Guiding recombinant antivenom development by omics technologies

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Abstract

In this review, the different approaches that have been employed with the aim of developing novel antivenoms against animal envenomings are presented and discussed. Reported efforts have focused on the use of innovative immunization strategies, small molecule inhibitors against enzymatic toxins, endogenous animal proteins with toxin-neutralizin capabilities, and recombinant monoclonal antibodies. Harnessing either of these approaches, antivenom development may benefit from an in-depth understanding of venom compositions and which toxins that are essential to neutralize in an envenoming case. Focus is thus also directed towards the different omics technologies (particularly venomics, antivenomics, and toxicovenomics) that are being used to uncover novel animal toxins, shed light on venom complexity, and provide directions for how to determine the medical relevance of individual toxins within whole venoms. Finally, techniques for assessing antivenom specificity and cross-reactivity are reviewed, with special focus on antivenomics and high-density peptide microarray technology.

Keywords	Toxinology; recombinant antivenom; toxicovenomics; antivenomics; high-density peptide microarray technology;

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April 12th, 2017
Dr. Mike Taussig
Editor-in-Chief
New Biotechnology

Dear Dr. Mike Taussig

Thank you for your encouragement to publish in the journal. Please find attached the manuscript entitled '*Guiding recombinant antivenom development by omics technologies'*, to be considered for publication as a review article in New Biotechnology.

In recent year, there has been an increased focus on how to overcome the challenge of snakebite and bring novel antivenoms to poor rural parts of the tropical world. A few reviews are already available in the field (of which I have co-authored some), however, these are focused on the individual efforts and specific toxin-neutralizing molecules, antibodies, or sera, and fail to provide an overview of the omics technologies employed to guide their development. Here, I have aimed to provide such an overview, and bring more clarity to how omics technologies interplay with toxinology and how these may be employed to guide medicinal chemistry and biotechnological efforts within antivenom development. It is my personal experience that too many research efforts have been unsuccessful, as they have attempted to develop toxin-targeting molecules without taking the complexity of venom and the often inverse relationship between toxicity and immunogenicity into account. It is my belief that an overview of how omics technologies can be harnessed in antivenom development will be useful for particularly drug development researchers (and graduate students) who are new in the field, and who need to gain an understanding of venoms and how these may be viewed and analyzed as drug targets.

I therefore hope that you will take this article into consideration.

Sincerely yours,

Dr. Andreas H. Laustsen

Guiding recombinant antivenom development by omics technologies

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Abstract

In this review, the different approaches that have been employed with the aim of developing novel antivenoms against animal envenomings are presented and discussed. Reported efforts have focused on the use of innovative immunization strategies, small molecule inhibitors against enzymatic toxins, endogenous animal proteins with toxin-neutralizin capabilities, and recombinant monoclonal antibodies. Harnessing either of these approaches, antivenom development may benefit from an in-depth understanding of venom compositions and which toxins that are essential to neutralize in an envenoming case. Focus is thus also directed towards the different omics technologies (particularly venomics, antivenomics, and toxicovenomics) that are being used to uncover novel animal toxins, shed light on venom complexity, and provide directions for how to determine the medical relevance of individual toxins within whole venoms. Finally, techniques for assessing antivenom specificity and cross-reactivity are reviewed, with special focus on antivenomics and high-density peptide microarray technology.

35 **Introduction**

36 Among the tropical diseases, snakebite envenoming remains one of the most neglected, causing
37 mortality and morbidity to thousands of victims worldwide each year [1–3]. In addition,
38 envenomings by other species, particularly scorpions and spiders, also constitute a medically
39 important challenge for public health [4–7]. Modern approaches based on biotechnology and
40 medicinal chemistry are starting to see the light of day through neutralization of animal toxins by
41 monoclonal antibodies (mAbs) and small molecule inhibitors [8,9]. Yet, serum-based antivenom
42 derived from immunized animals is currently the only therapeutically effective treatment option
43 against most animal envenomings [10]. Antivenom is thus one of the few biological therapies that
44 have not yet entered the modern era of biologics, despite the presence of an overwhelmingly large
45 patient population. Although part of the explanation for the lack of innovation within antivenom
46 development may be attributed to the poor financial incentive for investment in the field, the sheer
47 complexity of animal venoms may also hold part of the answer. Not only is each venom a complex
48 mixture of toxins, but venoms are highly diverse across the known 725 venomous snake species [8],
49 2000 scorpion species, and 44,000 spider species [9]. As an example and rough estimation, it has
50 previously been suggested that between 19,000-25,000 toxins may exist in the venoms of the two
51 snake families, Elapidae and Viperidae, that contain the species of the highest medical relevance for
52 human health [8]. Although neutralization of many of these toxins and toxins from other animal
53 species may not individually be medically essential in every envenoming case [11], such numbers
54 strongly support the notion that animal venoms are among the most complex drug targets known to
55 man. In this review, novel strategies for developing modern envenoming therapies are reviewed
56 with special focus on how omics (particularly venomics [12] and toxicovenomics [13]) technologies
57 can be employed to guide discovery of antibodies capable of neutralizing medically relevant toxins.
58

59 **Neutralization of animal toxins**

60 Animal venom toxins are proteinaceous and have evolved primarily to subdue prey, as well as to
61 deter predators. Animal toxins can exert a myriad of different pathophysiological effects in victims
62 of envenoming, including systemic neurotoxicity, haemotoxicity, myotoxicity, and cytotoxicity,
63 manifesting clinically as flaccid paralysis, involuntary muscle contraction, various coagulopathies,
64 nephrotoxicity, and local tissue damage including necrosis [14,15]. Different approaches have been
65 pursued in the attempt to combat different animal venoms and toxins, including the use of novel
66 immunization methods, small molecule inhibitors, endogenous toxin-neutralizing animal proteins,
67 and antibodies. These will be presented in the following. It is, however, beyond the scope of this
68 article to provide an exhaustive review on all the examples of individual antitoxins and novel
69 antisera that have been reported to date, as these can be found elsewhere [2,6,8,9].

70

71 ***Optimizing antisera by next generation immunization technology***

72 Two of the challenges in current antivenom production include procurement of venoms and
73 obtaining a balanced response against the medically relevant toxins within a venom [16].
74 Additionally, intraspecies venom variation is a common phenomenon [17,18], which may further
75 complicate design of effective immunization mixtures. Although differences in antibody responses
76 also occur among immunized animals for antivenoms, variation in response can to some extent be
77 controlled using standardized immunogens, such as (multi)epitope DNA strings, synthetic peptides,
78 or recombinant toxins [6]. Using such molecules also has the advantage that the immunization
79 mixture can be designed to contain only those immunogens of medical relevance to humans. A
80 prominent example of the successful use of DNA strings for immunization was reported in 2006,
81 where antisera against toxins of the two vipers, *Echis ocellatus* and *Cerastes cerastes*, were
82 successfully raised by immunizing mice in the epidermal layer of their abdominal region with a

83 multiepitope DNA immunogen using a GeneGun [19]. Using a synthetic peptide containing
84 continuous and discontinuous epitopes derived from the *Centruroides noxius* (scorpion) Cn2 toxin,
85 researchers in another study succeeded in raising efficacious antiserum in rabbits [20]. Similarly,
86 rabbits have also been immunized with a recombinant, non-toxic version (a toxoid) of the TsNTxP
87 toxin from *Tityus serrulatus* venom, which could protect rabbits against 20 LD_{50}s of whole venom
88 from this scorpion [21]. Nevertheless, despite these and many more examples of the successful use
89 of such modern immunization technologies, the final products of their use are still serum-based
90 medicines that are costly to produce and may be immunogenic to human recipients due to their
91 heterologous nature.
92
93 ***Using small molecule inhibitors against animal toxins***
94 A few dozen molecules have been reported to show inhibitory effects against various toxins from
95 spiders, scorpions, and snakes [8,9]. Common to all these small molecule inhibitors is that they
96 target enzymatically active toxins, either by mimicking the natural substrate or scavenging an
97 important co-factor for the apoenzyme. Examples of toxin-inhibiting small molecules include
98 varespladib, which effectively neutralizes phospholipase A_2 activity of many snake venoms [22],
99 batimastat and EDTA, which chelate Zn^{2+} ions and thereby inhibit metalloprotease activity [23],
100 and heparin, which may inhibit hyaluronidase activity [24–26]. However, no toxin-targeting small
101 molecule is currently in clinical use. More details and examples of small molecule inhibitors
102 (particularly from plants) is beyond the scope of this review and can be found elsewhere [27].
103
104 ***Harnessing natural toxin-neutralizing proteins from animals***
105 Non-antibody proteinaceous molecules capable of neutralizing animal toxins have not so far been
106 reported outside the field of snake venoms. However, for snake venoms the presence of protective

107 proteins has been described in a range of different animal species, including the South American
108 opossum, *Didelphis marsupialis* [28–30], snakes themselves [31–35], and ground squirrels
109 (*Spermophilus beecheyi*) [36]. Some of these factors have even shown greater toxin-neutralizing
110 capacity than commercial antivenoms, exemplified by the 97 kDa protein and the antibothropic
111 complex derived from *Didelphis marsupialis* serum [30]. These proteins were 4 and 6 times more
112 potent than the commercially available antivenom against *Bothrops lanceolatus* venom and *B.*
113 *jararaca* venom, respectively [30]. Although such results are fascinating and may even seem
114 promising, two major obstacles are likely to prevent the use of these types of molecules from being
115 used as envenoming therapy. Firstly, the expression of non-standard protein formats is not always a
116 trivial matter. Given the molecular diversity of the different naturally occurring toxin-neutralizing
117 proteins, eventual expression/production would be resource-consuming to standardize (though not
118 necessarily in the distant future). Secondly, none of the toxin-neutralizing proteins are of human
119 origin; combined with the fact that many of the toxins are quite large (> 50 kDa) proteins, their
120 heterologous nature is likely to inflict adverse reactions in human recipients due to the likelihood of
121 high immunogenicity. Possibly, however, if a promising scaffold protein were to be developed with
122 low immunogenicity and able to be engineered easily to target a multitude of different toxin
123 families, it is conceivable that a molecular platform and discovery strategy could be employed for
124 development of non-antibody-based toxin-neutralizing proteins. Examples of such platform
125 technologies might include DARPins [37,38], Armadillo repeat proteins [39], affitins [40–42],
126 adhirons [43], anticalins [44], and various other protein scaffolds [45], although these molecular
127 formats are yet to be tested for their applicability for antitoxin development.
128
129 ***Employing monoclonal antibodies to neutralize animal toxins***

130 Various different monoclonal antibody (mAb) formats have been discovered and developed against
131 toxins from different animal species, including snakes, scorpions, spiders, and bees [8,9,46]. The
132 first use of a mAb capable of neutralizing a toxin was reported in 1982 using hybridoma technology
133 [47]. This IgG antibody targets toxin α from the Black-necked spitting cobra, *Naja nigricollis*.
134 Since then, 63 additional murine IgG mAbs targeting toxins from snakes, spiders, and scorpions
135 have been reported [8,9]. However, this number is unlikely to increase significantly, given the
136 prospects of using transgenic (humanized) animals capable of producing human IgGs more suitable
137 for human therapy and the advent of phage display technology. This is one of the most promising
138 avenues for development of novel recombinant antivenoms [48,49]. Most commonly, phage display
139 selection has been used to develop human single-chain variable fragments (scFvs) with important
140 examples including the development of Serrumab against the toxins, Ts1 and Ts2, from the
141 Brazilian yellow scorpion (*T. serrulatus*) [50,51], Afribumab targeting melittin and phospholipase
142 A_2s in Africanized bee (*Apis mellifera*) venom [46], the human scFv P2B7 capable of neutralizing
143 myotoxicity from *Bothrops jararacussu* venom [52], and ER-5 against β-neurotoxins from
144 *Centruroides* scorpion venoms [53]. Other formats, including diabodies [54,55], camelid V_HH [56–
145 60], bispecific V_HH [61], and V_HH-Fc fusions [62] have also been reported to successfully
146 neutralize a range of different toxins, particularly from scorpions and snakes. The prospect of using
147 (mixtures of) human mAbs against animal envenomings has gained increasing interest in recent
148 years. The reasons for this include the versatility of the (human) antibody scaffold, its compatibility
149 with the human immune system, the success of human antibodies in other fields [63], and the
150 demonstration that recombinant antivenoms may be produced cost-competitively by mammalian
151 cell cultivation [64,65] with future production costs of antibodies likely to decrease even further
152 [66]. Despite the existence of several feasible discovery approaches (particularly using humanized

153 transgenic animals, phage display, or even combined approaches), a need remains to elucidate
154 which animal toxins to focus antivenom research efforts on.
155
156 **Using omics technologies to establish order in venom complexity**
157 Independent of the molecular scaffold and discovery strategy employed for developing the next
158 generation of antivenoms against animal envenomings, it is critical to have a thorough
159 understanding of venom complexity and which toxins to target. For this purpose, different omics
160 technologies are increasingly being used to uncover novel animal toxins and help guide antivenom
161 development.
162 To date, genomes have been reported for two snakes (King cobra, *Ophiophagus*
163 *Hannah*, and Burmese python, *Python molurus bivittatus*) [67,68], three spiders (African social
164 velvet spider, *Stegodyphus mimosarum*, Brazilian white-knee tarantula, *Acanthoscurria geniculate*,
165 and House spider, *Parasteatoda tepidariorum*) [69,70], one scorpion (Manchurian scorpion,
166 *Mesobuthus martensii*) [71], and the honey bee (*Apis mellifera*) [72]. In addition, a multitude of
167 transcriptomics studies has been performed, particularly on the venom glands from snakes,
168 scorpions, and spiders [8,9]. These have all provided important evolutionary insight into the biology
169 of venomous animals and may be used to uncover novel toxins with unique functionalities. In
170 general, however, correlation between mRNA transcripts and protein expression may not always be
171 high [73], which has on several occasions shown to be the case for certain snake venoms from both
172 the viper and elapid families [74–76]. Therefore, genomic and transcriptomic studies may benefit
173 from combination with proteomics to establish a full overview of a venom.
174 The state of the art for elucidation of venom composition is based on the venomics
175 approach, combining venom fractionation by chromatography and gel electrophoresis with mass
176 spectrometry (see Figure 1) [49,77,78]. These techniques can be used to quantitatively estimate

177 venom proteomes [79,80]. Venomics can be performed both by bottom-up [81] and top-down
178 approaches, with the latter having the benefit of being able to distinguish between closely related
179 toxin isoforms [82]. By mid-2015, approximately 140 snake venoms had undergone a venomics
180 analysis [8]. Since then, this number has been growing steadily with new studies on venomous
181 snakes from Asia, Africa, Australia, Europe, and Latin America [82–89]. Fewer venomics studies
182 have been performed on venoms from spiders, scorpions [9], bees, and insects, owing to the
183 amounts of venom needed for estimating quantitative venom proteomes, and the scarcity of venoms
184 from most of these rather small creatures. Therefore, most of the proteomic studies performed on
185 venoms from these smaller species are qualitative in nature and only provide a catalog of toxins
186 present in the venoms with unknown abundances.

187 Venomics provides a good descriptive overview of venom. However, to fully gain an
188 understanding of which toxins in a venom are important for an envenoming case, functional studies
189 are needed. One such approach is toxicovenomics (see Figure 1), which was introduced in 2015 in a
190 study of Black mamba (*Dendroaspis polylepis*) venom with the aim of identifying key toxins to be
191 neutralized by effective antivenom [90]. In this study, the Toxicity Score [11] was employed which
192 can be calculated for venom toxins and fractions (containing toxin mixtures) based on their
193 abundance and potency. The Toxicity Scores can be used to rank the toxins or fractions according
194 to their medical importance (typically judged by lethality in mice), although the difficulty of
195 isolating certain toxins, such as Snake Venom Metalloproteases (SVMPs) somewhat hinder its use
196 for some venoms [11]. Geographical and intraspecies variation may give rise to slightly different
197 estimates of quantitative venom proteomes, which may, in turn, affect the Toxicity Scores for toxins
198 in venom. Additionally, the presence of toxin synergism [91] in some snake venoms (such as the
199 Green mamba, *Dendroaspis angisticeps* [92]) may further obscure the identification of which toxins
200 are key neutralization targets. However, when used conservatively to select the toxins which are

201 essential to neutralize in order to abrogate overall venom toxicity, the toxicovenomics approach
202 may provide a robust roadmap for antitoxin discovery. So far only elapid snake species from the
203 *Naja, Dendroaspis*, and *Aipysurus* genera have been investigated by toxicovenomics [18,90,92–94].
204 However, researchers from Mexico have recently employed a similar approach for selecting which
205 toxins from Mexican scorpions to focus antibody discovery efforts on [53].
206
207 **Understanding cross-reactivity by antivenomics and high-density peptide microarray**
208 **technology**
209 In addition to employing proteomics tools to gain a more complete understanding of whole venoms
210 and which toxins to neutralize with novel antivenom components, it may be beneficial to harness
211 omics technologies to assess antivenoms and antitoxins to guide their development. A particularly
212 important feature for toxin-neutralizing components is their ability to (selectively) cross-neutralize
213 entire (sub)families of venom toxins, as this allows for a decreased number of antitoxins needed in a
214 novel antivenom. Traditionally, most studies focusing on cross-reactivity and cross-neutralization
215 have been based on ELISA, enzymatic *in vitro* assays, and *in vivo* rodent assays. However, new
216 technologies emerging within the antibody field allow for more holistic and high-throughput
217 assessments of cross-reactivity.
218 One of the main issues with assessing antibodies by ELISA is that cross-reactivity
219 often correlates poorly with cross-neutralization. As a solution to this, "antivenomics" has been
220 developed based on the same methodology as venomics [95,96]. In the antivenomics approach,
221 whole venom is pre-incubated with beads coated with antivenom antibodies, before both bound and
222 unbound venom components are analyzed by Reversed-Phase High-Performance Liquid
223 Chromatography (RP-HPLC) and compared with the RP-HPLC chromatogram and proteomic
224 analysis of the whole venom (see Figure 2) [97]. This approach, which has so far only been used for

225 serum-based antivenoms, allows for a holistic assessment of the binding capacity of antivenoms
226 against different venom components, which may further be used to assess (potential) cross-
227 reactivity to toxins from other snake species that the antivenom was not raised against [78,96].
228 Nevertheless, the methodology should, in theory, be just as useful for assessing cross-reactivity of
229 mAbs. Antivenomic studies have been performed on antivenoms against a range of different snake
230 species from Europe, Asia, Oceania, and Latin America [87,98–104]. They are yet to be performed
231 on non-snake species, possibly owing to the scarcity of venoms from smaller venomous animals.

232 To obtain a more detailed molecular view of cross-reactivity, immunoreactivity of
233 antivenoms has also been evaluated using synthetic linear peptides derived from amino acid
234 sequences of spider, scorpion, and snake toxins [105–109]. Generally, these studies have been quite
235 meticulous and low throughput, but recently a novel high-throughput approach was introduced
236 based on high-density peptide microarray technology (see Figure 3) [110]. In this pioneering study,
237 the cross-reactivity of three different antivenoms against sub-Saharan African snake species was
238 assessed and the linear elements of epitopes for all 82 reported neurotoxins from the *Dendroaspis*
239 and *Naja* genera mapped simultaneously in one experiment. Although both the previous approaches
240 and the more recent high-throughput techniques have so far only been employed in the assessment
241 of serum-based antivenoms, their applicability to characterization of mAbs is known from other
242 fields [111,112].

243 The feasibility of assessing both monoclonal and oligoclonal antibodies using both
244 antivenomics and high-density peptide microarrays may possibly be instrumental for *in vitro*
245 assessment of novel recombinant antivenoms, to reduce cost, guide development, and reduce the
246 amount of preclinical work needed.

247

248 **Conclusion**

Envenomings by venomous animals are one of the therapeutic areas, where medicines based on animal sera are still in use. Different approaches have been tested and reported in the pursuit of developing fundamentally novel antivenoms. These approaches have involved design of novel immunogens (synthetic epitope DNA strings, synthetic linear peptides, and recombinant toxins and toxids), the use of small molecule inhibitors against enzymatic toxins, isolation and preclinical testing of endogenous animal proteins with toxin-neutralizing capabilities, and various recombinant antibody formats. Other binding proteins exist that could be harnessed, but these are yet to be employed within antivenom development. The most promising approach is likely to be the use of mixtures of (human or camelid) mAbs due to their versatility, high target affinity, low immunogenicity, and decreasing cost of production.

Irrespective of the approach employed, antivenom development efforts may benefit significantly by being guided by different omics technologies. Genomics and transcriptomics are excellent tools for uncovering novel animal toxins and investigating venom evolution. However, the state of the art remains to be venom proteomics (particularly venomics and toxicovenomics), which provides a better overview of venom compositions and what toxin targets are essential to neutralize in envenoming cases. Finally, the use of antivenomics and high-density peptide microarray technology may be exploited to gain in-depth understanding of antivenom and antibody specificity and cross-reactivity.

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620

622 **Figure legends**

623

624 ***Figure 1.*** *Schematic overview of venomics and toxicovenomics. Following the venomics approach,*
625 *venoms are fractionated by HPLC and gel electrophoresis, and the different fractions are*
626 *enzymatically digested and analyzed by MALDI-TOF-TOF and bioinformatics for identification of*
627 *toxins. Using chromatographic data and SDS-PAGE densitometry, it is possible to quantitatively*
628 *estimate the venom proteome. Toxicovenomics builds upon venomics and includes in vivo toxicity*
629 *data, which can be used to obtain an overview of venoms as pharmacological targets for antitoxin*
630 *development and determine which toxins are essential to neutralize with antivenom in an*
631 *envenoming case.*

632

633 ***Figure 2.*** *Schematic overview of the antivenomics approach.* ***A)*** *First venom is passed through a*
634 *column containing resins that are coated with antivenom. Unbound toxins are analyzed by*
635 *Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC).* ***B)*** *Bound venom toxins*
636 *are then eluted and likewise analyzed by RP-HPLC.* ***C)*** *Combined with the chromatogram and a*
637 *proteomic analysis of the whole venom, the two analyses provide an overview of which venom*
638 *toxins are readily recognized and bound by a given antivenom, and which toxins are not.*

639

640 ***Figure 3.*** *Schematic overview of how high-density peptide microarrays are designed and used for*
641 *the study of epitope-paratope interactions between antivenoms and animal venom toxins.*

642

Highlights

- nvenoming by venomous animals remain a major public health challenge in the tropics
- Biotechnological approaches are being harnessed for development of novel antivenoms
- Genomics and transcriptomics are good for uncovering novel venom toxins
- Venom proteomics is useful for identifying key toxins for neutralization by novel antivenoms
- High-density peptide microarray technology is emerging as a valuable tool in antivenom research