Toxiconology provides multidirectional and multidimensional opportunities: A personal perspective

R. Manjunatha Kini

Protein Science Laboratory, Department of Biological Sciences, Faculty of Science and Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

ARTICLE INFO

Keywords:
Accelerated evolution
Gene duplication and neofunctionalization
Tissue-selective expression
Regulation of toxin expression
Targeted drug delivery

ABSTRACT

In nature, toxins have evolved as weapons to capture and subdue the prey or to counter predators or competitors. When they are inadvertently injected into humans, they cause symptoms ranging from mild discomfort to debilitation and death. Toxins are produced by living organisms that interfere in the physiological processes of the victim, which could be a prey, predator or a bystander. Venoms are complex mixtures of tens or hundreds of toxins and are produced in specialized organ. In venomous organisms, the toxins are actively administered into the victim by a bite or sting. In contrast, toxins produced in poisonous organisms enter the victim passively when the victim takes bite of the organism. In both situations, the victim suffers from mild to severe disturbances in various physiological processes leading to debilitation and death depending on the toxin dose. Toxins have evolved as the part of either the defensive strategy against predators or the offensive strategy to capture and subdue the prey. In poisonous organisms, as they do not actively ‘hunt’, toxins are produced as a deterrent against predators. Because of the importance of toxins for their survival, venomous/poisonous organisms have evolved multiple times throughout the phylogenetic tree resulting in myriads of toxins with varied structures, functions and targets in distinct physiological systems (Casewell et al., 2013). Toxins are substances produced by living organisms that interfere in the physiological processes of the victim, which could be a prey, predator or a bystander. Venoms are complex mixtures of tens or hundreds of toxins and are produced in specialized organ. In venomous organisms, the toxins are actively administered into the victim by a bite or sting. In nature, toxins have evolved as weapons to capture and subdue the prey or to counter predators or competitors. When they are inadvertently injected into humans, they cause symptoms ranging from mild discomfort to debilitation and death. Toxins are produced by living organisms that interfere in the physiological processes of the victim, which could be a prey, predator or a bystander. Venoms are complex mixtures of tens or hundreds of toxins and are produced in specialized organ. In venomous organisms, the toxins are actively administered into the victim by a bite or sting. In contrast, toxins produced in poisonous organisms enter the victim passively when the victim takes bite of the organism. In both situations, the victim suffers from mild to severe disturbances in various physiological processes leading to debilitation and death depending on the toxin dose. Toxins have evolved as the part of either the defensive strategy against predators or the offensive strategy to capture and subdue the prey. In poisonous organisms, as they do not actively ‘hunt’, toxins are produced as a deterrent against predators. Because of the importance of toxins for their survival, venomous/poisonous organisms have evolved multiple times throughout the phylogenetic tree resulting in myriads of toxins with varied structures, functions and targets in distinct physiological systems (Casewell et al., 2013).
2. Antivenom research

Conventional antivenoms comprise antibodies or antibody fragments derived from the plasma of larger mammals (typically horses) that have been immunized with venom(s) (Laloo and Theakston, 2003; Theakston et al., 2003; Gutiérrez et al., 2011). Quick and timely treatment of the victims through intravenous administration of antivenoms is the only accepted treatment of the severe medical emergency and concomitant multiple organ failure due to envenoming. Antivenomics helps to evaluate the quality and neutralizability of antivenoms and identifies toxins that exhibit poor antigenicity/neutralizability (Lomonte et al., 2008; Calvette et al., 2018). These studies indicate that despite best efforts, typical antivenoms contain only 5–46% toxin-binding antibodies (Rawat et al., 1994; Segura et al., 2013; Herrera et al., 2014; Sanz et al., 2018). These data along with others supporting the inherent drawbacks of antivenom use (Kini et al., 2018) provides impetus for the development of next generation strategies to replace more than a century old technology (Calmette, 1894a, 1894b; Calmette, 1896). Since the first Food and Drug Administration (FDA)-approved therapeutic monoclonal antibody (mAb) OKT3 (muromonab) in 1986 (Hooks et al., 1991), particularly human therapeutic mAbs and their fragments, have become the mainstay in the development of biologics for acute and chronic treatments of various human diseases (Walsh, 2014, 2018). The works of George P. Smith and Gregory P. Winter “for the phage display of peptides and antibodies” (Parnell and Smith, 1988; McCafferty et al., 1990) together with that of Frances H. Arnold “for the directed evolution of enzymes” (Chen and Arnold, 1991, 1993; Arnold, 1992) laid the foundation for the development of therapeutic antibodies. All three shared the 2018 Nobel Prize in Chemistry. Phage display for panning and selection combined with directed evolution as a reliable optimization algorithm have made selection and maturation of therapeutic antibodies somewhat routine (Hoogenboom et al., 1998; Rodi and Makowski, 1999; Sidhu, 2000). Recent improvements in antibody-expression yields and manufacturing processes have made mAbs as a cost-effective option (US$ 20–80/g) for the treatment of various diseases (Klutz et al., 2016; Pollock et al., 2017; Yang et al., 2019). Thus, there has been a dramatic rise in the use of therapeutic mAbs, with 78 mAbs approved by the FDA or the European Medicines Agency (EMA) and the annual global mAb market reached US$100 billion in 2017 (Hooft van Huijssduijnen et al., 2020). In 2018, 11 more mAbs were approved by the FDA (Mullard, 2019; Baedeker et al., 2019) and over 570 mAbs are in clinical development and this number of mAbs were approved by the FDA (Mullard, 2019; Baedeker et al., 2019). With these considerations, we and over 570 mAbs are in clinical development and this number of mAbs include compatibility with human victims, enrichment of toxin-neutralizing antibodies, consistent and reproducible quality, tailor-made mAbs and their fragments with optimal pharmacokinetics and pharmacodynamics, better safety and cross reactivity with related toxins (For details, see Kini et al., 2018). BOA can possibly be combined with repurposed small molecule, synthetic enzyme inhibitors (Builfone et al., 2018; Bryan-Quiros et al., 2019; Lewin et al., 2018a, 2018b; Salvador et al., 2019; Albulescu et al., 2019). Thus, venomics helps expanding our abilities to develop next generation antivenoms.

3. Toxin evolution

Venoms have evolved as chemical means for prey capture and competitor and/or predator deterrence (Fry et al., 2009). In the complexity of a food web, these indirect interactions control entire ecosystems. The sequence information obtained through proteomics, transcriptomics and genomics, in addition to providing the toxin profiles of venoms, opens new avenues in understanding the evolutionary history of toxins and in resolving these predator-prey interactions. Most accepted hypothesis for the evolutionary origins of toxin families suggests the duplication of nontoxic cognate genes expressed in other tissues, and subsequent change in tissue-specific expression (leading to venom gland expression) and neofunctionalization through modification of the structure and function. This hypothesis was supported by the structural similarity between various toxins with nontoxic proteins. With the initial protein sequence information, that evolution of protease inhibitors and ribonuclease (Strydom, 1973a,b) and phospholipase A2 (PLA2) (Halpert and Eaker, 1975) as snake venom toxins were proposed. Subsequent sequence studies have indicated that elapid and vipersid PLA2s originated from pancreatic and synovial (platelet) PLA2 lineages, respectively (Heinrikson et al., 1977; Seilheimer et al., 1989). With more sequence information of the toxins and their related proteins, it became clear that several other snake venom toxins originated from cognate genes encoding proteins that played roles in normal physiological functions. These included sarafotoxins/endothelins (Kociba et al., 1982, 1993; Weiser et al., 1984; Wollberg et al., 1988; Yanagisawa et al., 1988), cobra venom factor (Vogel et al., 1984), natriuretic peptides (Schweitz et al., 1992), nerve growth factor (Cohen and Levi-Montalcini, 1956; Inoue et al., 1991; Kostiza and Meier, 1996), vascular endothelial growth factor (Komori and Sugihara, 1990; Komori et al., 1999; Yamazaki et al., 2003), snake venom metalloproteases (Takeya et al., 1990; Hite et al., 1992, 1994; Au et al., 1993; Paine et al., 1992, 1994; Bjarnason and Fox, 1995; Jia et al., 1996), neurotoxin/Ly-6/lynx1 (Fuse et al., 1990; Fleming et al., 1993; Chang et al., 1997; Lyukmanova et al., 2011; Miwa et al., 1999) and acetylhicholinesterase (Cousin et al., 1998). Based on the protein sequences, we showed that venom prothrombin activators are structural and functional homologues of blood coagulation factors; group D prothrombin activators are similar to factor Xa (Joseph et al., 1999; Rao et al., 2003a), while group C prothrombin activators are similar to factor Xa-factor Va complex (Rao and Kini, 2002; Rao et al., 2003b). Some of the toxins exhibited significant diversification, while others showed minor structural and functional changes. Thus, toxins were thought to be evolved from endogenous genes regulating in normal cellular pathways (Ohno et al., 1998; Fry, 2005; Fry and Wüster, 2004; Kini and Chan, 1999). Recent genomes of venomous snakes (Vonk et al., 2013; Yin et al., 2016; Shibata et al., 2018; Perry et al., 2018; Schield et al., 2019; Suryamohan et al., 2020)
have confirmed many of these assertions. Phylogenetic analyses of toxin families also indicate the possibility of reverse recruitment of toxin genes back to physiological roles (Casewell et al., 2012). With increasing volumes of data such duplication, neofunctionalization, and recruitment events will be resolved.

Whole genome duplication (WGD) is considered as a potent force for evolution (Ohno, 1970). WGD and its impact on the emergence of evolutionary novelties and complexity, both functional and morphological, are supported by high-throughput sequence data. In vertebrate evolution, there have been two WGD or polyploidization events that are fixed over 500–600 million years (Dehal and Boore, 2005). In snakes, only certain class of toxin genes appear to have duplicated multiple times. For example, in cobras three-finger toxin genes have undergone multiple duplication events (Vonk et al., 2013; Suryamohan et al., 2020; and many transcriptome and proteome studies). In contrast, in crotalids serine protease and metalloprotease genes have undergone multiple duplication events (Yin et al., 2016; Shibata et al., 2018; Schiedl et al., 2019). Such unusual duplications cannot be explained by WGD.

In addition to WGD, there are four mechanisms of gene duplication. In ectopic recombination, crossing over occurs at non-homologous loci leading to dramatic chromosomal rearrangement, which is generally harmful to the organism. At times, it could lead to chromosomal evolution and rapid speciation (Bush et al., 1977). Transposable elements provide sequence similarity for this non-homologous recombination. The densities of transposable element correlate negatively with ectopic recombination, but the strength of this correlation depends on transposable element (Kent et al., 2017). Replication slippage occurs during DNA replication and produces duplications of short (2–3 bp) genetic sequences. During replication, DNA polymerase dissociates from the DNA and replication stalls. Due to misalignment during reattachment, it copies the same DNA segment again leading to duplication (Vigueria et al., 2001). Replication slippage is due to direct repetitive DNA sequences but requires only a few bases of similarity. The slippage efficiency is inversely proportional to the length of the direct repeat (Vigueria et al., 2001). Retrotransposition involves reverse transcription of mRNA transcripts to DNA and their insertion in the genome as retrogenes (Nauffer et al., 2019). Retrogenes usually lack introns and often contain poly-A sequences. They display changes in gene regulation leading in novel functions. Aneuploidy results in an abnormal number of chromosomes and gene dosage (Birchler et al., 2001). It is detrimental to the organism (Dey, 2004; Newman et al., 2019) and is unlikely to spread through populations. I hypothesize that the ectopic recombination or a distinct new mechanism may explain duplication of toxin genes.

Duplicated genes have three different fates: (a) non-functionalization (pseudogenization) is the most likely fate of duplicated genes based on the assumption that a deleterious mutation is a much more likely outcome than a beneficial mutation (e.g., Dowell et al., 2016); (b) subfunctionalization is a process by which mutations in their regulatory or coding sequences would lead to change in their expression patterns or altered functions, respectively, of the duplicated genes. This can occur because of the relaxation of selection pressures or constraints (e.g., Hargreaves et al., 2014); and (c) neofunctionalization, where one paralogue acquires a new function through mutations that are beneficial for function in regulatory or coding regions, while the other paralogue retains its ancestral function. As beneficial mutations are rare events, neofunctionalization is a rarer event than non-functionalization or subfunctionalization. Mutations in noncoding regulatory regions are more common and widespread, and duplication in gene-coding regions are rarer. Thus, it is still controversial whether neofunctionalization can indeed be a potent source of evolutionary novelties (For details, see Moriya and Koshiba-Takeuchi, 2018). In the case of toxins genes, pseudogenization and neofunctionalization are well documented.

The mutations in the regulatory region results in the alteration of expression pattern of duplicated cognate gene from its “parent” tissue to the venom gland. This process is broadly and loosely termed as “recruitment” (Fry and Wüster, 2004; Fry, 2005; Fry et al., 2008). The molecular details in such recruitment have been identified in venom PLA2 and prothrombin activators (Figs. 2–4). Fujimi et al. analyzed several gene sequences encoding PLA2 from Laticauda semifasciata to determine the relationship between pancreatic and venom PLA2 genes (Fujimi et al., 2002a, b). They found insertions in the promoter and the first intron of group 1A (venom) PLA2 gene compared with group 1B” (pancreatic) PLA2 gene (Fujimi et al., 2002a, 2004) (Fig. 2). The 411-bp insert in the promoter region has two E box and one GC box binding sites and interrupts promoter region of pancreatic PLA2 gene (Fig. 2B). AG-rich inserts are ~1100 bp long in venom PLA2 genes compared to 400 bp AG-rich region of pancreatic PLA2 gene (Tamiya and Fujimi, 2006) (Fig. 2A). Similarly, there was one 264 bp insertion in the promoter and 3 insertions and 2 deletions in intron 1 of the TroD gene (prothrombin activator gene expressed in the venom gland) compared to the TrFX gene (blood coagulation factor X gene expressed in the liver) (Reza et al., 2005, 2007) (Fig. 5). The promoter insert disrupts the cis elements controlling the liver-specific expression and contributes to expression of TroD gene in the venom gland. Therefore, we named this insert in TroD promoter region as venom recruitment/switch element (VERS2) (Reza et al., 2007). The AG-rich regions in second and third inserts in TroD intron 1 appear to act as the silencer in restricting its expression to venom glands (Described below) (Fig. 4). These two molecular evidences suggest that inserts in promoter and intron regions alter the tissue-specific expression of duplicated cognate genes from various “parent” tissues to venom gland. In these examples, the promoter inserts are distinct, but contain several cis elements. Such promoter inserts that are responsible for “recruitment” be named as “Prins” (Promoter inserts). In contrast, the intron inserts are AG-rich silencers. It will be interesting identify other Prins segments and respective silencers responsible for “recruitment” other toxin families. Genomic data will
also help in identifying whether these elements are disrupted through mutations or deletions during reverse “recruitment” (Casewell et al., 2012); in both instances, one could identify the evolutionary trajectory of such cognate genes.

The DNA as well as protein sequence information helps in mutation analyses within the toxin isoforms expressed by a single species, or across multiple species in a genus or even across various genera. Analysis of the cDNA sequences of Protobothrops (formerly, Trimeresurus) flavoviridis (habu snake) venom PLA2 enzymes indicated that the 5′ and 3′ untranslated regions are highly conserved (98% and 89%, respectively) compared to the protein-coding regions (67%) (Ogawa et al., 1992). Further, mutations appeared to have accumulated at similar rates for the three positions of codons. Comparison of the gene sequences indicated that the introns are much more conserved than the protein-coding regions of exons apart from the signal peptide-coding region (Nakashima et al., 1993). The nucleotide substitutions at non-synonymous site (Ks) are close to or larger than mutations at synonymous site (Ks) indicating Darwinian positive selection and accelerated evolution of protein-coding regions. Similar observations in other venom PLA2s, serine proteases and C-type lectin-like proteins suggested that accelerated evolution of exons appears to be universal in toxin isoforms (Nobuhisa et al., 1996; Deshimaru et al., 1996; Ogawa et al., 2005). These accelerated mutations were thought to neofunctionalize the toxins and lead to regional variations with suitable specificity and selectivity towards their target receptor, ion channel or enzyme through positive Darwinian selection (Lynch, 2007; Juárez et al., 2008). Our studies showed that such accelerated mutations appear to target the molecular surface of Class I (elapid) and Class II (crotalid) venom PLA2 enzymes (Kini and Chan, 1999). Interestingly, this adaptive evolution of elapid PLA2 enzymes is associated with speciation events and adaptation of the arsenal to target novel prey species (Lynch, 2007). Using nucleotide sequences of genes for fast-evolving toxins and human hereditary diseases, we showed that specific nucleotide sequences appear to determine point mutation rates (Kini and Chinnasamy, 2010). We classified triplets (not just codons) into stable, unstable and intermediate groups. The relative distribution of stable and unstable triplets is correlated with the accelerated evolution of exons of toxin genes (Kini and Chinnasamy, 2010). An analysis of gene sequences of Sistrurus catenatus edwardsi three-finger toxins (3FTxs) indicated that some segments in the exons changed to distinctly different segments compared to corresponding regions of the isoforms (Doley et al., 2008). Such a “switching” of segments in exons may result in drastic alteration in the molecular surface and, hence, the molecular target of these 3FTxs. Thus, we proposed that the phenomenon of accelerated segment switch in exons to alter targeting (ASSET) may play an important role in the evolution of toxins (Doley et al., 2008). ASSET appears to contribute to functional evolution of 3FTxs to a greater extent compared to other toxin families (Doley et al., 2009). In snake venom serine proteases, ASSET contributes to changes in three surface segments, including the segment near the substrate binding region. Interestingly, these “exchanges” occurred only in the molecular surface of toxins not affecting the structural integrity of toxins (Doley et al., 2008, 2009). Following drastic change through ASSET, accelerated point mutations probably contribute to fine-tuning of target specificity. Evolutionary fingerprint analyses of various groups of 3FTxs indicated that most residues in neurotoxic 3FTxs are under negative selection, while κ-neurotoxins and cytotoxic 3FTxs are under negative selection (Sunagar et al., 2013). The authors proposed Rapid Accumulation of Variations in Exposed Residues (RAVERS) or focal mutagenesis are responsible for the functional evolution of 3FTxs. Systematic analyses of venom disintegrin/metalloprotease genes indicated a new mechanism in the evolution through exonization and intronization (Kini, 2018). In the evolution of RTS/KTS disintegrins, a new 34 bp exon (10a) is formed in intron 10. Exon 10a originated from a non-repetitive element, unlike >90% new exons that are from repetitive elements in introns. In this case, simultaneous exonization and intronization occur within a single gene. This new mechanism introduces drastic changes to the molecular surface and alters the function of toxins (Kini, 2018). Increased sequence information combined with systematic analyses will help in fine-tuning some of these mechanisms of accelerated evolution and discover several others.
Toxins are produced in a specialized gland and their expression is tightly regulated (Kerchev et al., 2004, 2008; Luna et al., 2009; Viana et al., 2017; Yamanouye et al., 2004). Toxin expression is highest when the venom gland is emptied, as in the case of “milking”, and it slows down when the gland is full (Rotenberg et al., 1971; Oron and Bdolah, 1973, 1978; De Lucca et al., 1974). It is also apparent in the columnar and squamous statures of the gland epithelial cells that is prominent in crotalid and viperid snakes. Further, the transcriptional activity in vipers leads to a sharp increase on Day 3, after a 2-day lag period after milking, compared to the respective cognate genes. Both inserts 2 and 3 (on the minus strand) have significant number of AG-rich motifs.

### 4. Toxin expression

In general, toxin genes are selectively expressed in high quantities in the venom gland tissue compared to other tissues including the “parent” tissue. For example, venom group IA PLA2 gene is expressed 100–300 times (based on mRNA contents) higher in the venom gland compared with pancreas, while pancreatic group IB PLA2 gene is expressed at similar, low levels in both tissues (Fuji et al., 2004). However, using mass spectrometry the authors could not detect group IB PLA2 in the venom of *L. semifasciata*. Prothrombin activators are also expressed in significantly higher amounts in the venom gland. The expression of trocarin D gene in the venom gland is ~30 times higher compared with that of the TrFX gene in liver (Reza et al., 2007). Similarly, genes encoding psueratin C catalytic and nonenzymatic subunits are specifically expressed 80- and 280-fold in the venom gland compared to blood coagulation factor X and V genes in liver, respectively (Minh Le et al., 2005; Reza et al., 2006). Such a venom gland-selective expression is due to promoter and other cis elements and corresponding transcription factors.

The TATA box, the first eukaryotic core promoter motif identified (Lifton et al., 1978), regulates expression of 20% genes in yeast (Basehoar et al., 2004) and 24% genes in humans (Yang et al., 2007). It is located 25–35 bp upstream of the transcription start site. Genes containing the TATA-box are more highly regulated when compared to TATA-less genes (Basehoar et al., 2004; Bae et al., 2015). The TATA structure of neurotoxin, that of erabutoxin c, showed the presence of 32-TATAAA-28 in its promoter region (Fuse et al., 1990). Similarly, TATA boxes were found in the promoter regions of snake venom toxin genes, including 3FTx genes (for example, Chang et al., 1997, 1999; Lachumanan et al., 1998; Affiyian et al., 1999), PLA2 genes (for example, Nakashima et al., 1995; Nobuhisa et al., 1996; Jeyaseelan et al., 2000; Fujii et al., 2002b; Armugam et al., 2004; Suryamohan et al., 2020), and snake venom serine proteases (for example, Itoh et al., 1988; Reza et al., 2007). Some of these toxin genes have more than one TATA boxes (Lachumanan et al., 1998; Affiyian et al., 1999; Reza et al., 2007). The role of TATA boxes and associated other cis elements in toxin expression has been characterized in PLA2 genes (Jeyaseelan et al., 2000; Fujii et al., 2004), 3FTx genes (Gong et al., 2001; Ma et al., 2001, 2002) and prothrombin activator (factor X subunit) gene (Kwong et al., 2009) (Fig. 3C and D). Deletion and site-directed mutation of the above promoters indicate that all these cis elements enhance the expression of reporter enzymes in cell culture. Although all TATA boxes are functional, one of them is the strongest promoter element.

As mentioned above, promoter inserts (Prins) are responsible for venom gland specific expression and the “recruitment”. In the group IA PLA2 (*Laticauda semifasciata* venom LsPLA2GL1-1, LsPLA2GL2-1, and LsPLA2GL5-1) genes, there is a 411 bp insert (Prins) between the TATA box and TATA box (Fuji et al., 2002b) compared to the group IB PLA2 (*L. semifasciata* ‘pancreatic’ LsPLA2GL1-6 and Protobothrops elegans pancreatic PLA2 gene) (Fujii et al., 2004; Chijiwa et al., 2015) (Fig. 2B). This Prins segment has one GC box and two E boxes. The full length Prins enhances the expression of the reporter gene by ~7-fold (Fujii et al., 2004). By systematic deletion, the authors identified the segment ~232 to ~162 (containing both E boxes and GC box) that enhances expression and the segment ~410 to ~382 (unidentified) that encoding CTX, the most abundant toxin, increases rapidly from 0 to 24 h, while those of PLA2 and NTX increase gradually. The mRNA content for all three toxins is maintained at the elevated levels from two to eight days (Lachumanan et al., 1999). These changes in mRNA content correlates well with the production of respective toxins. Around eight days, once the tubular lumina of the venom glands are filled with venom, toxin synthesis slows to a minimal level (Lachumanan et al., 1999). Interestingly, in viperid snakes it takes about three weeks to reach this equilibrium (Oron and Bdolah, 1978). Such a minimal toxin production status is thought to be due to the enhanced hydrostatic pressure and the amount of venom present in the lumen of the gland, which act as the main regulatory factors for the toxin synthesis (Sells et al., 1989).
strongly suppresses reporter gene transcription. Similar Prins segments have been found in *Naja sputatrix* (venom acidic and neutral PLA2) (Jeyaseelan et al., 2000), *Bungarus multicinctus* (A1, A2 and A8 chains of β-bungarotoxins) (Chu and Chang, 2002), *Pseudonaja textilis* (venom PLA2 with pancreatic loop Pt-PLA1 and Pt-PLA2) (Armugam et al., 2004), and *Naja naja* (venom PLA2 Nana 39244 and Nana 39246) (Suryamohan et al., 2020) genes. Although the data is incomplete at the S’ end of Prins segments in *N. sputatrix*. *B. multicinctus* and *P. textilis* venom PLA2 genes, this segment varies across different snake genera (data not shown). All Prins segments in venom PLA2 genes contain the GC box. Interestingly, one E box is missing in *B. multicinctus* venom PLA2 genes (data not shown). Deletion mutants of *N. sputatrix* promoter showed cell-specific regulation of expression in CHO and HepG2 cells (Jeyaseelan et al., 2020).

The PL22 Prins elements enhanced expression of reporter enzymes by 3- to 7-fold (Jeyaseelan et al., 2000; Fujimi et al., 2004). In contrast, VERSE (prothrombin activator Prins element) enhances the expression by 19- to 49-fold in HepG2 (liver), HEK293T (kidney) and CHO.K1 (ovary) cell lines (Kwong et al., 2009). It also enhanced expression in primary venom gland cells. This 264 bp VERSE segment has three TATA boxes (TLB), one GATA-4 and one Y-box. As TLB1 is downstream of the transcription initiation site (TIS), we concluded it as non-functional and mutated all other predicted cis-elements. The mutants of the GATA-4, Y-box and TLB2 show 11–51%, 42–70% and 36–62% lower reporter enzyme activity compared to the VERSE promoter, respectively. In contrast, TLB3 mutant shows 14–25% increased activity (Kwong et al., 2009). By a series of deletion mutants, one suppressor and two enhancer cis elements were identified (Fig. 3D). Since VERSE was able to drive the constitutive expression in non-venom gland cells without any specific induction, it does not contain the cis-elements responsible for inducible and tissue-specific expression (Kwong et al., 2009). Subsequently, we showed that AG-rich motifs which act as silencers in intron 1 (Han et al., 2016). These AG-rich motifs are part of two large inserts found in the venom trocarin D and psuetarin C catalytic subunit genes but not in coagulation factor X genes (Reza et al., 2005, 2006) (Fig. 4). Transcription factors YY1, Sp3 and HMGB2 bind to these AG-rich motifs and silence gene expression in mammalian cells. When knockdown these transcription factors, the silencing effects of AG-rich motifs are relieved (Han et al., 2016). Two key features distinguish a silencer from a repressor; a classical silencer exhibits a position-independent repressive activity and it inhibits transcription from heterologous promoters (Ogbourne and Antalis, 1998). As expected of true silencers, constructs containing AG-rich motifs silence heterologous cytomegalovirus (CMV) promoter (Han et al., 2016). AG-rich motifs are found in other toxin (3FTx, PLA2, and metalloprotease) genes but not in their physiologic counterparts (Han et al., 2016). Thus, AG-rich motifs contribute to regulation of expression of toxin genes.

Tissue-specific expression is determined by both cis-elements and transcription factors (Trans elements). Northern blot studies showed that CTX-2 gene was expressed in venom gland, liver, heart and muscle but not in brain (Ma et al., 2001). Interestingly, the liver, heart and muscle predominantly express ~1 kb mRNAs, while venom gland expresses 0.5 kb mRNA. The liver also expresses a minor transcript of 0.5 kb. Further, transcription initiation and splicing also appears to be affected in various tissues. In the venom gland, there are three TISs – the major TIS is A (+1), while two minor TISs are A (-3) and C (-2) (Ma et al., 2001). In contrast, the major liver-specific TIS is T (-25) and other minor sites are G (-37), G (-98), C (-124) and T (-154). The liver-specific CTX-2 transcripts are lower (~20-times) compared to the venom gland-specific CTX-2 transcripts. In CHO (Chinese Hamster Ovary) cells, although both liver and venom gland-specific TISs are functional, the major TIS is at C (-124). Further, only two CTX-2 transcripts, a full-length 408 bp transcript with 26 bp longer 5’ UTR compared to venom gland transcript and a 289 bp transcript with unspliced intron 1 that encodes truncated product with 21-residue signal peptide and a mature tripeptide (Ma et al., 2001). The latter transcript is the major CTX mRNA in the liver. Although the authors found ~1 kb mRNA in their Northern blot experiments, no transcripts matching this size were obtained during 5’ and 3’ rapid amplification of cDNA ends (RACE) analysis (Ma et al., 2001). Thus, the TIS and splicing that affect the expression and function of the protein product, can be different in various tissues. Therefore, careful attention must be paid towards tissue-specific expression, as erroneous observations could be misleading. Systematic analyses of the transcripts along with appropriate quantitative PCR may help resolve some of these issues.

Previously, we evaluated the expression of prothrombin activator and the corresponding coagulation factor genes in the venom glands and liver (Minh Le et al., 2005; Reza et al., 2006, 2007). Venom prothrombin genes are expressed exclusively in the venom gland but not in the liver, while corresponding coagulation factor genes are expressed exclusively in the liver but not in the venom gland. Interestingly, trocinrin D promoter and the VERSE (a 264 bp Prins segment) alone induced expression of reporter enzyme in HepG2, an immortal cell line of polarized human hepatocytes (Kwong et al., 2009). Such a differential expression in the liver and cultured hepatocytes could be due to epigenetic differences the liver DNA compared to newly introduced plasmid DNA. Further, systematic studies may help in understanding the regulation of tissue-specific expression.

Although cardiotoxin and neurotoxin genes are similar, their expression levels are significantly different in the venom gland (Lachumanan et al., 1999). Cardiotoxins account for 60%, while neurotoxins constitute only about 3% of the total proteins in *Naja sputatrix* venom (Tan, 1983). Despite high similarity, the neurotoxin promoter shows several key differences with the cardiotoxin promoter (Ma et al., 2002). The third TATA box (~28 to ~33), which is important for the enhanced expression of CTX-2, has A (-32G) mutation. Further, an Ikaros element TCCC is found only in the neurotoxin promoter. The deletion of first 52 bp from the S’ end results in a sharp eight-fold increase in reporter enzyme activity compared to the whole promoter (Ma et al., 2002). This segment shows three single nucleotide substitutions, one nucleotide deletion and one nucleotide insertion compared to corresponding CTX promoter segment. By D/Nase 1 footprinting and gel retardation assays, the 23 bp segment (~678 to ~655) was identified as the silencer (Ma et al., 2002). NTX silencer, in both orientations, inhibits mouse mammary tumor virus promoter and it is 4-fold stronger than CTX silencer (Ma et al., 2002). Systematic evaluation of the existing and newly collected data will help in enhancing our understanding of regulatory processes involved in tissue-specific expression of toxin and related cognate genes.

5. Toxin characterization

As mentioned above, snake venom toxins are structurally and functionally similar to cognate proteins that are expressed in various non-venom gland tissues and play important roles in normal physiological processes. Thus, cognate proteins are essential for our survival. In contrast, with some modification to the structure and accessibility toxins cause devastating debility and death in the prey or victim. Therefore, understanding the structure-function relationships and mechanisms of toxins help us understand the subtle changes that affect their pathophysiological properties.

A classic example for subtle mutation leading to dramatic change is that of sickle cell anemia. Normal red blood cells have a biconcave disc shape. Their elasticity allows them to deform and pass through capillaries, leading to vessel occlusion (for a review, see Serjeant, 2010; Carden et al., 2019). Their fragile nature leads to hemolysis and anemia.

---

_R.M. Kini_  
Toxicon: X 6 (2020) 100039
The sickled cells last 10–20 days, while normal cells function for 90–120 days. Sickle cell disease causes acute and chronic complications in various organ systems, and the patients suffer from severe pain, anemia, bacterial infections and stroke (Rees et al., 2010; Yawn et al., 2014). The fragile nature of sickle cells provides an adaptive advantage to the heterozygote against malaria infection. Malaria parasite Plasmodium fails to reproduce in the red blood cells with defective hemoglobin prematurely rupture. The hemoglobin polymerization also affects its digestion by the parasite. Thus, sickle cell trait increases people’s chances of survival in malaria-stricken areas (Kwitkowski, 2005).

Similar small sequence changes drastically affect structure and function of toxins. Endothelins and sarafotoxins (SRTX), strong vasoconstrictor peptides, are excellent examples. Endothelins are endogenous vasoconstrictor secreted by primarily in the endothelium to maintain the blood pressure (Davenport et al., 2016). Neutral endothelins-decapeptide (NEP or Npeplisin), a zinc-dependent metalloprotease cleaves two peptide bonds at the amino side of hydrophobic residues (Ser5-Leu6 followed by Asp18-Ile19) and inactivates endotherlins. The removal of C-terminal Atp-21 through the second cleavage inactivates endotherlins (Kimura et al., 1988). SRTXs isolated from the venom of Atractaspis engaddensis (side-stabbing snakes, stiletto snakes, mole vipers) are structurally and functionally similar to endothelins. Interestingly, SRTXs are resistant to inactivation by nephrilysin, probably due to substitutions at both peptide bonds (Skolovskiy et al., 1990). Several natural isoforms of SRTXs has been characterized for their function. These studies have helped in understanding subtle structure-function relationships. SRTX-d have two conserved substitutions (Ser2Thr and Ser5-Leu6 followed by Asp18-Ile19) and inactivates endothelins. The hemoglobin polymerization also affects its heterogeneous against malaria infection. Malaria parasite Plasmodium engaddensis (VNPs) are structural and functional homologues of physiologic NPs that play important roles in blood volume-pressure homeostasis. Human NPs, ANP, BNP and CNP, at low concentrations induce diuresis/natriuresis and at slightly higher concentrations, they also induce vasodilation. All three NPs have a conserved 17-residue NP ring structure, but signaling regulates vasodilation, increased renal excretion of water and electrolytes, increased endothelial permeability and inhibition of renin-angiotensin-aldosterone system and sympathetic nervous systems (Sasaki et al., 1985; Maack et al., 1986; Brenner et al., 1990). CNP/NPR-B signaling, on the other hand, is mainly involved in tissue remodeling, reproduction and brain functions. It also induces vasodilation and mild hypertensive effects (Chusho et al., 2001). Since the discovery of first NP from Dendroaspis venom (DNP; Schweitz et al., 1992), VNP are found in both elapid and vipers venoms. These VNP exhibits distinct structural and functional attributes. Compared with ANP, DNP exhibits 60-fold higher resistance to proteolysis and 30-fold lower affinity to NPR-C binding (both involved in clearance of NPs) due to its 14-residue long C-terminal tail (Schweitz et al., 1992). A chimera of CNP and DNP (ring of CNP and C-terminal tail of DNP) called CD-NP has a longer half-life (Dickey et al., 2008; Lisy et al., 2008). CD-NP elicits natriuresis and diuresis through NPR-A activation using its C-terminal tail and vasodilation through NPR-B activation using its N-terminal ring (Dickey et al., 2008). Thus, CD-NP offers the beneficial effects of both NPR-A and NPR-B signaling and avoids severe hypertension by the sole and excessive activation of NPR-A (Zakeri and Burnett, 2011). We identified and characterized a usual krait NP (KNP) from Bungarus flaviceps with 38-residue long C-terminal tail, which has the propensity to form an α-helix (Siang et al., 2010; Sridharan and Kini, 2015, 2018). Systematic deletion studies showed that the presence of two pharmacophores that induce vasodilation through orthogonal pathways in KNP: K-Ring elevates intracellular cGMP levels through activation of NPR-A and Helix uses NO-dependent mechanisms (Sridharan and Kini, 2015). Infusion of K-Ring shows transient vasodilation at low doses and sustained vasodilation at high doses in anesthetized rats without affecting urine volume (Sridharan and Kini, 2015, 2018). As K-Ring induced only vasodilation with minimal or no diuretic effects, we used its structure-function relationships to delineate the determinants of hypertensive and diuretic functions in NPs (Sridharan and Kini, 2018). In ANP, conserved residues (F2, M6, D7, R8, I9 and L15) within the 17-membered ring and the C-terminal tail (NSFRY) are crucial for NPR-A binding and in vivo activity (Olins et al., 1988; Li et al., 1995; Ogawa et al., 2004, 2009). Within K-Ring, there are several substitutions (G3D, G4R, G10S, A11T, Q12H and G14D) compared with ANP. As residues at 10, 11 and 12 positions are variable among other NPs, we hypothesized that G3D, G4R and G14D substitutions and the shorter C-terminal tail may be responsible for the distinct functional differences in the hemodynamic and diuretic effects of ANP and K-Ring. By systematic substitution, we evaluated the importance of these three residues within the NP ring and C-terminal on the hemodynamic and diuretic effects in anesthetized rats (Sridharan and Kini, 2018). From these studies, we identified the residues responsible for hypertensive and diuretic effects in K-Ring and transferred these residues in to human ANP to create DGD-ANP, which is a diuretic peptide without vaso dilatory function, and DRD-ANP, which is a vasodilatory peptide without diuretic function (Sridharan and Kini, 2018; Sridharan et al., 2020). These peptides differ two or three residues from human ANP, respectively. These peptides were evaluated in conscious normal and heart failure sheep models (Rademaker et al., 2020). In these instrumented sheep models, hemodynamic effects, diuretic effects and neurohumoral changes were monitored after infusion of low, medium and high doses of both these peptides. DGD-ANP induces only diuretic and natriuretic effects, while DRD-ANP induces only hypotensive effects avoiding clear separation of pharmacological profile of these NPs that differ in a single amino acid residue (Rademaker et al., 2020). These first-in-class therapeutic peptides will be useful in the treatment of “Cold and Wet” and “Warm and Wet” acute decompensated heart failure patients (For details, see Sridharan et al., 2020; Rademaker et al., 2020).

There are several other examples of toxins that have ‘apparently’ small change through substitution/deletion/insertion but exhibit drastic change in functional profiles. Some of these subtle changes also lead to change in disulfide pairing and hence the conformation of the protein.
scaffold (Carlier et al., 2001; Kang et al., 2005, 2007; Kang and Kini, 2009; Ng and Kini, 2013; Carbajo et al., 2015).

6. Impact of toxin research

Toxin research has contributed to both fundamental and/or applied aspects of life and biomedical sciences. I will briefly enumerate a small number of examples which could inspire future toxinologists (Fig. 5).

6.1. Research tools

Highly selective interaction of toxins with their respective target receptor, ion channel or enzyme has helped in our understanding of normal physiological processes. α-Bungarotoxin, a neurotoxin isolated from *Bungarus multicinctus* venom (Chang and Lee, 1963) was used as a bait to isolate the nicotinic acetylcholine receptor (nAChR) (Changeux et al., 1970), the first receptor to be isolated and the most thoroughly characterized receptors. Subsequently, α-bungarotoxin and related neurotoxins were used as ideal ligands to probe the structure and localization of various subtypes of nAChRs. Thus, α-neurotoxins have significantly contributed to our understanding on neurotransmission. Botrocetin or venom coagglutinin (Read et al., 1978) is a C-type lectin related protein (snaclec) from the venom of *Bohadschia* species that forms a ternary complex with von Willebrand Factor (vWF) and its platelet receptor GPIb, causing platelet agglutination and aggregation. Thus, botrocetin is an excellent tool for examining interactions between platelets and vWF, for quantification of vWF and for detecting vWF- and GPIb-related diseases (Clementson et al., 2005; Marsh and Williams, 2005). Another snaclec, convulxin from *Crotalus durissus terrificus* venom (Prado-Franceschi and Brazil, 1981) binds to GPVI one of the two distinct platelet receptors for collagen and induces platelet aggregation (Jandrot-Peruus et al., 1997), while rhodocetin from *Calloselasma rhodopea* venom binds to the second collagen receptor α2β1 and inhibits platelet aggregation (Eble et al., 2001). Thus, snacles are used as molecular scalps in dissecting various stages of platelet aggregation and blood coagulation. For details on snake venom proteins used as research tools in hematology, see (Andrews et al., 2001; Clementson et al., 2001, 2007; Morita, 2004). Nerve growth factor from *Agkistrodon piscivorus* venom contributed to our knowledge on cell growth regulation (Cohen and Levi-Montalcini, 1956). Stanley Cohen and Rita Levi-Montalcini received Nobel Prize in Physiology and Medicine in 1986 for their work on nerve growth factor and epidermal growth factor. For details, see Mc Cleary and Kini (2013).

6.2. Gene duplication and novel libraries

Gene duplication appears to play critical role in the multiplicity of toxin genes. Depending on the family, genera and species, distinct classes of toxin genes appear to have undergone severe duplications. For example, in elapid and viperid snakes, 3FTx genes and SVMP genes (as well as SVSP genes) have undergone multiple duplication events. In *Pseudechis* genus, PLA2 genes show severe multiple duplications. As of now, it is unclear why only certain toxin genes undergo rampant duplication. If we understand the basic mechanism of duplication, it may be possible to create novel libraries with large number of toxin genes producing extraordinary diversity of toxins. Combining this mode of library creation with accelerated evolution (natural or through error prone polymerases), it will be possible to create unimaginable large libraries of protein toxins in distinct scaffolds.

6.3. Accelerated evolution

Since the first discovery of accelerated evolution of PLA2 genes (Ogawa et al., 1992), it has become clear that almost all toxin genes appear to show accelerated evolution (discussed above). Several theories have been proposed to explain the mechanism of accelerated mechanism. If we successfully understand the mechanism of accelerated evolution, it will aid in the development of novel libraries of protein toxins (described above).

6.4. Protein chemistry

A large number of protein chemistry problems have been resolved through structure-function relationships of toxins. I will highlight three lessons we learnt from toxin research. While studying the structure-function relationships of snake venom PLA2s, we predicted that the myotoxic site consists of a characteristic cationic site next to a hydrophobic region (Kini and Iwanaga, 1986). This cationic site was absent in non-myotoxic PLA2s. Similar characteristic regions of cationic and hydrophobic sites were also found in non-enzymatic myotoxins isolated from snake venoms (Kini and Iwanaga, 1986). We extended this study to cytolysins isolated from phylogenetically unrelated organisms that play either offensive or defensive role (Kini and Evans, 1989a). These pore-forming cytotoxins showed the common features of a cationic site flanked by a hydrophobic surface (Kini and Evans, 1989a). When this positive charge of the cationic site is chemically modified to negative or neutral charges, the cytolytic activity of *Naja nigricollis* cardiotoxin was lost (Kini and Evans, 1989b). When these positive charges are retained by guanidination, cytotoxic activity was retained. Similar cytotoxic sites were identified in other toxins (Chen et al., 1997; Calderón and Lomonte, 1998, 1999). By systematic evaluation of over 1600 protein-protein interaction sites, we showed that proline is most commonly found in the flanking segments. We proposed that the unique chemistry of proline helps protect the integrity and conformation of the interaction sites and present the sites (Kini and Evans, 1995a). These hypothetical structural roles were inspired based on the RGD loop of kistrin (Adler et al., 1991). Based on the presence of proline residues, we developed a simple predictive method to identify protein-protein interaction sites directly from the amino acid sequence (Kini and Evans, 1996). Using this straightforward method, we identified the protein-protein interaction (or functional) sites of toxins (Kini et al., 1998; Srinivasan et al., 2001) and non-toxins (Kini and Evans, 1996; Tan et al., 2001; Venugopal et al., 2018). As proline residues in the flanking segments enhance the protein-protein interactions, we developed a novel method to the design and development of potent peptide drugs (Kini and Evans, 1995b). In this universal method, incorporation of proline residues on both sides of the minimum recognition site enhance the binding ability and concomitant biological activity of the bioactive peptide by 10- to 15-fold through a non-covalent strategy. Thus, using two orthogonal methods (for details, see Kini, 1998) we utilized our finding of the presence of proline residues as the common feature in the flanking segments of protein-protein interaction sites to develop a simple strategy to convert a large, unstable protein in to a small, stable and
easily manipulatable bioactive peptides. Our recent studies on OH9-1 showed that this group of 3FTxs bind to the acetylcholine binding of nAChRs, same site as the classical α-neurotoxins, but with distinct set of functional site residues (Hassan-Puttaswamy et al., 2015). Alanine scan mutations of 12 residues in all three loops of OH9-1 indicated that loop II plays an important role in binding to nAChRs; both sides of loop-II β-strand interact with α3β5ε, while only one side interacts with α3β3ε. Although structurally important residues for 3FTx fold are conserved in OH9-1, none of the functionally important residues found in α-neurotoxins are conserved. Thus, this group of toxins, named as Ω-neurotoxins, have evolved independently and are example of unusual convergent (functionally) evolution (Hassan-Puttaswamy et al., 2015). Similarly, Type III neurotoxins (Gong et al., 1999) do not have the functionally important residues found in either α- or Ω-neurotoxins. Subtle changes in the α-neurotoxins leads to interface selectivity with higher affinity for the α-δ site as compared with α-ε or α-γ interfaces (Utkin et al., 2019). Recently, we characterized a novel dimeric neurotoxin, named fuldotoxin from Micrurus fulvius (Eastern coral snake) (Foo et al., 2020). It has a distinct dimeric quaternary structure and exhibits potent but reversible neurotoxicity in chick biventer cervicis muscle. As with all classes of 3FTx neurotoxins described above binds to the acetylcholine binding pocket of nAChRs but shares none of the functional residues. Hence, we named this new group of dimeric toxins, all from Micrurus venoms, as Σ-neurotoxins. Thus, all these groups of 3FTx neurotoxins have evolved independently through unusual convergent functional evolution.

6.5. Protein folding

Protein folding is one of the most intriguing fundamental questions of structural biology. Although the amino acid sequence of a protein encodes proper folding and its native three-dimensional structure, the specific determinants involved have not been clearly identified. Disulfide bonds, which are typically non-promiscuous covalent linkages between Cys residues, contribute to correct folding and conformation in disulfide-rich proteins such as toxins. We showed that this high fidelity of correct disulfide linkages in multiple disulfide toxins or protein domains is well-preserved due to a couple of key amino acid residues (Kang et al., 2005, 2007; Ng and Kini, 2013). The globular conformation of α-conotoxins is maintained by the presence of Pro6 in intercysteine loop 1 and the C-terminal amide. Despite conserved Cys residues, χ/γ-conotoxins fold in ribbon conformation due to the replacement of Pro6 by Lys or Ser and the C-terminal amide by free carboxylic acid (For details about these conformational switches, see Kang et al., 2005, 2007). Similarly, a single conserved aromatic, hydrophobic residue in intercysteine loop 3 is key to the formation of hydrophobic core and folding of EGF domains (Ng and Kini, 2013). When this aromatic residue is replaced by hydrophilic residue (Y25T) leads to disruption of the hydrophobic core and EGF fold. Thus, subtle changes in key structural elements alters the disulfide linkages and hence, the disruption of the protein fold.

6.6. Targeted drug delivery

Toxins have high specificity and selectivity towards a specific receptor, ion channel or an enzyme. They breach various barriers in the prey to reach the target tissue or cells. The knowledge of these intricate mechanisms and the protein structures that reaches a specific site in the body will help us develop simple but sophisticated strategies to deliver ‘cargoes’ to the target tissue. We purified a highly selective neurotoxin, candoxin from Bungarus candidus venom that binds reversibly to peripheral nAChR but irreversibly to neuronal α7 nAChR (Nithanathan et al., 2002). Intracerebroventricular injection of candoxin causes apoptosis and death of glial cells, which express α7 nAChR (Pachiappan et al., 2005). Candoxin also induces apoptosis of human glial cells (Hs 683 cells) indicating it is highly specific to glial cells. A 16-mer peptide CDX designed based on the loop II (functional site) of candoxin binds to α7 nAChR as well as primary rat brain capillary endothelial cells (Zhan et al., 2011). CDX labeled micelle carrying paclitaxel extends the survival time of mice with glioblastoma. These vesicles also deliver near infrared fluorescent dye DiR to the brain (Zhan et al., 2011). Using red blood cell derived nanoparticles labeled with 6CDX (synthesized using all D-amino acids with reversed sequence of CDX) created a brain-targeted delivery system for doxorubicin administration (Chai et al., 2017). Thus, based on the candoxin a NACHr-mediated brain drug delivery system was designed. 3FTxs exhibit distinct pharmacological activities by selectively binding to various targets including nAChRs, muscarinic acetylcholine receptors, GABA receptor, adrenergic receptors, Ω1βδ3 integrin, L-type calcium channel, acid-sensitive ion channel, acetylcholinesterase, and several blood coagulation complexes (for details, see Kini, 2002; Fry et al., 2003; Kini and Doley, 2010). In addition, there are at least 20 classes of orphan 3FTxs whose target proteins are yet to be identified (Fry et al., 2003). Similarly, snake venom PLA2 enzymes exhibit a wide variety of pharmacological effects including presynaptic and postsynaptic neurotoxicity, myotoxicity, hemorrhagic activity, anticoagulant and antiplatelet effects due to their specific interaction with various protein targets (For details, see Kini and Evans, 1988c; Kini, 2003). Based on the functional sites of 3FTxs, PLA2s and other toxins, the postal addresses of various tissues or cells, a number of tissue-selective delivery systems can be developed (Kini, 2003). While writing this manuscript, a new drug delivery system that reduces the systemic corticosteroid exposure. By using a cysteine dense particle (CDP) based on a potassium channel blocker from scorpion venom, a highly targeted delivery of arthritis drugs to joints (Cook Sangar et al., 2020). Thus, toxins provide ample opportunity to develop excellent drug delivery systems.

6.7. Diagnostic agents

Based on their effects on blood coagulation and platelet aggregation, several snake venom toxins are being used in diagnosis of hereditary diseases. Some of the standard blood clotting time measurements, such as Stypyen time (Hougie, 1956), Ecarin time (Kornakil et al., 1979), dilute Russell viper venom time (Thiagarajan et al., 1986), textar time (Triplett et al., 1993), Taipan snake venom time (Rooney et al., 1994), and CA-1 method using carinactivase (Iwahashi et al., 2001), are initiated by snake venom factors. These clotting time measurements help in identifying the defects in various blood coagulation factors as well as lupus anticoagulants and oral anticoagulants. Estimation of protein C (Hubbard, 1988), protein S (Han and Pradham, 1990), Factor V Leiden (Sayinalp et al., 2004; Linz et al., 2006), similarly, several snake venom proteins affecting platelet aggregation (Andrews et al., 2001; Clementson et al., 2001, 2007; Morita, 2004) are used to resolve hereditary defects in platelet aggregation. For example, botrocetin is used in understanding the von Willebrand factor and glycoprotein Ib diseases (Brinkhous et al., 2001, 2007; Morita, 2004) are used to resolve hereditary defects in platelet aggregation. As Stypven time of mice with glioblastoma. These vesicles also deliver near infrared fluorescent dye DiR to the brain (Zhan et al., 2011). Using red blood cell derived nanoparticles labeled with 6CDX (synthesized using all D-amino acids with reversed sequence of CDX) created a brain-targeted delivery system for doxorubicin administration (Chai et al., 2017). Thus, based on the candoxin a NACHr-mediated brain drug delivery system was designed. 3FTxs exhibit distinct pharmacological activities by selectively binding to various targets including nAChRs, muscarinic acetylcholine receptors, GABA receptor, adrenergic receptors, Ω1βδ3 integrin, L-type calcium channel, acid-sensitive ion channel, acetylcholinesterase, and several blood coagulation complexes (for details, see Kini, 2002; Fry et al., 2003; Kini and Doley, 2010). In addition, there are at least 20 classes of orphan 3FTxs whose target proteins are yet to be identified (Fry et al., 2003). Similarly, snake venom PLA2 enzymes exhibit a wide variety of pharmacological effects including presynaptic and postsynaptic neurotoxicity, myotoxicity, hemorrhagic activity, anticoagulant and antiplatelet effects due to their specific interaction with various protein targets (For details, see Kini and Evans, 1988c; Kini, 2003). While writing this manuscript, a new drug delivery system that reduces the systemic corticosteroid exposure. By using a cysteine dense particle (CDP) based on a potassium channel blocker from scorpion venom, a highly targeted delivery of arthritis drugs to joints (Cook Sangar et al., 2020). Thus, toxins provide ample opportunity to develop excellent drug delivery systems.

6.8. Therapeutic agents

The toxicity of venoms is one of the main reasons for our fascination with snakes. Over many centuries, scientists focused on understanding this toxicity and the ways to neutralize it. The discovery of bradykinin potentiating peptides in Brazilian pit viper (Bothrops jararaca) venom (Ferreira, 1965; Ferreira et al., 1970a, 1970b) followed by the development of non-peptidic inhibitors from peptides led to the synthesis of
captoxiril (Cushman et al., 1977). This changed the paradigm from “toxin as a villain” to “toxin as a savior”. Similarly, discovery of disintegrins from Trimeresurus gramineus (Indian green pit viper) and Echis carinatus (saw scaled viper) venom (Huang et al., 1987, 1989; Gan et al., 1988) culminated in the development of potent antiplatelet agents, epitfibatide (Scarborough et al., 1993; Phillips and Scarborough, 1997; Scarborough, 1999) and tirofiban (Barrett et al., 1994; Lynch et al., 1995). Vipeptide, a folded peptidomimetic is being developed as partial antagonist of α2(1) integrin and an antithrombotic agent based on a snacelce from Vipera palabininae venom (Arlinghaus et al., 2013; Momic et al., 2015). As described above, we are developing DRD-ANP and DGD-ANP for the personalized treatment of acute decompensated heart failure patients (Sridharan et al., 2020; Rademaker et al., 2020). There many other laboratories have been working towards developing several novel therapeutic agents for pain, cancer and antimicrobial function. There are several reviews on various aspects of drug development from based on toxins.

7. Conclusions and prospects

In the recent years, Omics technologies have effectively and significantly contributed in obtaining tremendous amount of information in toxicology. It will continue to provide more information in the coming years, with a much lower price tag. The database is expanding in a geometric progression and this growth showing healthy positive exponential. The data available has provided an excellent foundation and opportunities to all toxinologists. Although I have only identified four distinct directions, there are substantially more directions. In each of these directions, finding answers requires multidimensional approaches. These directions are also to an extent interconnected. As of now, we have just started scratching the surface and future expansion will lead us to more interesting questions and is limited only by our imagination. Toxicology provides excellent opportunities for fully intertwined research in fundamental and biomedical sciences. Over the last couple of decades, we have made some inroads but future holds much greater exciting and bright opportunities.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

R. Manjunatha Kini: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Writing - original draft, Writing - review & editing.

Acknowledgments

I thank thousands of toxinologists who have contributed this field and their contributions helped me guide my own research career. Many of their works, I had the pleasure to read and cite. I apologize to much bigger group of scientists whose work I have not cited here due to my own knowledge limitation combined with the space limitation. I thank my teachers and mentors, who contributed directly: Professors T. Veerabasappa Gowda (University of Mysore, India), Sadaaki Iwanaga (Kyushu University, Japan) and Herbert Evans (Virginia Commonwealth University, USA). I thank Dr. Cassandra Modahl for discussions and reading the manuscript. I thank Ms. Jasmine Jia Min Lim for excellent artwork used in the graphic abstract. I acknowledge the financial support by academic grants by the Ministry of Education, Singapore and the National University of Singapore.

References

Vonk, F.J., Caswell, N.R., Henkel, C.V., Heimberg, A.M., Jansen, H.J., McCreary, R.J., Kerckman, W., Vos, R.J., Rosetti, I., Calvete, J.J., Wüster, W., Woods, A.E., Logan, J.M., Harrison, R.A., Castoe, T.A., de Koninck, A.P., Pollock, D.D., Yandell, M., Calderon, D., Renjifo, C., Currier, R.B., Salgado, D., Pla, D., Sanz, L., Hyder, A.S., Ribeiro, M.J., Arntzen, J.W., van den Thillart, G.L.E., Boetzer, M., Pirovano, W., Ribeiro, D.P., Spank, H.P., Evans, R.M., Kini, R.M., Richardson, M.K., 2013. The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. Proc. Natl. Acad. Sci. USA.110 20651–20656. https://doi.org/10.1073/pnas.1207011110. Epub 2013 Dec 2.

A broth, F., Guénoche-Besbes, T., Tilouche, N., Elatrous, S., 2020. Scorpion envenomation: state of the art. Intensive Care Med. 46, 401–410. https://doi.org/10.1007/s00134-020-05924-8. Epub 2020 Mar 3.

Adler, M., Lazarus, R.A., Dennis, M.S., Wagner, G., 1991. Solution structure of kistatin, a potent platelet aggregation inhibitor and GP IIb-IIIa antagonist. Science 253, 445–448.

Ailtiyam, F., Armugam, A., Tan, C.H., Gopalakrishnakone, P., Jeyaanel, K., 1999. Postsynaptic alpha-neurotoxin gene of the spitting cobra, Naja naja sputatrix: structure, organization, and phylogenetic analysis. Genome Res. 9, 259–266.

Albuluescu, L.-O., Hale, M., Ainsworth, S., Alsolaiss, J., Rademaker, A., 2020. The work reported in this paper.

R. Manjunatha Kini: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Writing - original draft, Writing - review & editing.

Acknowledgments

I thank thousands of toxinologists who have contributed this field and their contributions helped me guide my own research career. Many of their works, I had the pleasure to read and cite. I apologize to much bigger group of scientists whose work I have not cited here due to my own knowledge limitation combined with the space limitation. I thank my teachers and mentors, who contributed directly: Professors T. Veerabasappa Gowda (University of Mysore, India), Sadaaki Iwanaga (Kyushu University, Japan) and Herbert Evans (Virginia Commonwealth University, USA). I thank Dr. Cassandra Modahl for discussions and reading the manuscript. I thank Ms. Jasmine Jia Min Lim for excellent artwork used in the graphic abstract. I acknowledge the financial support by academic grants by the Ministry of Education, Singapore and the National University of Singapore.

References

Vonk, F.J., Caswell, N.R., Henkel, C.V., Heimberg, A.M., Jansen, H.J., McCreary, R.J., Kerckman, W., Vos, R.J., Rosetti, I., Calvete, J.J., Wüster, W., Woods, A.E., Logan, J.M., Harrison, R.A., Castoe, T.A., de Koninck, A.P., Pollock, D.D., Yandell, M., Calderon, D., Renjifo, C., Currier, R.B., Salgado, D., Pla, D., Sanz, L., Hyder, A.S., Ribeiro, M.J., Arntzen, J.W., van den Thillart, G.L.E., Boetzer, M., Pirovano, W., Ribeiro, D.P., Spank, H.P., Evans, R.M., Kini, R.M., Richardson, M.K., 2013. The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. Proc. Natl. Acad. Sci. USA.110 20651–20656. https://doi.org/10.1073/pnas.1207011110. Epub 2013 Dec 2.

A broth, F., Guénoche-Besbes, T., Tilouche, N., Elatrous, S., 2020. Scorpion envenomation: state of the art. Intensive Care Med. 46, 401–410. https://doi.org/10.1007/s00134-020-05924-8. Epub 2020 Mar 3.

Adler, M., Lazarus, R.A., Dennis, M.S., Wagner, G., 1991. Solution structure of kistatin, a potent platelet aggregation inhibitor and GP IIb-IIIa antagonist. Science 253, 445–448.

Ailtiyam, F., Armugam, A., Tan, C.H., Gopalakrishnakone, P., Jeyaanel, K., 1999. Postsynaptic alpha-neurotoxin gene of the spitting cobra, Naja naja sputatrix: structure, organization, and phylogenetic analysis. Genome Res. 9, 259–266.

Albuluescu, L.-O., Hale, M., Ainsworth, S., Alsolaiss, J., Rademaker, A., 2020. The work reported in this paper.

R. Manjunatha Kini: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Writing - original draft, Writing - review & editing.

Acknowledgments

I thank thousands of toxinologists who have contributed this field and their contributions helped me guide my own research career. Many of their works, I had the pleasure to read and cite. I apologize to much bigger group of scientists whose work I have not cited here due to my own knowledge limitation combined with the space limitation. I thank my teachers and mentors, who contributed directly: Professors T. Veerabasappa Gowda (University of Mysore, India), Sadaaki Iwanaga (Kyushu University, Japan) and Herbert Evans (Virginia Commonwealth University, USA). I thank Dr. Cassandra Modahl for discussions and reading the manuscript. I thank Ms. Jasmine Jia Min Lim for excellent artwork used in the graphic abstract. I acknowledge the financial support by academic grants by the Ministry of Education, Singapore and the National University of Singapore.
Ohno, S., 1970. Evolution by Gene Duplication. Springer, ISBN 978-3-642-86661-6, pp. 1–160.
Ohno, S., Menez, R. Ogawa, T, Danse, J.M, Shimogishahi, Y, Fromen, C, Ducancel, P, Zinn-Justin, S, Le Duf, M.H, Boulain, JC, Tamiya, T, Menez, A, 1998. Molecular evolution of snake toxins: is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? Prog. Nucl. Acid Res. Mol. Biol. 59, 307-364, Oli, G.M., Patton, D.R., Mather, R.A., 1989. A linear approach to the study of natriuretic peptide (ANP) discriminates guanylate cyclase-coupled ANP receptors from non-coupled receptors. J. Biol. Chem. 263, 10989-10993. PMID:2990882.
Oron, U., Bodelah, A., 1973. Regulation of protein synthesis in the venom gland of viperid snakes. J. Cell Biol. 56, 177-190.
Oron, U., Bodelah, A., 1980. Intraacellular transport of proteins in active and resting secretory cells of the venom gland of Vipera palatinae. J. Cell Biol. 78, 488-502.
Pachippapan, A., Thwin, M.M., Manikandan, J., Gopalakrishnakone, P., 2005. Glial inflammation and neurodegeneration induced by candoxin, a novel neurotoxin from Bungarus candidus venom: global gene expression analysis using microarray. Toxicon 46, 883-899.
Paine, M.J., D/L Judson, H.P., Theakston, R.D.G., Crampton, J.M., 1992. Purification, cloning, and molecular characterization of a high molecular weight hemoglobin metallotransferrine, jararhagin, from Bothrops jararaca venom. Insights into the disintegrin family gene. J. Biol. Chem. 27, 2269-22876.
Paine, M.J.I., Moura-De-Silva, A.M., Theakston, R.D.G., Crampton, J.M., 1994. Cloning of metalloproteinase genes in the carpet viper (Echis pyramidum leakeyi). Further members of the metallo- protease/disintegrin gene family. Eur. J. Biochem. 224, 483-488.
Parney, S.F., Smith, G.P., 1988. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene 73, 305-318.
Peruch, A.M., Wilmer, M., 2010. Diagnostic use of snake venom components in the coagulation laboratory. In: Kini, R.M., Clemetson, K.J., Markland, F.S., McLane, M.A., Clemetson, T. (Eds.), Thrombosis and Haemostasis: from Bench to Bedside, Springer, Dordrecht, pp. 747-766, 2010.
Perry, B.W., Card, D.C., Mcglothin, J.W., Pasquesi, G.L.M., Adams, R.H., Schild, D.D., Schuler, M., Hales, N.R., Corbin, A.B., Demuth, J.P., Hoffmann, F.G., Vandelee, M.W., Schueler, K.R., Bhatnagar, N., Chang, N., Ohno, M., Raff, C.A., 1989. Structural similarity to mammalian coagulation factor V. Blood 102, 1347
Pinto, P., Pring, D., Rippin, D., Hales, N.R., Catterall, W.A., 1993. Design of heart failure antagonists. Peptide antagonists with high specificity for glycoprotein IIb-IIIa. J. Biol. Chem. 268, 1066-1073.
Schraer, D.R., Card, D.C., Hales, N.R., Perry, B.W., Pasquesi, G.M., Blackmon, H., Adams, C.F., Corbin, A.B., Chang, N., Chang, N., 1995. Inflammation and neurodegeneration induced by candoxin, a novel neurotoxin from Vipera palaestinae (Ophidia, Reptilia). Biochem. J. 121, 190.
Sanz, L., Quesada-Berna, S., Chen, P.Y., Lee, C.D., Chang, B.S.W., Casewell, N.R., Whiteley, G., Reyes-Amez, A, 1998. Molecular affinity purification of target genes. Gene 73, 305.
Schewitz, H., Vigne, P., Kotrla, J., Moinier, D., Frelin, C., Lazdunski, M., 1992 Jul 15. A new α1b-hANP (hANP) in rats. Eur. J. Pharmacol. 109, 281.
Schweitz, H., Vigne, P., Moinier, D., Frelin, C., Lazdunski, M., 1992 Jul 15. A new member of the natriuretic peptide family is present in the venom of the green mamba (Dendroaspis angusticeps). J. Biol. Chem. 267 (20), 13928-13932.
Schweitz, H., Vigne, P., Kotrla, J., Moinier, D., Frelin, C., Lazdunski, M., 1992 Jul 15. A new α1b-hANP (hANP) in rats. Eur. J. Pharmacol. 109, 281.
Sells, P.G., Hommel, M., Theakston, R.D.G., 1989. Venom production in snake venoms and its significance for cell culture. Venom cells cultured in vitro. Toxicon 27, 1249-1257.
Schröder, M.T., Scott, N.J.A., Koh, C.Y., Kini, R.M., Richards, A.M., 2020. Natriuretic factor 2 in the venom of Combretum oppositifolium. J. Thromb. Haemostasis 34, 612.
Segura, A., Herrera, M., Vargas, M., Gutierrez, J.M., Leoni, G., 2013. Assessment of snake antivenom purity by comparing physicochemical and immunological methods. Biologicals 41, 93-97. https://doi.org/10.1016/j.biologicals.2012.11.001. Epub 2012 Nov 26.
Seilheimer, J.J., Pruzanki, W., Vadas, P., Plant, S., Miller, J.A., Kloss, J., Johnson, L.K., 1989. Cloning and recombinant expression of phospholipase A2 present in rhesomalbumin arthritic synovial fluid. J. Biol. Chem. 264, 5335-5338.
Sells, P.G., Hommel, M., Theakston, R.D.G., 1989. Venom production in snake venoms and its significance for cell culture. Venom cells cultured in vitro. Toxicon 27, 1249-1257.
Schröder, M.T., Scott, N.J.A., Koh, C.Y., Kini, R.M., Richards, A.M., 2020. Natriuretic factor 2 in the venom of Combretum oppositifolium. J. Thromb. Haemostasis 34, 612.
Schröder, M.T., Scott, N.J.A., Koh, C.Y., Kini, R.M., Richards, A.M., 2020. Natriuretic factor 2 in the venom of Combretum oppositifolium. J. Thromb. Haemostasis 34, 612.
Schröder, M.T., Scott, N.J.A., Koh, C.Y., Kini, R.M., Richards, A.M., 2020. Natriuretic factor 2 in the venom of Combretum oppositifolium. J. Thromb. Haemostasis 34, 612.
Suga, S., Nakao, K., Hosoda, K., Mukoyama, M., Ogawa, Y., Shirakami, G., Arai, H., Saito, Y., Kambayashi, Y., Inouye, K., 1992. Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130, 229–239. https://doi.org/10.1210/endo.130.1.11390930.

Suga, S., Itob, H., Komatsu, Y., Ogawa, Y., Hama, N., Yoshimasa, T., Nakao, K., 1993. Cytokine-induced C-type natriuretic peptide (CNP) secretion from vascular endothelial cells – evidence for CNP as a novel autocrine/paracrine regulator from endothelial cells. Endocrinology 133, 3038–3041. https://doi.org/10.1210/endo.133.6.8243323.

Sunagar, K., Jackson, T.N., Undheim, E.A., Ali, S.A., Antunes, A., Fry, B.G., 2013. Three-fingered RAVRs: rapid accumulation of variations in exposed residues of snake venom toxins. Toxins 5, 2172–2208. https://doi.org/10.3390/toxins5112172.

Suryanarayan, K., Krihsnakunty, S.P., Guilloy, J., Jevit, M., Schroder, M.S., Wu, M., Kuriakose, B., Mathew, O.K., Perumal, R.C., Koludarov, L., Goldstein, L.D., Senger, K., Dixon, M.D., Velayutham, D., Vargas, D., Chaudhuri, S., Muralideenham, M., Goel, R., Chen, Y.J., Ratan, A., Liu, P., Faherty, B., de la Rosa, G., Shibata, H., Baca, M., Sagolla, M., Ziai, J., Wright, G.A., Vucic, D., Mohan, S., Antony, A., Stonin, K., Kirkpatrick, D.S., Hannoush, R.N., Durinck, S., Modrzan, Z., Stawiiski, E.W., Riley, K., Raudsepp, T., Kin, R.M., Zacharias, A., Seshagiri, S., 2020. The Indian cobra reference genome and transcriptome enables comprehensive identification of venom toxins. Nat. Genet. 52, 106–117. https://doi.org/10.1038/s41588-019-0559-4. Epub 2020 Jan 6.

Takayanagi, R., Kitazumi, K., Takasaki, C., Ohnaka, K., Aimoto, S., Tasaka, K., Suga, S., Nakao, K., Hosoda, K., Mukoyama, M., Ogawa, Y., Shirakami, G., Arai, H., Saito, Y., Kambayashi, Y., Inouye, K., 1992. Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130, 229–239. https://doi.org/10.1210/endo.130.1.11390930.

Viana, L.G., Valente, R.H., Heluany, C.S., Souza-Imberg, A., Luna, M.S., Perales, J., Yamanouye, N., Kerchove, C.M., Moura-da-Silva, A.M., Carneiro, S.M., Markus, R.P., Williams, D.J., Faiz, M.A., Abel-Ridder, B., Ainsworth, S., Bullone, T.C., Nickerson, A. D., Habib, A.G., Junghans, T., Fan, H.W., Turner, M., Harrison, R.A., Warrell, D.A., 2019. Strategy for a globally coordinated response to a priority neglected tropical disease: snakebite envenoming. PLoS Neglected Trop. Dis. 13, e0007059. https://doi.org/10.1371/journal.pntd.0007059.

Weiser, E., Wollberg, Z., Kochva, E., Lee, S.Y., 1984. Cardiotoxic effects of the venom of the burrowing asp, Atractaspis engaddensis (Atractaspididae, Ophidia). Toxicon 22, 767–774.

Williams Jr., D.L., Jones, K.L., Pettibone, D.J., Lis, E.V., Clinesmith, B.V., 1991. Sarafotoxin 56c: an agonist which distinguishes between endothelin receptor subtypes. Biochem. Biophys. Res. Commun. 175, 556–561.

Yamanouye, N., Kerchove, C.M., Moura-da-Silva, A.M., Carneiro, S.M., Markus, R.P., 2004. Long-term primary culture of secretory cells of Bothrops jararaca venom gland for venom production in vitro. Nat. Protoc. 1, 2763–2766.

Yamazaki, Y., Takani, K., Atoda, H., Morita, T., 2003. Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of KDR (VEGF receptor 2). J. Biol. Chem. 278, 51985–51988.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

Yan, C., Bolotin, E., Jiang, T., Shlak, F.M., Martinez, E., 2007. Prevalence of the P10 mutation in the precursor of the human natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130, 229–237. https://doi.org/10.1210/en.2006-08243. Epub 2007 Oct 30.

Ye, Z., Yang, C., Bolotin, E., Jiang, T., Sladek, F.M., Martinez, E., 2007. Prevalence of the P10 mutation in the precursor of the human natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130, 229–237. https://doi.org/10.1210/en.2006-08243. Epub 2007 Oct 30.

Ye, Z., Yang, C., Bolotin, E., Jiang, T., Sladek, F.M., Martinez, E., 2007. Prevalence of the P10 mutation in the precursor of the human natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130, 229–237. https://doi.org/10.1210/en.2006-08243. Epub 2007 Oct 30.

Ye, Z., Yang, C., Bolotin, E., Jiang, T., Sladek, F.M., Martinez, E., 2007. Prevalence of the P10 mutation in the precursor of the human natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130, 229–237. https://doi.org/10.1210/en.2006-08243. Epub 2007 Oct 30.