difficult drug targets are becoming the normal course of business in drug discovery, sometimes due to large interacting surfaces or only small differences in selectivity regions. For these, a different approach is merited: compounds lying somewhere between the small molecule and the large antibody in terms of many properties including stability, biodistribution and pharmacokinetics. Venoms have evolved over millions of years to be complex mixtures of stable molecules derived from other somatic molecules, the stability comes from the pressure to be ready for delivery at a moment's notice. Snakes, spiders, scorpions, jellyfish, wasps, fish and even mammals have evolved independent venom systems with complex mixtures in their chemical arsenal. These venom derived molecules have been proven to be useful tools, such as for the development of antihypotensive Angiotensin Converting Enzyme (ACE) inhibitors and have also made successful drugs such as Byetta, Integrilin and Echistatin. Only a small percentage of the available chemical space from venoms has been investigated so far and this is growing. In a new era of biological therapeutics,

venom peptides present opportunities for larger target engagement surface with greater stability than antibodies or human peptides. There are challenges for oral absorption and target engagement, but there are venom structures that overcome these and thus provide substrate for engineering novel molecules that combine all desired properties. Venom researchers are characterising new venoms, species, and functions all the time, these provide great substrate for solving the challenges presented by todays difficult targets.

Contents

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Key Words

Macrocycle, venom, toxin, peptide, polyamine, Drug Discovery

1. Introduction to venoms and historical perspective

1.1 Evolution

Evolution of a venom system presents a species with a powerful advantage in terms of ability to catch prey, evade predators and even compete for a mate. This has led to

many independent, unrelated venom systems developing. [1,2] Venoms evolved from other somatic proteins through co-option of genes [3,4], protein neofunctionalisation [5,6] and gene duplication. [7–9] This means that they already possess, inherited from the ancestral proteins, functionally active groups for engaging other biological proteins.

Venoms have been studied from an evolutionary perspective and because they are of great medical importance. Snakebite envenomation alone kills more than 100,000 people per year and causes morbidity in more than 400,000 people each year. [10] It is estimated that scorpion stings cause 3000 deaths a year worldwide [11] but it is rare for a human to die from a spider bite. [12] Morbidity and mortality are essentially due to large doses of pharmacologically active components delivered together. Here is where the interest to drug discovery lies.

1.2 Venom sources and pharmacology

Physiologically, venom components are expressed and stored in the venom gland and reside there until delivered by the organism into another, via an injection system, when required. This has led to selection of stable folds to maintain peptide components in active conformation [13–15] ready for use. Thus venom orthologues are more stable than their somatic orthologues. For example, natriuretic peptides from mambas (*Dendroaspis* spp.) venom are stable for many hours in rat plasma whereas brain and atrial natriuretic peptides are mostly degraded in under an hour [16]. The glucagon like peptide, exendin-4, from the Gila monster *Heloderma suspectum* has a tenfold greater plasma half-life than human GLP1(17) making this peptide extremely useful for drug discovery.

Animals from a wide range of species utilise venoms for hunting and as a defence mechanism, and examples of the evolved diversity are shown in Figure 1. These venoms can have a varied range of consequences on the victim such as a cytotoxic, neurotoxic, myonecrotic, haemolytic, cardiotoxic, necrotic, nephrotoxic, hypotensive, haemorrhagic or algogenic effects based on their biological activities and unique origins.

1.2.1 Terrestrial Reptiles

The venom systems of reptiles, now correctly known as non-avian reptiles due to the genetic inability to separate them from birds (the direct decedents of the dinosaurs), were the first to be characterised. The majority of snake species are non-venomous, however those that are, include vipers, rattlesnakes, mambas, cobras [18] and many species of sea snakes, will be discussed later. The helodermatid lizards are also venomous, these are the Beaded lizard (*Heloderma horridum*) and the Gila Monster (*Heloderma suspectum*). The venom system in these lizard may have evolved independently from the snake system as initially proposed. [19] However later work by Fry and colleagues in 2012 proposed the Toxicofera clade, which has largely been adopted, suggesting a single venomous reptile ancestor and loss of venom from the other suborders. [20] The debate continues on as to the true evolution of venom systems in non-avian reptiles as either a single event [20] or multiple convergent events as restated. [21]

Snake venoms are frequently described as being composed of a complex mixture of proteins and peptides which typically make up 90-95% of their dry weight. By function these include three-finger toxins, disintegrins, phospholipase A2s (PLA2s), metalloproteinases, hyaluronidases, cholinesterases, L-amino acid oxidases, serine

proteases, lectins, and growth factors. Other components include inorganic cations, such as calcium, zinc, magnesium, sodium, and potassium. [22] Envenomation can cause the recipient of snake bite to be exposed to a wide range of effects from pain and necrosis to haemorrhage or coagulation. Despite this complexity many snake venoms contain a few major protein classes with lots of minor components [22] and the evidence for this can be seen in the ion exchange chromatogram Figure 2a. In snakes, venom proteins have also been found in extracellular vesicles (SVEVs) within the venom of four different species of snake (*Agkistrodon contortrix contortrix*, *Crotalus atrox*, *Crotalus viridis* and *Crotalus cerberus oreganus*) [23] but this has not yet been reported in species other than snakes.

1.2.2 Arthropods

A wide range of arthropods is venomous. [24] This includes the major groups of Arachnida including spiders (Araneae). [25] Spiders are one of the largest venomous clades [26] but not all of these species produce venoms that are harmful to humans. Typical spider venom is estimated to comprise over 400 venom peptides [27] and the majority of these fall within a small size range of 8-11 KDa [28]. This similarity is demonstrated through reverse phase HPLC in Figure 2. The vast majority of spiders use their venom in a predatory manner but the neotropical jumping spider *Bagheera kiplingi* actually feeds mainly on plants. [29,30] Spider venoms have different levels of toxicity to humans and other animals. Funnelweb and widow spiders (*Latrodectus hasselti and L.mactans*), can kill a human, although a successful antivenom means there are few fatalities [31], whereas British native spiders are of no risk to mammals at all but are very effective at killing their prey item, which could be an insect or another

arthropod. Examples of the latter would be the giant house spider *Eratigena atrica* (Figure 1H) and the European spider *Tegenaria agrestis* . [32]

The main active components in brown recluse spider (Loxosceles) venom are inhibitor cystine knot (ICK) peptides, phospholipases-D and astacin-like metalloproteases. Also found in the venom are serine proteases, serpins, hyaluronidases, venom allergens, and a translationally controlled tumour protein (TCTP) [33,34]. Spiders of the genus *Latrodectus* (widow and red back spiders) have venom that contains latrotoxins which are neurotoxic and are the main components which cause intense pain to the recipient of a bite. [26] There are insect-selective neurotoxins from the venom of the Blue mountains funnel-web spider *Hadronyche versuta* called Janus-faced atracotoxins (J-ACTXs) [35] of use to crop science. There are many other neurotoxic peptides such as; hainantoxin-III and hainantoxin-IV from Theraphosidae spiders. These toxins were initially isolated from the venom of the Chinese bird spider *Seleconosmia hainana.* [36] Later confirmed through genomics and proteomics although taxonomic changes meant they were assigned to *Haplopelma* (*Ornithoctonus*) *hainana.* [9] At the time of writing the official *toxin nomenclature is* μ-theraphotoxin-Hhn2a and μ-theraphotoxin-Hhn1b reflecting the Haploplema genera, however recent mitochondrial genome sequencing confirms this spider as *Cyripagopus hainanus.* [37]

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Other arachnids that are also venomous including the Scorpions (Scorpiones) [37] and pseudoscopions (Pseudoscorpiones); the latter have an average venom yield of 5 nL [38]. It was also once questioned whether the ticks (Parasitiformes) were venomous but they are, according to the latest definitions of a venomous animal, i.e. the animal actively injects its toxins [39].

Centipedes (Chilopoda) [40] and Remipedes (Remipedia: *Speleonectes tulumensis*) [41] are also venomous and so are blood worms, such as *Glycera dibranchiata, Glycera fallax* and *Glycera tridactyla.* [42] A wide range of Insects (Insecta) are also venomous including Hemiptera or the true bugs and an example of these are the assassin bugs [43]. Hymenoptera (bees, wasps, and ants) produce venom and while these are the best known, there are also many other insects that possess venom. [43] Adult moths (Lepidoptera) are not venomous themselves, but moth caterpillars can be venomous [44]. An example of Dipterans (true flies) that produce complex venom, rich in peptides, (in particular disulfide rich peptides) is the superfamily Asilidae, known as Assassin flies, such as *Dolopus genitalis* . [14]

1.2.3 Mammals

There are a number of venomous mammals including the slow loris species (*Nyciticebus* spp.) [45] and the male platypus (*Ornithorhynchus anatinus*). [46] The platypus uses its venom in competition as well as in defence, [46] and has paired venom glands that are connected to spurs on each hind leg. [47] Echidna were once thought to be venomous but they do not fit the definition of a venomous animal, [46] however some other insectivores are truly venomous. From the order Eulipotyphla the Hispaniolan Solendon *Solenodon paradoxus* [48] Figure 1 and a number of shrews including *Neomys fodiens* the Eurasian water shrew are a venomous. [49] Blarina toxin (BLTX) has been isolated from the venom of the short tailed shrew *Blarina brevicauda* and it is suggested that its toxicity is due to a kallikrein-like activity of the venom. [50] Blarinasin is also a tissue kallikrein-like protease found in the same shrew. [51]

1.2.4 Cone snails (Conoidea)

Like scorpions, cone snails also use their venom for both defensive and predatory purposes. It contains peptides called conotoxins that have neurotoxic effects on the victim. These peptides have evolved to adapt to new prey. [52] The geography cone (*Gastridium geographus*) and the piscivorous cone snail (*Conus tulipa*) are thought to be the most deadly species of cone snail [53] to humans, probably due to their diet of vertebrates, compared with other cones. There is also another lineage of less well studied cone snails belonging to the *Gemmula* genera. [54]

1.2.5 Sea Snakes

Many other venomous creatures inhabit the ocean, including sea snakes. An example being the spine-bellied sea snake (*Hydrophis curtus*) whose venom shows myotoxicity against a human skeletal muscle cell line and is selective for skeletal muscle cells compared to cardiac muscle [55]. The elegant sea snake (*Hydrophis elegans*), olive sea snake (*Aipysurus laevis*) and the Australian beaked sea snake (*Hydrophis zweifeli*) possess venoms which habe a higher myotoxic effect compared to the spine-belled sea snake. These snakes are passive in their natural ocean habitat and only really present a hazard to humans when caught in nets or washed up.

1.2.6 Fish

There have been fewer studies on fish venoms compared with other venomous animals even though more than fifty percent of venomous vertebrates are fish. [56] Fish have convergently evolved venom systems multiple times [57,58] leading to multiple lineages. The scorpionfish (*Scorpaena plumieri*) has venom containing cytolysins which cause cardiotoxic, neurotoxic and inflammatory effects. [59] The

lionfish (*Pterois volitans*) (Figure 1C), has venom which causes algogenic and inflammatory effects [60]. One of the most dangerous venomous fish is the reef stonefish (*Synanceia verrucosa*). The venom glands are in the dorsal spines and cause necrosis, haemorrhage and inflammatory cell infiltration when tested in rats. [61] Humans fall victim to this fish when walking in shallow reef waters and their sting has been shown to even penetrate flipflops by several television presenters. Stingrays, unrelated to the other venomous fish as they are cartilaginous (Chondrichthyes)and more closly related to sharks, deliver envenomation, typically to the lower limbs which causes pain, swelling and necrosis. [62,63]. Also, several species of sharks are venomous [58], although this property has been poorly studied compared with other taxa.

The greater (*Trachinus draco*) and lesser (*Echiichthys vipera*) weever fish have some unique venom components but these haven't been studied in detail [64]. Some have been shown to cause specific effects. For example Dracotoxin in the greater weever fish causes membrane depolarising activity and haemolysis [65]. Components of the venom of the lesser weever fish venom cause cell lysis in erythrocytes, cell cycle arrest and tissue necrosis [66].

1.2.7 Cnidaria

The Cnidaria are a group of aquatic animals all containing subcellular stinging capsules called nematocysts. Coral and jellyfish venom is much understudied [67] but this field is growing as techniques improve. Members of this phylum include corals, which have interesting bioactive compounds [68], hydras, which have pore forming toxins called cytolysins [69], sea anemones [70] and jellyfish. [71] Sea anemones produce a number of peptide toxins that modulate ion channels. [72] The box jellyfish

Chironex fleckeri venom is neurotoxic and causes tissue necrosis [71] but also has haemolytic effects. [73] The Portuguese man-of-war (*Physalia physalis*) is actually a colony of polyps rather than a single organism, however, together they maintain stinging tentacles that cause cardiotoxic, necrotic and neurotoxic effects [74] in humans, even after death.

1.2.8 Other venomous species

The blue-ringed octopus (*Hapalochlaena fasciata*) (Figure 1A) has tetrodotoxin (TTX) in its venom which causes pain and neurotoxic effects (paralysis) by inhibiting signal transduction by nerve cells through sodium channel blockade [75]. This toxin is synthesised by bacteria and is also found in other several species, for example in pufferfish and salamanders. Even sponges can cause pain and swelling, fire sponges are an example (*Tedania* spp.). (76) Sea urchins trigger an inflammatory reaction and can cause tissue necrosis and muscular paralysis in some], and the crown-of thorns starfish (*Acanthaster planci*) venom has strong haemolytic activity(77).

1.2.9 Application to drug discovery

Convergent evolution from unrelated species has led to extremely varied compositions of venom across a range of species lineages. For drug discovery, this means there is a very diverse compound library of potentially millions of compounds from an estimated 220,000 venomous species currently known [1]. The Australian funnel web spiders have the most complex venoms venom discovered to date, the venom of the Darling Downs funnel-web spider (*Hadronyche infensa*) contains over 3000 different peptides. [6] Cone snails have over 1000 venom peptides. [79] Platypus venom has

83 peptides from 13 different families. [47] In contrast the Northern short-tailed shrew has much less complex venom with only seven proteins isolated so far. [5]

So if venoms cause high mortality and morbidity in so many people why would venom components be considered as potential drugs? Looking at the problem from a pharmacological stand point the opportunities are obvious, and it is not a surprise that receipt of a random dose of a complex mixture of pharmacologically active compounds is dangerous. The clear action of these compounds led to early experimentation and application as traditional medicines with variable results [80] but now, in the hands of drug discovery scientists, their true potential is being realised .

2 Approved drugs from venoms

There are a number of currently approved drugs that have their origins in venom components, and these range from small molecules to peptides and proteins. Looking specifically at peptide therapeutics there are a number that have been derived from venoms which have been approved for clinical use [81] with origins ranging from snakes and cone snails to Gila monsters (Figure 1J).

2.1 Hypertension

Capoten (Captopril) an angiotensinogen converting enzyme (ACE) inhibitor was the first successful therapeutic to have been derived from a venom component. [18,82,83] It is a synthetic small molecule that was derived from work investigating the short, proline-rich bradykinin potentiating peptides isolated from the venom of the Brazilian or arrowhead pit viper *Bothrops jararaca*. Bradykinin potentiating peptides act by blocking the production of angiontensin II by inhibiting somatic ACE and are important in blood circulation physiology; their effects originally discovered from studying

patients envenomed by *Bothrops jararaca.* Investigative work has examined the possibility of using these peptides as direct therapeutics to control blood pressure but they were not developed due to poor oral bioavailability. Instead, the key features of the peptides were used to screen small organic molecule libraries leading to the identification of 1-((2s)-3-mercapto-2-methylpropionyl)-1-proline (Captopril) Figure 3. The significantly increased potency of this molecule attributed to the orientation of the molecule's proline residue within the substrate binding pocket of ACE together with the thiol moiety coordinating with the active site Zn. Improved versions of the original drug have been developed, including Ramipril which is currently prescribed to treat hypertension. [84]

2.2 Angina

A number of venom-derived drugs have been approved to treat unstable angina, in patients that are at high risk of death from myocardial infarction. Angina drugs Aggrestat (tirofiban) and Integrilin (eptifibatide) [18] are disintegrins that block the aggregation of platelets by binding to the aIIbb3 receptor thus preventing fibrinogen binding. These were developed from the small disulfide-rich venom proteins echistatin, isolated from *Echis carinatus* (the saw-scale viper), and barbourin, from *Sistrusus miliarius barbouri* (the south-eastern pygmy rattlesnake) (Figure 3). These mimic the short amino acid sequence Arg-Gly-Asp (RGD) and Lys-Gly-Asp (KGD) respectively, that are key to binding to the receptor, and it has been found that the substitution of the lysine for arginine in the barbourin pharmacophore increases specificity for the aIIbb3 receptor.[84] The RGD motif within echistatin is located away from the disulfide-constrained core of the protein in a loop region. The development of tirofiban from echistatin involved altering the spacing of the amino acid RGD pharmacophore

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side chains within the venom pharmacophore. In contrast eptifibatide was developed from barbourin as a small cyclic disulfide-constrained peptide containing the barbourin KGD motif.

2.3 Anticoagulants

Viprinex (ancrod) is derived from the Malayan pit viper (*Calloselasma rhodostoma*) and was developed as an anticoagulant and for use in the treatment of acute ischaemic stroke. [85,86] It is not currently approved for clinical use in any country although its effects on stroke have been studied in clinical trials [87,88].

Exanta™ (ximelagatran) from cobra venom was a promising anti-coagulant peptide [89] developed by Astra Zeneca to try to replace warfarin as an anticoagulant. The problem with warfarin is that it carries a high risk of haemorrhage at therapeutic concentrations and its therapeutic window is narrow. Exanta was hailed as a wonderful new drug and was already being used in a number of different countries when unfortunately it had to be withdrawn in 2006 because of serious liver injury displayed by raised levels of liver enzymes. However development of this drug showed that a specific oral thrombin inhibitor was a possibility and led to the development of non-vitamin K antagonist oral anticoagulants (NOACs). [90]

Defibrase has been developed from the enzyme component batroxobin found in toxin from the Brazillian Lancehead snake (*Bothrops moojeni*) which acts as a defibrinogenating agent and is used to treat thrombosis [91]. It is registered for use in China and Japan. Reptilase is the trade name for a thrombin-like serine protease thathas been similarly developed but from the Common lancehead (*Bothrops atrox*). This work was first published in 1958 [92] and reptilase was later used in patients to breakdown fibrin clots to restore blood flow [93] and reduce bleeding post cardiac

surgery [94]. It is also used in a blood test used in the UK's National Heath Service to investigate normal clotting time even in preference to heparin [95]. Hemocoagulase (Botroclot®), used in the prevention of bleeding and treatment of surgical bleeding (haemorrhage), is derived from *Botrops jararaca* . [94]

Alfimeprase, a fibrolase, is a fibrinolytic enzyme isolated from southern copperhead snake (*Agkistrodon contortrix contortrix)* venom which has very effective thrombolytic activity. It looked very promising *in vivo* but this enzyme did not get past Phase III trials as needed endpoints of the trial were not met. [96]

Refludan® (lepirudin) and Hirulog® (bivalirudin) are anticoagulant drugs for patients who have developed a reaction to other anticoagulants. Their structures are based on hirudin (Figure 3), a direct thrombin inhibitor (DTI), isolated from the saliva of leech *Hirudo medicinalis.* [100] Refludan® was discontinued in 2012, not due to safety concerns but because of discontinuation of manufacturing. [101] Bivalirudin was developed as a synthetic peptide and was approved for use in 2000 as an anticoagulant in patients with unstable angina undergoing percutaneous transluminal coronary angioplasty, and in patients at risk of heparin-induced thrombocytopenia (HIT) undergoing percutaneous coronary intervention (PCI). [81]

2.4 Analgesics

Chronic pain is another area in which successful drugs have been derived from venoms. Prialt (ziconotide) has been developed from a peptide from the magical cone snail (*Conus magus*) venom ω-conotoxin MVIIA [97] (Figure 3) and is a blocker of voltage gated calcium channel $Ca_V2.2$. Leconotide, an even more selective drug

based on the same toxin, the displayed side effects in clinical trials and was not developed further. [81,98] CAROL

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Byetta (Exanatide) was developed to treat type 2 diabetes from a peptide called exendin-4 (Figure 3) from *Heloderma suspectum* (Gila Monster) (Figure 1 J). This peptide mimicks glucagon-like peptide 1 (GLP-1) by stimulating the glucagon-like peptide 1 receptor (100). Later it was found that these is an increased risk of having pancreatitis with this drug. Further developments in this area have led to improved drugs being produced and these are Bydureon® (exenatide extended-release) and Lyxumia® (Lixisenatide) although Bydureon® may increase the risk of developing thyroid cancer.

3 Composition of venoms, an overview of evolutionary complexity

Unlike synthetic compound libraries the nomenclature of biological compounds from venoms relies on taxonomic descriptions of the species. This work has focused on the protein components but the growing adoption of rational nomenclature pushes the focus towards functional activity [102–104]. Universal adoption of this nomenclature with its Greek prefix denoting activity will greatly aid in the uptake of venom proteins in drug discovery. Further work is on-going on reinforcing uptake of rational nomenclature to describe diverse venomous groups; this will further open up the utility of venom compounds to drug discovery scientists not familiar with the animals and systems the toxins come from.

3.1 Proteinaceous venom components

3.1.1 Enzymes

Large enzymes predominate in viper venoms (Serpentes, Viperidae) [105] and on the face of it are far from drug like molecules; however some have made it as successful drugs. Reptilase, mentioned above, is an example of a thrombin like serine protease from the lance head viper (*Bothrops* spp.). Snake venom serine proteases act as pro-coagulant through conversion of soluble fibrinogen to insoluble fibrin [106], but also act to breakdown clots through continued catalysis of fibrin into fibrinopeptides which are uncoagulable resulting in afibrinogenemia [107]. Those deposited in UniProt have a molecular mass of 27.7 (+/- 2.5) KDa. Serine proteases are common in viper venoms [106–108]. They are also commonly reported in Cnidarian venoms [69,109–112], which is a clear case of convergent evolution as there is no venomous ancestor between Cnidaria and Serpentes taxa. Other marine venomous species containing serine proteases include Stingrays (Elasmobranch) [113], and the Squid family (Coleoidae). [114] Hemodynamic serine proteases are also reported in wasps and ants (Hymenoptera) (114–116), theraphosids (Theraphosidae) [118], true spiders (Araneidae) [119] and scorpions (Buthidae) [120,121] but have not been used therapeutically. Evolution by natural selection is the famous blind watch maker and thus mutations in some serine proteases have also led to them inhibiting ion channels [122] a feature also common in phospholipase A2 enzymes. [123]

Phospholipase enzymes are almost ubiquitous in venoms and have been described from a wide diversity of taxa, including; snakes [124], true spiders [125], scorpions [126], jellyfish [110], fish [127], snails [128], bees and wasps. [129] They have only been described from a couple of Theraphosidae genera *Pamphobeteus* [130] and *Lasiodora* [131], however the authors believe they are more prevalent than the literature suggests. (Venomtech unpublished work) Phospholipase enzymes such as PLA2 act as spreading factors [132], like but are also cytotoxic, especially PLD [133],

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and are responsible for neuro- and hemo-toxic effects [124]. The catalytic site of action of PLA2 enzymes is at the *sn*-2 position of sphingolipids such as those found in cell membranes.

Hyaluronidase is another enzymes frequently recruited into venom gland expression and is found in such diverse species as cobras [134], spiders [135], centipedes [136] and even the duck-billed platypus [137], which have all evolved venom independently. Phosphodiesterase (PDE) enzymes as drug targets gained popularity in 1996 with the publication of a potent and selective inhibitor of PDE5, sildenafil (Viagra™), for male erectile dysfunction [138]. These enzymes were first purified from snake venom in 1963 [139] and have since been used for identification of sildenafil analogues [140]. Priapism has been noted in patients envenomed by Brazilian wandering spiders (Phoneutria spp.) [141] and scorpions [142], however these effects are independent of PDE enzymes. More recently the first ever case of priapism from snake venom was reported from an envenomation case involving a juvenile Russel's Viper (*Daboia russelii*). [142] The mechanism is currently unknown but could be through PDE inhibition. Phosphodiesterases have also been discovered in scorpion venoms [125] and stonefish venom. [143]

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ADPase, ATPase and 5'-nucelotidase activities are commonly found in viper venoms containing phosphodiesterase components. [144] These enzymes are considered one of the minor protein families in venoms [22], but at this moment are also poorly studied. DNase and 5'-nucleotidase enzymes are common in viper venoms(144) and stone fish venom(143) where they act much like other enzymes of this class degrading polynucleotide chains and free nucleosides. However these venom proteins have had a critical role in phosphodiesterase drug discovery as the PDE assays rely on 5'-

nucleotidase from (*Crotalus atrox*) Western Diamondback rattlesnake venom to function. These enzymes appear to be restricted to snake venoms, however. There is one report of nuclease activity in lizard venom [145] and one in the assassin fly [146], however both of these are transcriptomic and proteomic rather than confirmation of functional activity. The most interesting nuclease activity is from the crown of thorns starfish (*Acanthaster planci*) venom as it is cell and nuclear penetrant where it causes hepatocyte apoptosis through DNA degradation [146]. Phosphatases, both alkaline and acid, are also known from snake venoms.

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Metalloproteases are a major component of viper venoms and are particularly common in rattlesnake venoms where they compose up to 43% of the venom and are also responsible for major muscle damage after envenomation [147]. These enzymes are also inhibited by cation chelation and non-selective metalloprotease inhibitors which are being investigated as potential snakebite envenomation treatments. [147,148] Previously thought only to be present in viper venoms, these enzymes have more recently been discovered in recluse spiders (*Loxosceles* spp.) [149], bark scorpions (*Tityus* spp.) [121], jellyfish [69] and even centipedes. [150] Many venom metalloprotease transcripts are translated with their associated disintegrin domains, but these are not as well represented in the venom proteomes [22].

L-Amino Acid Oxidase enzymes (LAAO) are a major component of snake venoms and are particularly abundant in pit vipers, including rattlesnakes (Crotalinae) and contribute to the yellow colouration of such venoms as a result of the isoalloxazine ring system contained within the flavin cofactor [151]. Though thought to be restricted to snake venoms, there is a recent report of an LAAO found within a scorpion (*Tityus serruatus*) venom (125) and, if the yellow colouration is indicative of the presence of

LAAO, then it is likely that these enzymes will be discovered in the venoms of the Indian tiger or ornamental spiders (Genus *Poecilotheria*) (figure 1 D). Although there are numerous reports of the antimicrobial action of snake venom LAAO, it is highly unlikely that these will yield any useful compounds in the fight against infection due to the probable mode of action being release of H_2O_2 (152). The presence of LAAO activity in venom suggests that there could be selection pressure either to inhibit this enzyme in the venom gland, or for the presence of D-amino acids that are resistant to it. Peptide isomerases have so far only been reported from a few species including duck-billed platypus [153] and funnel web spiders [154]. D-amino acids are well known in cone snail venoms [155] and reported from funnel web spiders [156]. It is therefore possible that isomerisation is underreported in venoms and could potentially account for differences in synthetic versions of natural peptides.

3.1.2 Cysteine-Rich Secreted Proteins

Cysteine Rich Secretory Proteins (CRiSP) are predominantly found in snake venoms and some arthropods, but also in the venomous saliva of monitor lizards [146]. In mammals these proteins play a major role in the mammalian reproductive system, such that the venom versions may have utility in reproductive medicine. These 20-30KDa proteins consist of over 200 amino acids, with sixteen cysteines, of which ten are present in the C-terminal producing the characteristic cysteine-rich motif [157]. CRiSPs are highly conserved throughout evolution with proteins deposited in UniProt from tomatoes to turkey vultures and drosophila to dogs, so it's no surprise that they are also present in a wide range of unrelated venom systems. Of these, they have been predominantly studied in non-avian reptiles [157]. Snake venom CRiSPs are some of the largest ion channel blocking proteins and act on cyclic nucleotide gated

ion channels [158] and L-type calcium channels. [157] L-type calcium channel block results in blockade of smooth muscle contraction. The diversity of these proteins in the natural world and their subsequent neofunctionalisation in venom systems presents a great opportunity to the drug discovery scientist to find structurally improved stability in a molecule by studying the venom orthologue of the CRiSP of interest.

3.1.3 Other large proteins

Cobra venom factor (CVF) is one of the largest molecules present in venoms at approximately 180KDa, depending on species. It is a structural and functional mimic of mammalian complement C3 but with a significantly longer half-life [159]. Through persistent activation of the complement system the net result is complement depletion and thus it is used as a tool for this purpose *in vivo* studies such as ischemic reperfusion injury. [160]

3.1.4 Membrane attack complexes pore forming (MACPF) proteins

These large proteins (>35 KDa) are part of the membrane attack complex pore forming (MACPF) proteins family and are related to perforins. These genes are present throughout the natural world, being predominantly expressed in the immune system. MACPF proteins bind to cellular membranes and form large pores for cellular destruction [161]; this has clearly made them ideal as weapons and thus they have been repurposed in venom systems. They are a major component of aquatic venoms, in Cnidaria they have evolved into a gene family of venom MACPF proteins called actinoporins which are toxic to fish and crustaceans [162]. Other aquatic venoms including those from fish and cone snails that have also repurposed these toxins [163]. With the high risk of internal damage from such toxins when they are stored in the

venom glands, there is a very high likelihood of important inhibitors of the immune system membrane attack complex being present within venoms and these could potentially dramatically improve the lives of patients with overactivity disorders of the immune system.

3.1.5 Cystatins

Cystatins are a group of poorly studied C1 Cysteine protease inhibitors of around 140 amino acids, predominantly found in snake venoms [164] and some others where they have no known toxic activity. However they have potential as anti-metastatic agents for cancer. [165]

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3.1.6 Peptide toxins

Unlike the large enzymes the smaller protein and peptide components of venoms present the drug discovery scientist with a wealth of biotherapeutic potential. The delineation of peptide from polypeptide and protein hasn't been formally decided. Typically in venom research, and in this chapter, non-catalytic polypeptide chains of approximately less than 100 amino acids are referred to as peptides, and those larger as proteins. The largest peptides are the three-finger neurotoxins and natriuretic peptides which have many members around and slightly over the 100 amino acids. However, sizes range down to small cationic and other linear peptides of less than ten amino acids.

Inhibitor cysteine knot (ICK) peptides, also called knottins, dominate the venom drug discovery literature as these amino acid chains are stably folded with typically three to six disulfide bonds and inhibit a wide range of ion channels across a wide range of species including mammals and insects. Thus, they have utility in crop protection as

insecticides as well as in human and veterinary drug discovery. They are predominantly found in arachnid and cone snail venoms, but such is the taxonomic distance between these that it is unsurprising to discover recruitment in other species such as robber flies. [14] Although initially discovered as an ion channel inhibiting motif, the knottin family of peptides have a wide range of specific activities including GPCR agonism [165], intracellular stimulation of ryanodine receptors [166] and ion channel activation. [167] The stable confirmation of disulfide bonds allows mutation of the loops for fine tuning pharmacology, both by evolution and human intervention, without significantly altering the tertiary structure. This makes the knottin superfamily probably the most useful class of venom peptides for drug discovery. ICK peptides have also been found to inhibit venom proteases [168] and are expected, to have utility at many other targets.

Another class of enzyme inhibitors from venoms contains the Kunitz fold typical of mammalian trypsin inhibitors. Kunitz peptides are among the most common peptides in snake venoms [22] but are also found in arachnids including the tiny Pseudoscorpions [38,169] whose venom, like those of snakes, is rich in proteases. It is likely that these serine protease inhibitors evolved due to the need to protect the venom glands from internal damage from venom enzymes and later evolved further for action in prey such as clot prolongation from plasmin or kallikrein inhibition. [170]
To date no phosphodiesterase inhibitors found in venoms have been published, but their presence is postulated due to the prevalence of PDEs in venoms.

Other enzyme inhibitors include the well characterised bradykinin potentiating peptides from the lancehead vipers (*Bothrops* spp.) which exert their hypotensive effect though inhibition of angiotensin converting enzyme (ACE). Discovery of this

action inspired the ACE inhibitor class of small molecule drugs on the market today. These peptides have been discovered in diverse classes of pit vipers and some scorpions. They are very short (5-13 amino acids) and possess a post-translationally modified pyroglutamic acid. The peptides have already shown great utility in drug discovery but the smallest, at five amino acids and 611.7 Da, could be useful in its own right. There is also a potential for cyclisation through the N-terminal cleavage of the pyroglutamic acid. [171]

Bothrops natriuretic peptides are located on the bradykinin potentiating peptide transcripts and post-translationally cleaved into the active forms along with the many isoforms of BPPs. [172] However this does not appear to be the case for the elapid, lizard and scorpion venom natriuretic peptides, but could be the case for those from the duck-billed platypus. [173]

Three-finger toxins (3FTX) are almost exclusively restricted to elapid snakes, where they make up a large percentage of the venom proteome and are found across all the Elapid taxa. There are a few exceptions found in other snakes such as vipers and rear-fanged colubrids. Three disulfide bonds hold these peptides in a stable structure resembling a thumb and first two fingers of a human hand when held apart. Three-finger toxins are responsible for the majority of the neurotoxic and cardiotoxic actions of these snakes and the cytotoxic actions of the spitting cobras. These incredibly diverse toxins represent another class of venom peptides with great drug potential due to their extreme pH and thermal stability, coupled with selective target engagement such as beta adrenergic receptor antagonism [174], muscarinic receptor antagonism [175] and vasopressin antagonism. [176]

Snaclecs are a class of C-type lectins found specifically in snake venoms where they modulate prey and predator haemodynamics, but they are also very useful as drug discovery tools [177] and have made it to licenced drugs. [22] Lectins are also found in Cnidarian venoms and a few other unrelated species where they act in a similar way.

Cationic peptides are commonly found within venom systems, both short and long forms, however, the long forms are still only about 40 amino acids. These short non-disulfide linked peptides exist structurally as cationic helices that interact with membrane lipids, some form pores causing lysis which can be specific for bacterial cells. [178] Others, like crotamine, are cell penetrant and are being investigated as potential cancer therapeutics. [179] These alpha-helical cationic peptides are very common in scorpion venoms where there main target appears to be intracellular calcium signalling through ryanodine receptors but their pharmacology can be engineered out making them useful intracellular shuttle vectors. [180] Probably the most well characterised alpha helical peptide is melittin from honeybees (*Apis melifera*) but similar peptides are found in many hymenoptera where they have similar antimicrobial and cell penetrant properties [181,182] and antiviral actions. [183]

3.1.7 Growth factors

Nerve growth factor (NGF) and other growth factors have been discovered to be components of venoms. Snake venom NGF is made up of a homodimeric complex of beta-subunits. The activity of NGF was first described using snake venom from the moccasin snake *Agkistrodon piscivorus*, the first source used to isolate NGF [184] and was later found in Viperidae, Crotalidae and Elapidae family venoms. The structures of these vary between species of snake. Vascular endothelial growth factor (VEGF)

is key in the formation of blood vessels but has also been found in snake venom. It varies in structure and function in different snake species and in particular around the C-terminal putative coreceptor-binding and receptor-binding loop regions of the proteins. The puff adder *Bitis arietans* has venom type VEGFs unique from other VEGFs. [185] Epidermal Growth Factor (EGF) repeat-containing transcripts were discovered in the rear fanged snake *Thamnodynastes strigatus.* [186] and in the venom of the giant red bull ant, *Myrmecia gulosa*, a peptide has been discovered which shares the same six cysteine containing structural domain as EGF. This peptide has been named MIITX2-Mg1a. [187] EGF peptide domains have also been found in sea anemones including *Stichodactyla gigantean.* [188] Some of these venom proteins are likely to be responsible for some of the effects seen on tyrosine kinase receptors. [189]

3.1.8 Non proteins

Tetrodotoxin is probably the most well-known of the non-protein venom components, although its presence in pufferfish (for which its most associated) is not associated with a venom system, it is a poison as the aggressor has to do the biting. Tetrodotoxin, palytoxin and saxitoxin are produced by zoanthids, dinoflagellates and cyanobacteria in aquatic environments and thus when found within venom systems the host must have acquired mutations to protect itself as in the blue ringed octopus [75] (Figure 1A). It is extremely useful in neuroscience research as it only binds to a select group of voltage gated sodium channels, sparing the pain relevant NaV1.8 and NaV1.9 along with the cardiac NaV1.5 channel [75]. Thus it is used frequently at the end of electrophysiology studies to confirm whether the channels being studied are TTX sensitive or resistant.

Scorpion venoms are unique in potentially containing mucopolysaccharides and mucoproteins [190] in their venom and also have an unusual prevenom which is rich in potassium ions [191]. Neither of these are presently considered of interest to the drug discovery world, and the high viscosity of scorpion venom is a constant challenge when collecting and purifying the components from these venoms.

Many venoms, especially those from true spiders (Araneomorphae) and insects contain a host of small non-peptide components such as acylpolyamines, free amino acids and biogenic amines which, until recently, have been difficult to study. A team from the University of Zurich, Switzerland, has produced a database of such toxins from spiders and a rational nomenclature to make the research of these toxins much easier [192]. The VenoMS database (www.venoms.ch) contains a host of identified small molecules from spider and wasp venoms that need to be taken into account when screening. Glutamate is also likely to be added to the list soon although not currently in the database. Snake venoms, and probably many other venoms, also contain polyamines and other metabolites but at this stage it is not clear if these are produced by the venomous animal or the newly discovered venom microbiome. [193,194] The other class of small molecules known from venoms are the alkaloids, principally restricted to ant venoms[195]. However, there are many erroneous references to toad and millipede venom, where in fact these are sequestered toxins acting as a poison as they lack the delivery system characteristic of a venom.

4 Drug like structures within venoms

Determination of the three dimensional structure of a venom component provides an insight into the molecule's interaction with its target, allowing the researcher to identify

critical parts of the molecule essential for activity and permitting the development of the compound to generate leads for potential therapeutics. Knowledge of the structure identifies moieties that can be modified to increase potency, selectivity or to introduce modifications to improve desirable drug-like properties, such as increased stability, solubility *etc*., just as in those studies performed with synthetic small molecules.

4.1 Structures found in venom components

Recent work that utilised a combined proteomic/transcriptomic approach to characterise venom from 191 different animal species has revealed over 20,000 venom toxin sequences. [165] From these some 3600 physical peptides were recombinantly produced for further characterisation, with a remarkable 8% found to possess activity against the human melanocortin receptor (hMCR4). Half of these peptides contain at least three disulfide bonds, with 23% having four. From a size perspective 28% of the peptides are around 31-40 amino acids in length, with 15% being 41-50 amino acids and 20% of peptides are between 10 – 30 amino acids in length. Of the possible post-translation modifications (PTMs), 38% of peptides are hydroxylated and 34% amidated. Other PTMs include pyroglutamic acid addition (in 21% of cases) or show addition of 4-carboxyglutamic acid.

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Peptide toxins can be broadly separated into two classes based upon their structure: disulfide-rich peptides and linear peptides. Disulfide-rich peptides have significant structural and functional variation whilst linear, non-disulfide bridged peptides tend to be helical in structure.

4.1.1 Disulfide-rich peptides

Within the disulfide-rich peptides class is the inhibitor cysteine knot (ICK) [196]. The ICK is a structural motif characterised by three disulfide bonds and arranged such that two of the disulfide bonds form a ring through which the third threads (Figure 4), while the main secondary structural element consists of a beta hairpin loop near the C-terminus. The cysteine knot is a very stable arrangement, and this structure is prevalent in many peptides from venomous organisms from all kingdoms. Further structural variations of this basic motif are also known that possess additional disulfide bonds, presumably to increase stability further.

A representative ICK peptide is the ProTX-III peptide from the green-velvet theraphosid *Thrixopelma pruriens*, which has been found to inhibit sodium channels and has potential for use in treating chronic pain. Structure/function relationships of this peptide reveal that the C-terminal region is responsible for interacting with the human $Na_V1.7$ sodium channel without affecting voltage sensing, and that possibly side chain orientations within this peptide are responsible for the distinct interactions and mechanism of action when compared to ICK peptides from other spiders. [197] It should be referred to as μ-theraphotoxin-Tp1a using the rational nomenclature system. [102]

Double knot peptides, where there are two independently-folded ICK motifs, were predicted to be found from amino acid sequences isolated from the Chinese bird spider or earth tiger tarantula, *Ornithoctonus huwena* [198] and were subsequently isolated from the funnel web spider *Hadronyche infensa*, (Hi1a peptide, Figure 4). [199] The Hi1a peptide has been found to target the acid-sensing ion channel 1a (ASIC1a) and has potential for development as a therapy for ischemic brain injury. Structure/activity studies revealed that both ICK domains need to be present for full potency of the

peptide revealing the importance of the ICK domains for activity. Other tools for ASIC channels come from unrelated venoms including sea anemones, black mambas and another spider (*Psalmopoeus cambridgei*). [28]

A derivative of the ICK motif has also been identified in the form of a disulfide-directed beta hairpin (DDH) and consists of a two disulfide bonds (connectivity 1-4 and 2–3) which may represent either an evolutionary precursor to the ICK motif or have arisen via a convergent evolutionary process. [35,200] A further structure identified within the ISTX-1 sodium channel inhibitor from the tick *Ixodes scapularis* [201] has the disulfide arrangement of the ICK motif but a spatial arrangement distinct from ICK or DDH.

There are many other disulfide-containing peptides present in arthropod venoms that are distinct from the ICK motif and its derivatives. A structurally diverse disulphide-containing peptide is found in chlorotoxin from scorpion venom. It possesses a complex CS alpha / beta structural motif which has been found to vary in the number of disulfide bonds and their respective connectivities (Figure 4). This toxin binds to chloride channels in the brain.

4.1.2 Three-finger toxins

Elapid snake venoms are rich in three-finger toxins (3FTXs) which range from 58 to 81 amino acid residues, and are folded into a three-finger structure stabilised by disulfide bridges. These non-enzymatic peptides bind post-synaptically at neuromuscular junctions to induce paralysis in victims [209]. This diverse group of peptides differ in length and the number of disulfide bridges and can be present as monomers, covalent- or non-covalent homo and heterodimers. [210,211]

4.1.3 Linear Peptides

Linear, non-disulfide bridged poly-cationic peptides predominantly occur in the venoms of hymenoptera (ants, bees and wasps) [202]. Poneratoxin from *Paraponera clavata* is a linear 25 amino acid peptide with an apolar *N*-terminal and a polar charged C-terminal helix connected by a beta turn resulting in a tertiary bent-conformation (Figure 4). In frog skeletal muscle, application of this peptide causes paralysis and appears to modulate sodium channel activity. [203] Melittin from the venom of the honey bee (*Aris mellifera*) is one of the most characterised examples of these linear peptides. Melittin itself is a 26 amino acid peptide with a C-terminal amidation. [204] It forms an alpha helix that contains a hydrophobic N-terminal region, a central region with both hydrophobic and hydrophilic faces and a C-terminal region that is hydrophilic (Figure 4). The peptide can bind to three other peptide units to form an antiparallel tetramer in a bent conformation. [181]

4.1.4 Large protein structures

In contrast to venoms from arthropods, snake venoms are more complex, exhibiting a broader range of pharmacological effects. They consist of a diverse array of larger proteins and peptides than the predominantly disulfide bonded peptides found in spiders, scorpions and cone snails[205]. Snake venom metalloproteinases (SVMP), snake venom serine proteases, phospholipases A2 (PLA2s) and three-finger peptides (3FTX) represent the major protein families present in snake venom. Additional protein components include cysteine-rich secretory proteins, L-amino acid oxidases, Kunitz peptides, C-types lectins, disintegrins and natriuretic peptides. [22,206,207]

Snake venom metalloproteinases are a major component of viper venoms. These zinc-dependent proteases range from 20-110 kDa in size and can be differentiated according to structure into three different classes; P-I, P-II and P-III. P-III proteins consist of a metalloproteinase and disintegrin-like domain together with cysteine-rich domains; P-IIs contain a metalloprotein and disintegrin domain, while P-Is contain only a metalloproteinase domain. The pharmacological effects of these multifunctional proteins are in influencing various steps of the blood clotting cascade causing systemic haemorrhage and incoagulable blood in prey/victims. The recombinant P-I metalloproteinase fibrolase with a modified N terminus was called Alfimeprase (Amgen) passed phase II for clearing peripheral artery occlusions, but failed to reach enpoints in phase III .[95]

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Snake venom serine proteases (SVSPs) range from 26-67 kDa and possess two distinct structural domains. SVSPs act by cleaving polypeptide chains after positively charged or hydrophobic amino acid residues resulting in protein degradation. Within the prey or victim, SVSPs cause a variety of effects, although the molecular mechanisms remain unclear. These include rupturing of capillary blood vessels, inducing edema and hyperalgesia, and hemotoxic effects. The thrombin-like serine protease batroxobin discussed above converts fibrinogen into fibrin through the cleavage and release of fibrinopeptide. Ancrod is also a serine protease that acts similarly to batroxobin in cleaving fibrinogen.

Phospholipase A2 cleaves the fatty acid tail from the glycerol backbone of phospholipids releasing the cleaved fatty acid as arachidonic acid. Subsequent downstream modification of the arachidonic acid produces eicosinoids which in turn mediate the inflammation response. Venom PLA2s range from 13-15 kDa and are

classified into four major sub-types of which Types 1 and 2 are a major component of venoms [208]. The single chain polypeptides typically contain 7 (sometimes 6 or 8) disulfide bridges and possess more alpha-helical secondary structure (50%) than beta-sheet (10%). The active site also acts as the binding site for the Ca^{2+} ion, an obligatory co-factor for catalytic activity. This basic structure is highly conserved between species but there are variations between isozymes in the number and arrangement of disulfide bonds, addition of a C-terminal extension or in substitutions of particular amino acids within the active site that affect catalytic activity.

4.2 Venom Small Molecule Structures

Small molecule or low molecular mass component toxins are significant components within a number of arthropod venoms. They are active against several biological targets including glutamate receptors, GABA receptors, NMDA receptors and monoamine oxidas. e[212–214] They vary structurally but the acylpolyamines dominate (Figure 5). Acylpolyamines, which are primarily antagonists of ionotropic glutamate receptors, are composed of an aromatic acyl headgroup linked via an amide to a polyamide backbone and ending with either a basic amino acid or amine tail. [215,216] In spiders, there are two separate classes; those that contain amino acids and non-amino acid containing acylpolyamines. [217] The non-amino acid acylpolyamines are more widely distributed across arthropod species and consist of an acyl head group attached to the polyamine backbone via a three to five carbon linkage composed of methylene units with hydroxylamines separating them. The amino acid acylpolyamines contain either one or two of the basic residues asparagine, ornithine or ω-N-methyllysine between the acyl headgroup and the polyamine

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backbone, and possibly up to three terminal amino acids. The polyamine backbone typically consists of methylene groups interspersed with amine (NH), methyl amine (NCH_3) or dimethyl $N(CH_3)_2$ amines and can vary between seven to forty three unit in length. [217]

Small structural differences can affect the mode of action of the acylpolyamines and therefore provide possibilities for development. As an example, Chiba *et al* (1996) purified two acylpolyamines (spidamine and joramine) from the orb weaver spider *Nephila clavata* and determined their structures by NMR spectroscopy. [218] On comparing the two molecules, joramine was found to differ from spidamine by the absence of an hydroxyl group on the C2 carbon of the aromatic headgroup. From an assessment of their activity, joramine was shown to reversibly block lobster neuromuscular synapses whereas spidamine was found to block them irreversibly. Structural studies suggest this hydroxyl group causes a conformation change in the molecule that may be responsible for this difference. [218]

Other low molecular weight molecules found in venoms with activity and potential for therapeutic development include purine derivatives of adenosine, inosine, guanosine and cytidine. HF-7, isolated from the corner funnel weaver or funnel web spider *Hololena curta* is a guanosine derivative that possesses an acetate ester attached to the C4 position of an alpha-linked guanosine molecule with two sulfate groups attached to the ribose sugar (Figure 5). HF-7 has been demonstrated to block kainite receptors and L-type calcium channels, albeit weakly. [219] Other molecules include the neuroactive nigriventrine from the wandering spider *Phoneutria nigriventer* which causes convulsions in rats, while alkaloids found in a variety of venoms have been shown to inhibit nitric oxide synthases (NOS). Solenopsins, a heterogenous group of

alkaloids isolated from fire ants, have a variety of biological activities, ranging from antibacterial and antibiofilm activity to interfering in neuromuscular transmission, mast cell histamine release, ATPase / sodium pump inhibition, phosphatidylinositol 3-kinase signalling and angiogenesis. [220] Two example solenopsins isolated from the venom of the fire ant *Solenopsis invicta* and the red ant *Solenopsis saevissima* are shown in Figure 5.

5. Drug like properties of venom components

Due to their extremely tight binding to cellular targets, peptide-based therapeutics can offer high specificity and low toxicity with this high specificity resulting from the large chemical space created by native amino acid side chain variation. By exploring this space with backbone and side chain combinations, peptide chemists can expect to select binders, agonists and antagonists of almost any biologically important target. Development of peptides as therapeutics requires a compromise between peptide length and pharmacologically useful levels of activity. There are numerous variables including size and accessibility of the ligand binding surface, the possible induced fit, ligand stability, and receptor residency time. Native peptide ligands are susceptible to proteolysis which can be reduced by chemically altering the amide bond and side chains. [221] However, even if cleaved, small fragments may still retain biological activity. [222] As many venom systems contain proteases or have evolved to be injected into other animals where proteases exist, they contain many modifications to greatly increase stability. [223] Mamba natriuretic peptides have a naturally longer plasma half-life to that of the mammalian peptides [16]. An additional problem remains that peptide ligands and their fragments are rapidly excreted through the kidneys giving short turnover times.

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Many of the toxin-derived drugs currently approved for clinical use have small volumes of distribution and exert their action at the site of administration. A small volume of drug distribution of current toxin-derived peptide drugs also indicates efficient clearance by the liver and kidney or by fairly rapid proteolysis, despite toxin adaptations. Together these clearance processes result in a short half-life value of peptide therapeutics. Depending on the therapy required, this may be advantageous or disadvantageous. For short acting drugs such as anti-clotting agents, rapid clearance may be desirable, but for therapies requiring prolonged exposure to the peptide, repeated administration or continuous drug infusion may be required.

Elimination of the peptide can be avoided by covalent modification using a wide variety of natural and synthetic polymeric conjugates that reduce the ease of proteolytic degradation and renal clearance. These conjugates include polyethylene glycol (PEG), hydroxyethyl starch (HES), human serum albumin (HAS), XTEN (a recombinant polyamide), PAS (a recombinant polypeptide made of proline, alanine and serine), polyglutamic acid and monoclonal antibodies.

Improving the access of therapeutic peptide to their targets across biological barriers can be achieved by adding moieties that are recognised by a cell's passive or active transport mechanisms. It has been found that the incorporation of positively charged amino acids at the termini of peptides improves tissue and cellular access [224,225] although large numbers of positively charged residues may destroy mammalian membranes. [178] An alternative is to conjugate the therapeutic peptide to ligands of cell surface receptors for receptor-mediated uptake. The most frequently used of these are sugar moieties coupled to peptides which can increase tissue penetration,

including transport across the blood/brain barrier and/or intestinal absorption while still maintaining activity. [226–229]

Typically, covalent modifications reduce potency but this is compensated for by a concomitant extension in half-life. The new covalently modified peptide would become a new chemical entity (NCE) and as such may have different pharmacokinetic, pharmacodynamics and immunogenic properties. [230] If the covalent modification is reversible then the NCE would take the form of a pro-drug, releasing the peptide as the active pharmaceutical ingredient (API). In this instance, the NCE would not need to interact with the target. However, if the covalent modification is irreversible, then the NCE must interact with the target.

The oral bioavailability of peptides remains an obstacle to their development as drugs outside a clinical setting. Peptides are rarely absorbed across the intestinal mucosa and various chemical modification strategies have been employed to try to address this, including the conjugation to passive and active transport enhances as discussed previously. A successful insecticide orally active to insects on crops has been developed from spider venom [13] engineering stability in the hydrogen bond structure of analgesic α-conotoxin Vc1.1 delivers oral bioavailability and target engagement. [231] However, peptides do not necessarily need to be administered orally and can be administered subcutaneously thereby avoiding potentially degradative intestinal mucosal enzymes and first passage through the liver.

Determination of the important pharmacological parameters for peptides including pharmacodynamic and pharmacokinetic properties (adsorption, distribution, metabolism and excretion, ADME) is challenging as they usually exhibit different patterns to those seen for more traditional small molecule pharmaceuticals. Due to

profound differences in structure and conformation, toxin-derived peptide drugs vary in their physicochemical properties and subsequently in their pharmacokinetics. This provides a wide substrate for chimeric peptides to maintain target engagement and improve PK.

The structural differences between peptides and peptide-derived drugs have a marked effect on their physicochemical properties. In particular the molecular weight of the proposed peptide drug is important as it directly affects its suitability for oral administration. Depending upon the site of action of the drug this may either be an advantage or disadvantage. For example the relatively high molecular weight peptide drugs linaclotide and plecanatide (Figure 3) act within the GI lumen and this together with their low water solubility ensures limited systemic absorption after oral administration, contributing to their selectivity and safety. [232] Molecular weight is not an issue if the drug is administered intravenously and locally, e.g. eptifibatide, and indeed could be advantageous in limiting the distribution of the drug to other locations in the body. Some examples of the pharmacokinetic properties of toxin-derived peptide drugs that are clinically approved for use are discussed in the following paragraphs.

As described previously, captopril is a small molecular weight drug that mimics a bradykinin-potentiating peptide from *Bothrops jararaca*. Its chemical structure and physicochemical properties ensure that is soluble in the aqueous environment, however absorbance is reduced across the GI tract (and other biological membranes). This means that around 60-70% of an oral dose of captopril is absorbed with peak blood concentration reached 45-60 minutes after oral administration although this reduces to 30-40% if co-administered with food [233]. Once in body fluids and plasma,

captopril's sulfhydryl group binds to albumin and other thiol-containing molecules (such as cysteine and glutathione). A disulfide bond can also form between two adjacent captopril molecules to form a dimer. Together, these compounds act as a reservoir of the pharmacologically active drug and prolong its pharmacological effects. Pharmacokinetic data for captopril and other drugs developed from venom toxins are shown in Table 1. Approximately 30% of captopril binds to plasma proteins in central circulation with a volume of distribution of 0.8 L/kg (corresponding to a 56 L in a 70 kg patient). Captopril is cleared at a rate of 49 L/h primarily through the kidneys where it undergoes active secretion in the tubuli of the nephron. This results in an elimination half-life of approximately 2 hours. [233]

Eptifibatide, derived from a disintegrin peptide from the pygmy rattlesnake (see previously) is administered as an intravenous bolus followed by infusion with doses adjusted to patient's weight (Table 1). The volume of distribution in patients with coronary disease is 185-260 mL/kg, being somewhat higher than that found in healthy individuals (220-270 mL/kg). Unlike captopril, eptifibatide remains largely unbound within the plasma and body fluids and has an elimination half-life of 2.5 h. Renal clearance accounts for 50 % of the total clearance with eptifibatide where the majority of the drug excreted is in the form of the original drug or its metabolites. [234]

Ziconotide derived from the ω-conotoxin MVIIA peptide (Figure 3) is administered intrathecally within an isotonic solution via a micro infusion device. [235] This special route of administration is not dependent upon patient related factors such as weight, gender or renal function. The volume of distribution within the cerebrospinal fluid is 155 mL/kg, similar to the estimate total cerebrospinal fluid volume [236] with clearance

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within the body via cleavage by protease enzymes. Any ziconotide that leaves the brain and spinal fluid exerts low systemic exposure due to extensive dilution in body fluids. Ziconotide has a terminal half-life of 4.6 h. [232]

6. Target Engagement

The majority of characterised venom peptides and proteins target interactions with extra cellular or cell surface proteins and lipids, however there are also cell penetrant peptides interacting with intracellular targets such as Ryanodine receptors. which will be discussed here. [237] Natural selection is a strong force for shaping pharmacologically active proteins and selecting those that optimally engage with the target proteins, as those that do not reach or engage with the target are of no benefit to the host.

6.1 GPCRs

G-protein coupled receptors are a well characterised family of cell surface receptors and represent one of the key target classes for drug discovery and modern pharmaceuticals. Published knowledge of venom components acting at these receptors has, so far, predominantly focused on glucagon like peptide receptor and muscarinic GPCRs. However, there are promising signs for other GPCR targets including adrenoceptors, vasopressin and somatostatin. Binding to lysophospatidic acid receptor (LPAR6) has also been hypothesised through molecular dynamics studies. [238] Conference posters have also been presented at The European Laboratory Research and Innovation Group's Drug Discovery meeting in 2017 which also demonstrated hits to orphan receptors GP120, GPR39 and vasoactive intestinal polypeptide receptor 1 (VIPR1). Although many interactions of venoms with drug

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discovery targets go beyond what we know about their evolutionary purpose, they are very useful for drug discovery.

Exendin-4 has been extensively mentioned due to the success of Byetta® and this is in a large part due to the peptide binding the glucagon like peptide receptor with a Ki 0.46 nM. Another subnanomolar GPCR ligand is AdTx1 from the green mamba (*Dendroaspis angusticeps*) which generates insurmountable inhibition of α1a adrenoceptor at sub-nanomolar concentrations K_i 0.35 nM. The slow on-rate of $6x10^6$ $M^{-1}min^{-1}$ is balanced by the long half-life of the receptor–toxin complex $t_{1/2}$ of 3.6 h. [239] The highly selective inhibition of α1a adrenoceptor occurs at an orthosteric binding site. [240]

Muscarinic acetylcholine receptors are important GPCR drug targets expressed in neuronal cells (M1 and M4) and smooth muscle (M2 and M3). Several toxins from African and Asian Elapid snakes have been discovered with low nanomolar Ki, good selectivity and slow dissociation. [175] Muscarinic toxins are another example of why a universally adopted toxin nomenclature is required [102] for snake venoms as for other taxa, with authors using m1 toxin and MT1 to identify separate muscarinic toxins from *Dendroaspis angusticeps.* [241] This has led to the UniProtKB entry (Q8QGR0) for muscarinic toxin 7 to list muscarinic toxin 1 as an alternative name. As previously discussed, venom genes are subject to multiple gene duplication events throughout their evolution and this gives rise to multiple, closely related toxins, which is the perfect substrate for structure activity relationship studies like those performed with small molecules. This is due to individual amino acid mutations which can lead to changes in selectivity, potency and/or binding kinetics, thus producing good substrates for SAR analysis. Reactive groups can be elaborated at the amino acid level meaning

synthesis of modifications is very straightforward. Site-directed mutagenesis or substitutions in solid state synthesis can be used to test hypotheses from such SAR assessments. With the muscarinic toxins these studies have identified that key residues at the tip of the middle finger of these three-finger toxins reach down into the orthosteric binding cleft of the receptor, [175] whereas those amino acids distal to the tip are responsible for the irreversibility of the binding. [241]

Venom from the iconic mamba snakes of Africa also contain GPCR-specific tool molecules such as mambaquaretin-1 which inhibits vasopressin 2 receptor with a K_i of 2.81 nM, but which is inactive on other GPCRs and cardiac ion channels at 1 uM. [176]

It is not just non-avian reptile venoms that have useful interaction with drug-relevant GPCRs; the cone snail cysteine knot peptides also possess GPCR activity and a good example of this is Conotoxin τ-CnVA which selectively blocks the Somatostatin receptor SST3 with K_i of 1.5 uM. [242] Another α-conotoxin is a competitive antagonist at LPAR6, blocking binding of oleoyl-L-α-lysophosphatidic acid. [238]

6.2 Ion Channels

Ion channels represent promising yet challenging drug targets, these challenges are often down to highly conserved regions within each family and thus limited substrate for discovery or design of potent and selective small molecule drugs. This is where the larger interaction surface of venom peptides provides key selectivity opportunities whilst maintaining drug like potency. Venom peptides bind to ion channel targets in three main locations; pore blocking [243], voltage sensor [244] and ligand binding site. [209] α-Toxins block inactivation and β-toxins block activation. However CRiSP toxins

with their large size are still able to act as pore blockers but interact with around 14 amino acids present in the major extra cellular loops of cyclic nucleotide gated ion channels. [158]

6.3 Ionotropic receptors

The group of α-neurotoxins that inhibit post synaptic nicotinic acetylcholine receptors on smooth muscle were some of the first venom actions to be discovered. Initially discovered from elapid snakes where they represent a dominant toxin class, these neurotoxins are poorly reversible, and sometimes irreversible, thus causing life threatening skeletal muscle paralysis which is a hall mark of envenomation from these species. [209] α-Bungarotoxin binding is now part of a standard assay when studying nicotinic acetylcholine receptors as it is a potent, high affinity (K_d 0.35 nM) and irreversible ligand at α7-nicotinic acetylcholine receptors. [245] Highly charged amino acids lysine and arginine in loop II of these three finger toxins are critical for target engagement. [209] There are also a wealth of nicotinic acetylcholine receptor tools from the cone snails which independently potently bind to different receptors producing a range of useful pharmacological tools. [246] Elapid three-finger neurotoxins are also able to potently inhibit GABA A binding with a K_i of 0.8 nM. [247]

7. Therapeutic areas and discovery phase leads from venoms

Discovery of new components in venom that have an effect on a range of disease targets continues and there are molecules from a various species currently being tested *in vivo*. There is also promising emerging data in recent literature

demonstrating the effect of venoms on a wider range of disease targets at the very early stages of drug discovery.

7.1 Cancer

There have been a number of developments in cancer research using whole and purified components of venom. A number of studies have been undertaken utilising scorpion venom. Iranian scorpion *Hemiscorpius lepturus* whole venom has been shown to inhibit tumour growth displaying an anti-proliferative effect via the Trp53/Bcl2/Casp3 pathway in the colon cancer cell line CT26, while showing low toxicity on a non-cancerous cell line. [248] Whole venoms from the scorpions *Androctonus crassicauda*, *Messobuthus eupeus and Hemiscorpius lepturus* have been used in a CT26 tumour mouse model to show that a strong *in vivo* anticancer immune response. [249] A protein component of the venom from the Indian black scorpion *Heterometrus bengalensis* (named Bengalin by authors) has been shown to have involvement in the MAPK pathway in human leukemic U937 cells. [250].

Contortrostatin, a snake venom disintegrin from the southern copperhead viper *Agkistrodon contortrix contortrix*, has been shown to reduce metastasis in breast carcinomas xenograft models in nude mice and also displayed antiangiogenic activity. [251] This contortrostatin has had the three amino acids from its C-terminal end replaced with part of another disintegrin, echistatin, to produce Vicrostatin and a delivery method for this drug has been established [252]. Vicrostatin can be recombinantly produced in an *Escherichia coli* expression system. *In vitro* and *in vivo* studies are ongoing in breast, prostate, ovarian and brain tumours. [253] Disintegrins in snake venom block integrin signalling on platelets and during metastasis leading to their investigation in cancer therapeutic discovery. [254]

Venoms and toxins have been shown to have effects on many of the hallmarks of cancer. [255] In breast cancer cell lines, it has been shown that whole venoms from a range of species can affect the phosphorylation of receptor tyrosine kinases which are targets of cancer treatments. [189] These targets include the epidermal growth factor Receptor (EGFR) which is overexpressed or mutated in a number of cancers. Theraphosidae and Elapidae venoms have also been shown to reduce phosphorylation of the Ephrin A6 receptor which is another anti-cancer target. Anti-oncogenic activity has been shown in toad secretions (not venoms) from the following species *Rhinella schneideri* and *Rhinella marina.* [256]

Samsum ant (*Pachycondyla sennaarensis*) venom has shown anticancer activities in cancer cell lines through a reduction in insulin growth factor 1 (IGF-1) mediated cell proliferation and reduction in phosphorylation of the kinases AKT and extracellular-signal-regulated kinase (ERK) which are also pathways involved in cancer [257]. This effect has also been shown in *in vivo* models in rats. [258] Cardiotoxin 3 (CTX-III) from the Taiwan or Chinese cobra *Naja atra* induces apoptosis in the lung cancer cell line A549 by inactivating EGFR, PI3K and JAK/STAT3 kinase pathways. [259]

The giant jellyfish *Nemopilema nomurai* venom has been shown to have anticancer properties in the human hepatocellular carcinoma cell line HepG2 and in the human breast adenocarcinoma cell line MDA-MB-231 by targeting the AKT and mTOR kinase signalling pathways. [260,261]

The peptide melittin mentioned earlier in this chapter is the main component found in the European honeybee *Apis mellifera* venom and this peptide exhibits anticancer affects in renal, lung, liver, prostate, bladder and leukemia. [182] As there are issues

with the cytotoxicity, degradation and haemolytic activity of melittin much work has been done to develop more targeted therapies with this peptide to produce anticancer agents. This has been undertaken utilising conjugation with hormone receptors, gene therapy or nanoparticle based delivery of melittin in tumours. [262]

In the venom of the lesser weaver fish (*Echiichthys vipera)* novel cytotoxic components have been discovered that have an effect on human colon cancer cells, but not yet identified due to limited information about this venom. [64,263]

7.1.1 Imaging tumours in surgery

The peptide chlorotoxin from the venom of the Israeli deathstalker scorpion (*Leiurus quinquestriatus*) has been widely studied due to its ability to bind selectively to tumour cells. It is being used as an imaging agent to label gliomas in surgery so they can be safely removed from the brain without removing as much healthy tissue. [264] This new drug diagnostic tool, currently in Phase II clinical trials is called Tozuleristide. [80]

7.2 Antimicrobial effects

Antibiotic resistance is a worldwide health problem [265] and to add to that the pharmaceutical development pipelines are lacking new antibiotics. A range of venoms from a number of different species have been shown to have an antimicrobial effect. [256,266] Venom peptides, lycotoxin (from the wolf spider) and cathelicidin (from the king cobra and banded krait) cause strong reversible inhibition of ATP synthase in *Escherichia coli* [267]. Cathelicidin, which is a synthetic peptide derived from snake venom also has also been shown to have wound-repair activity [266]. Ant venom has been shown to have antimicrobial, antiparasitic, anti-inflammatory and antioxidant activity [268]. As well as anti-cancer effects melittin

has been shown to have antiviral effects on a range of viruses including a number of studies with human immunodeficiency virus (HIV). [183] It has also shown antifungal and antiparasitic activity [262]. A modified cationic peptide from the Chinese swimming scorpion (*Lychas mucronatus*) called Mucroporin-M1 has shown effective inhibition of viruses such as measles (EC_{50} 7.15 µg/ml) and SARS CoV (EC_{50} 14.5 µg/ml). [269] There are also preliminary studies suggesting that venom peptides could block binding of SARS CoV2 to the human ACE2 receptor, although nothing has been published to date with peer review.

Melittin also has anti-bacterial effects. [270] A melittin related antibacterial peptide, RV-23 from the frog skin of *Rana draytonii*, which has lower cytotoxic effects than bee melittin, and another related peptide AR-23 which is from the skin of the frog *Rana tagoi* have also been studied. RV-23 was shown to have a higher lytic activity. [271]

7.3 Antiparastic effects

Snake venom has been shown to have an antiparasitic effect. The venom from the cobra *Naja sumatrana* showed antiprotozoan activity towards the ciliate *Tetrahymena pyriformis* and the effect of these cytotoxic peptides from the three-finger toxin family caused immobilisation and death in these organisms. [272]

Antileishmanial activity of venom from the common krait *Bungarus caeruleus* snake venom has been displayed in a mouse model [273]. Spider venoms have been also shown in mice to have an anti-parasitic effect when these mice were infected with tachyzoites from *Toxoplasma gondii*. Using spider venom from *Ornitoctonus huwena* showed a better survival rate in the mice than the control when treated. This has

potential in the treatment of toxoplasmosis and new drugs are urgently needed to the serve side effects of current drugs. [274]

7.4 Analgesics

Venoms can have an effect on a range of ion channel targets and act as an analgesic [28]. Spider venom-derived cysteine knot peptides have therapeutic value selectively targeting voltage gated sodium channels which could be used in epilepsy and chronic pain treatments. [275] This is a very active area of venom drug discovery.

7.5 Diabetes

The FDA approved drug Byetta (exanatide) was mentioned in the earlier section in the treatment of diabetes but since the development of this drug many more promising peptides have been discovered that act as pharmacological tools and therapeutics for this disease. These include platypus glucagon-like peptide-1 (pGLP-1) from the male platypus (*Ornithorhynchus anatinus*) which acts as an GLP-1 receptor agonist, a fast acting insulin analogue from the geography cone snail (*Conus geographus*), a blocker of voltage gated potassium channel $K_V2.1$, hanatoxin, from the Chilean rose tarantula (*Grammostola rosea*) and guangxitoxin-1 from the Chinese earth tiger tarantula (*Chilobrachys guangxiensis*). There is also a peptide called conkunitzin-S1 which is a blocker of voltage gated potassium channel $K_V1.7$ which has been isolated from the striated cone snail (*Conus striaus*), and, from the Eastern Indian red scorpion (*Buthus tamulus*), the peptide Iberiotoxin which is a large-conductance calcium-activated potassium (BK) channel blocker. [276]

An Echidna (*Tachyglossus aculeatus*) peptide Echidna GLP-1 (eGLP-1) is also a GLP-1 receptor agonist but this is outside the scope of this article as echidna are not defined as venomous [46].

7.6 Anti-coagulants and procoagulants

As shown by reptilase™ from *Bothrops atrox,* discussed earlier, snake venoms have procoagulant properties but some have anticoagulant properties as well. In a study of 28 different snake venoms from a range of families [277] the following snakes showed procoagulant activity: Russell's viper (*Daboia russellii siamensis*); fer-de-lance pit viper (*Bothrops asper)*; Brazilian lancehead pit viper (*Bothrops moojeni)*; and the Southern Pacific Rattlesnake *Crotalus oreganus helleri*. The Western diamond-backed rattlesnake (*Crotalus atrox*) and the red spitting cobra (*Naja Pallida*) demonstrated anticoagulant activity. Both activities can have useful medical applications.

7.7 Insecticides

Janus-faced atracotoxins (J-ACTXs) neurotoxins that could be developed into new insecticides, are found in the venom of the Australian funnel-web spider [35] targeting voltage-gated sodium channels. Sea anemones have also been shown to be a source of insecticidal peptides. [72]

7.8 Venoms that modulate ion channels

A range of venomd fromspecies including snakes, arachnids [278] and fish hunting molluscs have been shown to modulate voltage-gated calcium channels by peptide toxins [243]. These components could be used as tools to investigate new pathways

or as potential new drugs. Venom from *Ornithorhynchus anatinus* (the duck billed platypus) potently induces calcium ion influx in human neuroblastoma cells. [173] A number of sea anemone venoms also have an effect on voltage gated sodium channels targeting receptor site-3. [279] Centipede venoms are also a source of drug leads as they have an effect on neuronal ion channels and receptors. [40]

7.9 Autoimmune disease

A key therapeutic target for a range of autoimmune diseases is the voltage-gated potassium channel $K_V1.3$. [280] The peptide toxin ShK from the sea anemone (*Stichodactyla helianthus*) blocks this channel potently. It also blocks $K_V1.1$, $K_V1.4$ and $K_V1.6$ weakly [281] but has been synthesised and modified by just one amino acid (K18A mutation) to increase its selectivity for $K_V1.3$ and $K_V1.1$ which makes it a better lead for autoimmune disease targeting [282].

Dalazatide targets potassium channels and is a treatment for psoriasis and other autoimmune diseases. It was recently trialled in humans, but further larger studies are needed. [283] It is a 37-amino acid synthetic peptide which was originally derived from ShK and is a specific inhibitor of potassium channel $K_V1.3$ [284] and blocks the activation of the effector memory T-cells. Other peptides with $K_V1.2$ activity with similar sequences to Shk and BgK from *Bunodosoma granulifera* have been discovered from *Oulactis sp* sea anemones[285], this produces good substrate for SAR.

8. Strategies for Discovering Novel Actives within Venoms

The initial investigations into venoms dealt with the whole mixture as a starting point to discover why they were dangerous to humans and what could be done about it.

Cobras were thought to produce alkaloids and so called cobric-acid in their venoms as proteins were not thought then to be so toxic. The conclusion that toxic proteins were responsible and not alkaloids was discussed in the Indian Medical Gazette of 1887. [286] This is likely to be the first mention of proteins being potent and therefore toxic. At the turn of the 20th century the only published research on venoms was concerned with those of medical significance and how to limit their effects on human health. It was only in 1935 when the use of venom in drug discovery was first demonstrated in the scientific literature. [287] This was the use of whole Russell's viper (*Daboia russelii*) venom as procoagulant for haemophiliac patients during tooth extraction. [287] Whole venom is still used in clinical practice, for example the dilute whole Russell's viper venom clotting time (RVVT) is still the mandated test for lupus [288] and the venom was patented in 1992 as a procoagulant in vacutainers for blood collection. [289] So even though whole venoms are complex mixtures with many synergistic and even potentially antagonistic properties they are still used by many in early drug discovery research both *in vitro* [170] and *in vivo* [258]. This can yield good results if it is followed up through subsequent purification and identification of the active compounds [176]. Use of whole venoms in the initial screening is also useful in situations where there is no prior eveidence for venom activity such as P2X receptors [290] and receptor tyrosine kinases. [189] However there are many drawbacks with the use of whole venom due to the complexity of the mixtures, such that there is a high probability of confounding actions. The use of binary biochemical assays where the experimental system is a single binding event, reduces the problems associated with assessing complex mixtures, however these assays are also amenable to High Throughput Screening (HTS) and therefore could start with the purified components. The rise of automated biochemical and cell based screening, in particular automated

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ion channel screening, has revolutionised drug discovery biology. These advances came into wider use at the turn of the 21st centurary to keep up with the explosion of synthetic chemistry and ever larger compound libraries. Cell based assays are particularly sensitive to cytotoxins masking possible active molecules and thus screening of separated venom components is preferable.

As with all screening campaigns and thus compound libraries, the most efficient start point is a targeted library. Preparation of venom libraries begins with collection. The method of collection depends on the organism and ranges from a snake biting onto a paraflm topped container through to electrical stimulation of venom glands. The venom is then quantified and fractionated using HPLC)purification to separate it into its component parts. Figure 2 shows an example of an ion exchange chromatogram from spitting cobra *Naja nigricincta* venom which has a few dominant protein families. This is compared with a second reverse phase chromatogram from the theraphosid spider *Monocentropus lambertoni* which has many peaks of similar intensity. The complexity of the venoms often requires two orthogonal separation methods such as reverse phase and size exclusion or ion exchange to get the purest fraction library. This is a very practical approach as the most useful drug-like molecules in the venoms are very stable when treated this way. Once lyophilised into Society for Biomolecular Screening (SBS) standard assay plates of a chosen format they can be handled in a similar way to other compound libraries. Figure 6 shows an example workflow. Despite the trend of assay miniaturisation leading to multi-thousand well plates, the dominant work-horse sceening plates are still 384-well and, to a lesser extent, 1536-wells; this was nicely predicted by Mayr and Fuerst in 2008. [291] Thus it is not surprising that the 384-well and 384-well Low Dead Volume (LDV) assay format is currently dominant in venom peptide screening, although several labs are still using 96-well formats. Assay

format and thus well size, is key to the ability to utilise the venoms. Large snakes produce many milligrams of protein with each venom yield, whereas the yield is many orders of magnitude less when considering small scorpions, wasps or cone snails. Even more extreme are the negected venoms of many invertebrates such as pseudoscorpions, flies and gnathiid isopods, due to their small size of a few millimeters body length. [292] The first recoverable venom collection from a pseudoscorpion (*Synsphyronus apimelus*) produced 5 nl of venom on average. [38] Thus miniaturised wells allow for the most use from the smaller species. There are a few suppliers around the world who produce assay ready libaries of venom fractions which make them easily accessible to drug discovery.

While the native venom, collected recoverably from the source animals, represents the true power of the evolved molecules, there are many technical challenges to producing compound libraries this way, as discussed. The rise in gene sequencing throughput has allowed other methods of natural product library production, such as recombinant expression in model organisms or solid state peptide synthesis from known sequences [165,293]. Typically venom glands are terminally dissected from euthanised specimens [165,292] so that the RNA can be extracted for reverse transcription and subsequent recombinant expression. However one group do report surgically removing one of the pair of venom glands from a Heloderma lizard such that the animal could recover and still have a working venom gland, while the scientists could access the RNA[165]. Transcriptomics is a very powerful tool to characterise venoms through recombinant production or synthesis from gene sequence data, but it usually comes at the cost of the animal. However a seminal paper in 2012 identified the unusual stability of messegner RNA in whole venom [294] thus negating the need for sacrificing the animal or invasive surgery. But, to date, this ethical and practical

approach to obtaining venom transcripts repesents a minority, with only one paper on comparison of PLA2, readily found. [295] Recombinant and synthetic libraries have their benefits in hit follow up as the compounds have already been identified and are ready for scale-up. Also the toxins can be expressed with novel tethers to enable use of particular screening technologies such as for difficult ion channels like TRPA1. [296] However a major drawback is that the native post translational modification of the venom proteins, often important in their function and stability, are not replicated and thus the starting point may not be as good. [8,205,297]

However the venom component library is prepared, for the most part it can be screened with a similar methodology to standard compound libraries, thus enabling screening of the full range of targets used with other libraries. This process has been used to identify novel leads from venoms on difficult targets such as ion channels, GPCRs, kinases and protein-protein interactions. Automated patch clamp methods have proven useful for screening venom fractions to discover novel, selective, inhibitors of the pain relavent sodium channel $Na_V1.7$. [298] Another method used for high throughput screening of $Na_V1.7$ has been using a chimeric protein containing the $Na_V1.7$ voltage sensor domain fused to the more amenable $K_V2.2$ channel such that fluorescence can be measured. [244] One major benefit of the venom libraries is that they are soluble in assay buffers, negating the need for DMSO which is particularly problematic in cell based assays. Also, DMSO at standard assay concentrations of between 1% and 0.1% can alter the stability and other physical properties of the venoms [299], thus leading to potential complexities later on.

Once hits have been identified from the screen and confirmed in the usual way, hit follow up methods differ depending on the type of venom library used. Those from

native venoms can be identified by intact mass and digested peptide high resolution MS/MS techniques when the starting material hasn't been previously identified. These libraries are very efficient as only interesting hits need to be identified and produced, In contrast, those from synthetic or recombinant libraries are already known, due to the extra steps in the production. Either way the resulting information can easily be used to take into lead development to tune the hit into more drug like molecules such as by reducing the size, or changing selectivity or PK parameters by comparing with other venom compounds and developing structure activity relationships (SAR). Such approaches include screening venoms of closely related species where the combination of gene duplicaton and natural selection has produced a library of similar compounds with minor mutations providing good substrates for SAR work.

9. Utilising SAR with venom peptides

The classification of venom peptide toxins into groups permits study and understanding of the structure-function relationship of each individual group. Classification could be based on a number of different determinants: receptor or ion channel specificity or cellular target, peptide length, structural scaffold, number of disulfide bonds, mechanism of action *etc*. By using amino acid sequence similarities, positioning of key amino acid residues (i.e. cysteines in disulfide bonds) and comparing key structural folds, peptide venoms can be classified into each group and the knowledge gained used to engineer peptides with desirable pharmacology. In this regard, the approach is to use positional substitution of amino acids within the sequence (positional scanning) by producing the peptide analogue and measuring the resulting activity/binding specificity against the target. For example, to determine the contribution of a particular residue within a peptide, alanine scanning can be

performed whereby peptide analogues of the original peptide are produced in which alanine systematically replaces sequential amino acids within the peptide. Due to alanine's non-bulky methyl side chain, any structure within the peptide is likely to be preserved while removing any potential chemical activity (and hence potential functionality) of the original amino acid. Further positional substitutions with other natural and non-natural amino acids can also be used to determine the impact of changing charge, hydrophobicity, bulkiness *etc* at key positions within the peptide sequence as part of a rational design process.

As an example, extensive structure activity relationship studies have been performed on inhibitory cysteine knot (ICK) peptides from spider venoms that target voltage gated sodium channels (Na_V) and on binding act allosterically to modulate the channels [275]. These peptides have been classified into groups based on the sequence similarity and the position of the key cysteine amino acids that, when participating in the disulfide bond, make up the characteristic cysteine knot. The conserved ICK is composed of three disulfide bonds between cysteine residues C1-C4, C2-C5 and C3-C6 resulting in a peptide with a globular structure with four loops and a C-terminal tail. There are twelve distinct families of Na_V modulators (NaSpTx 1-12) and extensive SAR studies have resulted in a detailed study of the potency, selectivity and pharmacology of peptides from families NaSpTx 1, 3 and 7. [275]

SAR investigations using alanine scanning and substitutions of other amino acids have been carried out on several members of the NaSpTx 1 family of proteins. GpTx-1 from the theraphosid *Grammostola porteri* is a nanomolar inhibitor of $Na_V1.7$ (IC_{50} 10 nM) and alanine scanning revealed that amino acid residues Trp29, Lys31 and Phe34 were essential to this inhibition. As part of a rational design to create an optimized GpTx-1

peptide, additional positional substitutions Phe5Ala, Met6Phe, Thr26Leu and Lys28Arg produced a peptide that possessed a 6-fold enhanced potency for $Na_V1.7$ and a 1000-fold selectivity over $Na_V1.4$ and $Na_V1.5$. [300] Later scans with amino acids other than alanine revealed further residues critical to activity while structural models of GpTx-1 bound to the $Na_V1.7$ protein predicted interactions between critical residues in the peptide and those within the voltage sensor region of $Na_V1.7$. A summary of the structure activity relationships undertaken for this peptide is shown in Table 2.

By aligning the amino acid sequences of members of the NaSpTx family, the positions of the cysteine residues and regions of conservation can be identified [301]. Pro12, Trp31 and Lys33 are not buried and appear to be critical for activity as is the serine residue in position 26. [244,300] Zhang *et al* used this sequence conservation to engineer analogues of HNTX-1, a member of the NaSpTx 1 family, to engineer a gain-of-function analogue that was a potent blocker of the human $Na_V1.7$ channel. [301] HNTX-1 itself displays weak affinity for insect sodium channels and has no effect on mammalian channels but was used as a framework to engineer analogues which were subsequently tested for activity. Using the conserved sequence within the NaSpTx 1 family, amino acids were substituted at various positions within the peptide leading to the eventual analogue (HNTX-1:E1G-N23S-D26H-L32W) with potent human $Na_V1.7$ blocking activity and an I_{C50} of 0.036 +/- 0.007 µM. As part of the development of this analogue, a consensus sequence motif (X1X2SWCKX3) was identified and shown to be critical for suppression of the human $Na_V1.7$ channel.

The HNTX-1 three dimensional protein structure (PDB code 1NIX) revealed that the basic amino acid residues Arg25, Lys27 and Lys30 came together to form a positively

charged surface that would interact with Na_Vs by electrostatic interactions. Nearby residues Phe5, Tyr20, Trp28, Val31, Leu31 and Leu32 clustered to form a hydrophobic patch. Both these surfaces are highly conserved within the NaSpTx 1 family. Upon modelling the potent HNTX-1:E1G-N23S-D26H-L32W peptide onto the HNTX-1 template it was found that these amino acid substitutions strengthened the interactions: Asp26His increased the positive electrostatic interaction with the Na_V; Glu1Gly reduced the negative charge, while Leu32Trp strengthened the hydrophobic interaction and the Asn23Ser substitution was required to better orient the critical Trp29 and Lys31 residues correctly [301].

A similar approach has been used on other members of the NaSpTx 3 and NaSpTx 7 families, revealing key motifs and residues important for activity. ProTx-II, a NaSpTx-3 family member is a potent $Na_V1.7$ inhibitor (IC_{50} 0.3 nM) yet also exhibits potent inhibition of off-target $Na_V1.4$ and $Na_V1.5$. Alanine and glutamine positional scanning of recombinantly expressed ProTx-II analogues in SAR studies against Na_V channels revealed an active face composed of hydrophobic and cationic residues [302], with residues Trp5, Lys26, Lys27, Arg13 and Arg22 critical for potency (10 – 125-fold). Additional residues Met6, Trp7, Met19, Val20, Trp24, Leu29 and Trp30 also result in a 10-fold loss of potency while an additional study identified some of these residues critical for $Na_V1.5$ activity.

JZTX-III is a NaSpTx 7 ICK isolated from the Chinese theraphosid *Chilobrachys jingzhao* and is a potent inhibitor of $K_V2.1$ channels and TTX-R currents in rat cardiac muscle. SAR investigations against $Na_V1.5$ using alanine substitutions revealed residues Asp1, Glu3, Trp8, Trp9, Trp28 and Trp30 as critical for activity while

substituting arginine 13 for a glutamate residue enhanced $Na_V1.5$ inhibition 11-fold. JZTX-III does not inhibit the $Na_V1.7$ channel, which is attributed to an aspartate residue at position 816 in the $Na_V1.7$ channel. Substituting this aspartate for an arginine (D816R) within $Na_V1.7$ resulted in enhanced inhibitory properties of JZTX-III against $Na_V1.7$ [275].

Together with other similar studies on these peptides, optimised analogues and Na_V proteins, a consensus of prevalent features and key residues have been identified that are associated with activity. In the NaSpTx 1 family, amino acid residue positions 5 and 6 are typically occupied by the hydrophobic residue phenylalanine; similarly, in NaSpTx-3 these positions are occupied by tryptophan and methionine and are critical for activity. Increased potency can be achieved by reducing the negative charge at the N-terminus while modifications at the C-terminus by increasing positive charge or increased hydrophobicity enhance inhibition of Na_V channels.

Due to gene duplication events and subsequent natural amino acid mutations SAR tables can be assembled and analysed without the need for cloning or synthesis of a large library of systematic alanine substitutions. By utilising the natural mutations all the changes have a function as they have been subject to natural selection and thus the data is easily obtainable. Potassium channel toxins blocking Kv1.3 from bark scorpion (*Centruroides* spp.) venoms are just such a group containing intra and inter-specific mutations. Disulfide bonds have been conserved between Cys7-Cys29, Cys13-Cys34 and Cys17-Cys36 and thus the sizes of the loops between the cysteines are also maintained (as shown in Table 3) however other Kv1.3 toxins from other genera of scorpions do have different loop lengths. Linear regression analysis of the

amino acid sequences along with potencies at Kv1.3 allows for the identification of key residues driving potency. This can also be performed with selectivity or pharmacokinetic data to optimised lead peptides.

The application of SAR to optimise Na_V channel modulators and help develop treatments for channelopathies and chronic pain has been demonstrated in rodent models, where the ProTX-II peptide and its optimized analogue (JNJ63955918) successfully reversed neuropathic and inflammatory pain when administered intrathecally or locally. [303,304] Similarly, intraperitoneal administration of the NaSpTx 1 peptides HwTx-IV and HNTX-IV reversed neuropathic and inflammatory pain. [296]

Future Perspective

To unleash the full potential of drugs and tools from venoms, further crossover is required between evolutionary biology, biochemistry, and drug discovery chemistry. Through the building of this interdisciplinary approach, further novel tools are likely to be found to meet the demands of drug discovery in a world dominated by tough targets, new therapeutic modalities, and emerging diseases. The intrinsic nature of venoms being stable, repurposed versions of many protein families, will continue to aid protein therapeutic development. Biotechnological entrepreneurs have already taken the stable spider venom ICK motif and recombinantly added it to the binding domain of humanised antibodies, thus delivering the binding potency and selectivity of the ICK peptide with the pharmacokinetic properties of antibodies. However, these still lack the desired oral bioavailability required for the patients' preferred dose route. Many venom peptides demonstrate exceptional stability in gut relevant pH ranges,

protease stability and an ability to cross membranes. In addition as small cyclic peptides such as cyclosporin, have already shown oral bioavailability, the potential for oral bioavailability of more complex venom derived peptides is within in our grasp. Coupled with improvements in understanding of biotherapeutic delivery mechanisms, the ideal pharmacological properties of venom peptides could soon be making their way into the oral dose route. A greater use and understanding of venom peptides as therapeutics will open up the major classe of difficult targets for small molecules – the protein-protein interactions. It is often cited that these targets are tough to modulate with small molecules due to the large interaction surface and surface chemistry, whereas nearly all venom peptides exert their actions through such interactions and thus will prove a great resource for this underrepresented target class.

Coupled with this greater understanding of peptide chemistry is the continued discovery of new venom systems, and new components and actions within known venoms. Charles Darwin said that “endless forms most beautiful and most wonderful have been, and are being, evolved in the natural world” and thus it is clear that we have only just scratched the surface of the potential substrates for new biotherapeutics from venoms.

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Tables

Table 1: The major pharmacokinetic parameters of some of the clinically approved toxin-derived peptide drugs (in a typical adult 70-kg patient with normal renal function). Where Fu, fraction of drug unbound in plasma; t1/2, elimination half-life; Tmax, time taken to achieve maximum plasma concentration post administration; L, litres; h, hours. Adapted from Stepensky, 2018(233).

Drug	Absolute Bioavailability F	Volume of Distribtution V or Apparent V (V/F) L	f_u %	Clearence CL or Apparent CL (CL/F) L/h	$t_{1/2}$ h	T_{max} h
Captopril	60 - 75 % (PO)	56	65-70	49	2	0.75-1
Eptifibatide	10 - 89 % (variable, PO)	210-350	10	21 - 29	5-27	1.5-2
Ziconotide	50 % (intrathecal)	0.155	-	-	2.9 - 6.5	-
Lepirudin	100 % (IV)	12.2	-	9.8	-	1.3
Linaclotide	0 % (PO, local activity in GI - not absorbed systemically)	-	-	-	-	-
Plecanatide	1 % (PO, local activity in GI - not absorbed systemically)	-	-	-	-	-

Table 2: Structure-Activity Relationships analysis of GpTx-1 from *Grammastola porteri* selectively blocks human $Na_V1.7$, and has been improved through four mutations. Table produced from data published by Murray *et al*, 2015 [300] with amino acids shown by single letter codes..

Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	NaV1.7	NaV1.5	NaV1.4
wild type	D	C	L	G	F	M	R	K	C	I	P	D	N	D	K	C	C	R	P	N	L	V	C	S	R	T	H	K	W	C	K	Y	V	F	0.09 ± 0.01	>5	2.7 ± 1.2
F5A	D	C	L	G	A	M	R	K	C	I	P	D	N	D	K	C	C	R	P	N	L	V	C	S	R	T	H	K	W	C	K	Y	V	F	0.63 ± 0.12	27 ± 10	45 ± 3
M6A	D	C	L	G	F	A	R	K	C	I	P	D	N	D	K	C	C	R	P	N	L	V	C	S	R	T	H	K	W	C	K	Y	V	F	0.43 ± 0.05	>5	>4.6
T26A	D	C	L	G	F	M	R	K	C	I	P	D	N	D	K	C	C	R	P	N	L	V	C	S	R	A	H	K	W	C	K	Y	V	F	0.26 ± 0.05	>5	3.2 ± 0.1
K28A	D	C	L	G	F	M	R	K	C	I	P	D	N	D	K	C	C	R	P	N	L	V	C	S	R	T	H	A	W	C	K	Y	V	F	0.47 ± 0.05	>5	>4.9
optimised	D	C	L	G	A	F	R	K	C	I	P	D	N	D	K	C	C	R	P	N	L	V	C	S	R	L	H	R	W	C	K	Y	V	F	0.0016	>10	1.9

Table 3: Structure-Activity Relationships of Kv1.3 toxins form bark scorpions (*Centruroides spp*) with amino acids indicated by single letter codes. Linear regression analysis has identified five key residue positions driving potency with a correlation coefficient greater than 0.6 (black) and four less important substitutions with a correlation coefficient greater than 0.3 (grey). Linear regression performed on data presented by Olamendi-Portugal et al (2005) [305].

UniProt ID	Species	toxins	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	Kv1.3 (nM)
P40755	*Centruroides margaritatus*	KTx 2.2	T	I	I	N	V	K	C	T	S	P	K	Q	C	L	P	P	C	K	A	Q	F	G	Q	S	A	G	A	K	C	M	N	G	K	C	K	C	Y	P	H	0.23
P0C162	*Centruroides elegans*	KTx 2.9	T	I	I	N	V	K	C	T	S	P	K	Q	C	L	K	P	C	K	D	L	Y	G	P	H	A	G	A	K	C	M	N	G	K	C	K	C	Y	N	N	0.25
P0C161	*Centruroides elegans*	KTx 2.8	T	V	I	N	V	K	C	T	S	P	K	Q	C	L	K	P	C	K	D	I	Y	G	P	K	A	G	A	K	C	M	N	G	K	C	K	C	Y	N	N	0.71
P0C164	*Centruroides elegans*	KTx 2.11	T	I	I	N	V	K	C	T	S	P	K	Q	C	L	L	P	C	K	E	I	Y	G	T	R	A	G	A	K	C	M	N	G	K	C	K	C	Y	K	I	0.98
P08815	*Centruroides noxius*	KTx 2.1	T	I	I	N	V	K	C	T	S	P	K	Q	C	S	K	P	C	K	E	L	Y	G	S	S	A	G	A	K	C	M	N	G	K	C	K	C	Y	K	N	1
P85529	*Centruroides suffusus*	KTx 2.13	I	F	I	N	V	K	C	S	S	P	G	Q	C	L	K	P	C	K	A	A	F	G	I	S	A	G	G	K	C	I	N	G	K	C	K	C	Y	P	I	7.2
C0HJW6	*Centruroides tecomanus*	KTx 2.16	T	I	I	N	V	K	C	T	S	P	K	Q	C	L	K	P	C	K	D	L	Y	G	P	H	A	G	E	K	C	M	N	G	K	C	K	C	Y	P	I	26.2
P0C165	*Centruroides elegans*	KTx 2.12	T	I	I	N	V	K	C	T	S	P	K	Q	C	L	P	P	C	K	E	I	Y	G	R	H	A	G	A	K	C	M	N	G	K	C	H	C	S	K	I	69
P0C163	*Centruroides elegans*	KTx 2.10	I	F	I	N	V	K	C	S	L	P	G	Q	C	L	R	P	C	K	D	R	F	G	Q	H	A	G	G	K	C	I	N	G	K	C	K	C	Y	P	I	366
C0HJW5	*Centruroides tecomanus*	KTx 2.17	T	I	I	N	V	K	C	T	S	P	K	Q	C	L	L	P	C	K	Q	I	Y	G	P	H	A	G	A	K	C	M	N	G	K	C	H	C	S	K	I	1000
P0DL70	*Centruroides limpidus*	KTx 2.18	T	T	I	N	V	K	C	T	S	P	K	Q	C	L	P	P	C	K	E	I	Y	G	R	H	A	G	A	K	C	I	N	G	K	C	H	C	S	K	I	1000

Figure legends

Figure 1: Examples of the range of species that utilise venom for defence / feeding / competition. Examples shown are A) Blue ringed octopus *Hapalochlaena fasciata* B) Flat rock scorpion *Hadogenes trogloytes* C) *Pterois volitans* Lion Fish D) Indian ornamental theraphosid *Poecilotheria regalis* E) Monocled cobra *Naja kaouthia* F) European wasp *Vespula germanica* G) White spot jellyfish *Phyllorhiza punctata* H) Giant house spider *Eratigena atrica* I) Giant forest scorpion *Heterometrus mysorensis* J) Gila monster *Heloderma suspectum* K) Chinese red-headed centipede *Scolopendra subspinipes* L) Solenodon *Solenodon paradoxus.*

Figure 2: HPLC separation of the whole venom demonstrates the standard techniques and example chromatograms. A: Ion Exchange chromatography venom from the zebra spitting cobra *Naja nigricincta* performed on the ThermoFisher (Dionex) UltiMate3000, demonstrating dominant peaks of few protein families and many minor components. B: Reverse Phase chromatography of whole venom from Lamberton's Tarantula (Theraphosid), *Monocentropus lambertoni* demonstrating more even distribution of peaks.

Figure 3: Chemical and protein structures of toxin-derived compounds either used as clinically approved drugs or used as the basis of clinically approved drugs. Captopril was derived from a nonapeptide isolated from the venom of *Bothrops jararaca.* It is an angiotensin converting enzyme (ACE) inhibitor used to regulate blood pressure. Eptifibatide was derived from barbourin, a disintegrin protein isolated from *Sistrurus*

m. barbouri. Tirofiban is based on echistastin disintegrin isolated from *Echis carinatus* and a potent inhibitor of platelet aggregation. ω-conotoxin MVIIA isolated from *Conus magnus* is used as the basis for the synthetic peptide Ziconotide, a N-type calcium channel blocker. Recombinantly produced hirudin (lepirudin) is a potent thrombin inhibitor. Synthetically produced exanatide (exendin-4) is a glucagon-like peptide-1 receptor agonist and is used to treat diabetes mellitus type 2.

Figure 4: Basic Structures of disulfide bond containing and non-disulfide bond containing (linear) venom peptides. ICK inhibitor cysteine knot ProTx Tp1a from *Thrixopelma pruriens*; double knot peptide Hi1a from *Hadronyche infensa*; non-ICK disulfide bonded peptide chlorotoxin from *Leiurus quinquestriatus*; linear toxin poneratoxin from *Paraponera clavata*; linear toxin melittin from *Apis mellifera*.

Figure 5: Examples of small molecule components of venoms. Acylpolyamines joramine and spidamine from *Nephila clavata*; nigriventrine from *Phoneutria nigriventer* ; HF-7 from *Hololena curta*; solenopsin A from *Solenopsis saevissima*; dehydrosolenopsin B from *Solenopsis Invicta*.

Figure 6: Example workflow from venom extraction to assay. Venom is extracted and quantified in this case using a DS11 spectrophotometer (DyNovix). Venom is fractionated using high pressure liquid chromatography (HPLC) and this could be by a number of dimensions utilising reverse phase and size exclusion or ion exchange chromatography. The venom fractions are then standardised, plated into SBS

standard assay plates, lyophiilsed ready for screening against drug targets or cell lines.

Footnotes

none